NADPH Oxidases NOX-1 and NOX-2 Require the Regulatory Subunit NOR-1 To Control Cell Differentiation and Growth in *Neurospora crassa* " View Article Online |

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We have proposed that reactive oxygen species (ROS) play essential roles in cell differentiation. Enzymes belonging to the NADPH oxidase (NOX) family produce superoxide in a regulated manner. We have identified three distinct NOX subfamilies in the fungal kingdom and have shown that NoxA is required for sexual cell differentiation in *Aspergillus nidulans*. Here we show that *Neurospora crassa* NOX-1 elimination results in complete female sterility, decreased asexual development, and reduction of hyphal growth. The lack of NOX-2 did not affect any of these processes but led instead to the production of sexual spores that failed to germinate, even in the presence of exogenous oxidants. The elimination of NOR-1, an ortholog of the mammalian Nox2 regulatory subunit gp67phox, also caused female sterility, the production of unviable sexual spores, and a decrease in asexual development and hyphal growth. These results indicate that NOR-1 is required for NOX-1 and NOX-2 functions at different developmental stages and establish a link between NOX-generated ROS and the regulation of growth. Indeed, NOX-1 was required for the increased asexual sporulation previously observed in mutants without catalase CAT-3. We also analyzed the function of the penta-EF calcium-binding domain protein PEF-1 in *N. crassa*. Deletion of *pef-1* resulted in increased conidiation but, in contrast to what occurs in *Dictyostelium discoideum*, the mutation of this peflin did not suppress the phenotypes caused by the lack of NOX-1. Our results support the role of ROS as critical cell differentiation signals and highlight a novel role for ROS in regulation of fungal growth.

A significant amount of recent research has established that reactive oxygen species (ROS), long considered as harmful by-products, can play cell signaling roles (1, 7, 14, 18, 23). For many years, we have used the model organism *Neurospora crassa* to investigate the role of ROS in the regulation of asexual development (conidiation). In this fungus, synchronous conidiation is started when a liquid culture is filtered and exposed to the air. The hyphal cells in contact with the air aggregate and adhere to each other within 40 min and grow aerial hyphae after 2 h, and then asexual spores (conidia) are formed at the tips of branched aerial hyphae (aerial mycelium) after 8 to 9 h of air exposure (37, 43). The occurrence of a hyperoxidant state at the start of each of these morphogenetic transitions (hyphal adhesion, formation of aerial mycelium, and conidium formation) has been documented (1, 2, 19–21, 30, 44, 45). In addition, *N. crassa* develops multicellular fruiting bodies called perithecia, which contain the sexual spores or ascospores. Under nitrogen limitation conditions, a strain with either mating type (A or a) can differentiate a multicellular structure called a protoperithecium and function as a “female” or acceptor strain. A protoperithecium is fertilized through a specialized hypha called the “trichogyn” which fuses with a cell, usually a conidium, from the opposite mating type. Fertilized protoperithecia develop into mature perithecia (6, 34, 46).

We have proposed that ROS are key players in the regulation of cell differentiation in microbial eukaryotes (1, 19, 20). According to this hypothesis, the inactivation of antioxidant enzymes should increase cell differentiation processes whereas the inactivation of prooxidant enzymes should inhibit these processes. As NADPH oxidases (NOX) produce ROS in a regulated manner, we sought to examine the occurrence of NOX enzymes in fungi and their possible roles in cell differentiation (1, 24).

Superoxide generation by the phagocyte NOX involves the formation of an enzyme complex composed of the membrane-associated catalytic core gp91phox (Nox2) and p22phox subunits, as well as regulatory subunits p47phox, p40phox, p67phox, and the GTPase Rac2. Nox2 activation requires the phosphorylation of the p47phox “organizer subunit,” which then interacts with p22phox and the “activator subunit” p67phox, also recruiting p40phox to the complex. In addition, Rac2 interaction with Nox2 and p67phox is essential for the activity of this NOX (reviewed in references 4 and 31). Several Nox2 homologs (NOX1, -3, -4, and -5 and DUOXA1 and DUOXA2) have been identified in mammalian cells, while two organizer (p47phox and NOX01) and two activator (p67phox and NOX1) subunits have been reported. Some of these NOX have been involved in cell proliferation and apoptosis, clearly indicating the importance of ROS in cell signaling (see references 4 and 23 for recent reviews).
Functional NOX enzymes were not recognized in microbial eukaryotes until recently. NoxA, the only Nox2 ortholog present in the fungus Aspergillus nidulans, is involved in ROS production and is essential for the differentiation of sexual fruiting bodies (24). An exhaustive phylogenetic analysis of the NoxA orthologs indicated the presence of three NOX subfamilies (NoxA to -C) within the filamentous fungi (1, 24). Some members of these families have now been characterized and shown to regulate different aspects of fungal biology. In the saprophytic fungus Podospora anserina, disruption of the Panox1 gene drastically reduces but does not eliminate the development of sexual fruiting bodies, while inactivation of the Panox2 gene results in production of sexual spores that are nonviable or unable to germinate (29). Null noxA mutants from the fungus Epichloë festucae show unregulated growth in its plant host, changing the interaction from mutualistic to antagonistic, while deletion of the noxB gene does not produce a detectable phenotype (41). In the plant pathogen Magnaporthe grisea NOX1 and NOX2 mutants differentiate penetration structures called appressoria but are unable to penetrate the plant and therefore are nonpathogenic. In addition, NOX1 NOX2 double mutants show a drastic reduction in the production of asexual sporules or conidia (12). NOX genes have also been characterized or identified in the slime mold Dictosteli um discoideum (25), as well as in pluricellular and unicellular algae (22). D. discoideum contains three nox genes, and the inactivation of noxA, noxB, noxC, or a p22phox ortholog results in the same phenotype: arrested development and lack of asexual spores (25), indicating that the three NOX play partially redundant functions.

The subunit composition and regulation of NOX activity in microbial eukaryotes are still poorly understood. A p67phox ortholog was initially identified in D. discoideum (25), but its function was not evaluated. More recently, Takemoto et al. (40) identified p67phox orthologs in several fungi and showed that the E. festucae p67phox ortholog NoxR regulates NoxA, but only during association with the plant host. In addition, these authors showed that RacA, a mammalian Rac2 ortholog, shows specific interaction with NoxR. However, no clear orthologs of p22phox, p40phox, or p47phox have been yet identified in fungi (40).

The fact that NOX regulate developmental processes in different microbial eukaryotes suggests that ROS regulate cell differentiation and that this is a ROS ancestral role conserved throughout the eukaryotes. How ROS exert their functions and the identity of their downstream effectors are still unclear. Ca2+ signaling has been linked to NOX function in plants (15). Notably, the elimination of the alg-2b gene restored normal development in noxA- and noxB- but not noxC-null mutants in D. discoideum (25). In this organism, alg-2b encodes one of two calcium-binding penta-EF hand proteins, members of the peflina family (28). This suggests that ALG-2B inhibits a downstream effector of NOX signaling and indicates cross talk between ROS and Ca2+ signaling. The function of peflins or their possible interactions with NOX function have not been evaluated before in filamentous fungi.

Here we used a genetic approach to examine the role of NOX-encoding genes nox-1 and nox-2 in N. crassa growth and cell differentiation. We provide evidence showing that, although each NOX is involved in different aspects of growth and development, a single regulatory subunit, NOR-1, is required for the function of both NOX. In addition, we show that NOX-1 is required for the increased sporulation due to inactivation of the antioxidant enzyme CAT-3 (30). Furthermore, we characterize mutants lacking the only penta-EF calcium-binding protein, PEF-1, and show that this protein does not appear to be related to NOX signaling.

**MATERIALS AND METHODS**

*Neurospora crassa* strains and growth conditions. Strains used in this work are indicated in Table 1. General methodologies for growth and crossing have been reported previously (11). All strains were grown in Vogel's minimal medium supplemented with 1.5% sucrose. When needed, L-histidine (200 µg/ml) and myo-inositol (50 µg/ml) were added. For sexual crosses, synthetic crossing medium was inoculated with 5 × 10^6 conidia and incubated for 6 days at 25°C in the light. Cultures were fertilized with 10 µl drops of a conidial suspension (2.5 × 10^9 conidia/drop) from the opposite mating type, and incubation was continued. Between 12 and 14 days, the ascospores expelled from the asci were harvested in sterile water and incubated overnight at room temperature. Ascospores were activated at 60°C for 30 min; plated on Vogel's solid medium containing 2%
t-sorbos, 0.05% fructose, and 0.05% glucose; and incubated for 24 to 48 h at 30°C. Colonies were isolated and transferred to tubes containing Vogel's medium for propagation. For 2-furfuraldehyde treatment, ascospore suspensions were incubated at 30°C for 6 h to induce swelling of contaminating conidia and later incubated at 46°C for 1 h for partial ascospore activation and killing of swollen conidia (38). After this treatment ascospores were incubated with 1 mM 2-furfuraldehyde (Sigma-Aldrich Corporation, St. Louis, MO) for 15 min at room temperature and plated on solid medium with L-sorbose. For germination experiments in the presence of H2O2, ascospores were activated by heat shock at 60°C for 30 min and then incubated with 0, 1, 5, 10, and 100 mM H2O2 for 20 min. Alternatively, H2O2 was added to ascospores before heat shock activation or ascospores were treated only with H2O2.

Synchronous development of ascogonia and protoperithecia was induced according to the method of Biistis (5). Briefly, water-agar plates were inoculated in the center with 1 x 10³ conidia and incubated for 4 to 5 days at 25°C in the light. Next, four circles of solid crossing medium (4 mm in diameter) were transferred to the water-agar plate and placed equidistantly and near the limits of the colony. Ascogonia and protoperithecia were observed 24 and 48 h later, respectively.

Plasmid constructions. noc-1 and noc-2 deletion plasmids were constructed based on the strategy published by Pratt and Arayamo (35). A noc-1 deletion construct was generated as follows: first, a 5' noc-1 region was amplified by PCR, using N. crassa genomic DNA as template and primers 5'-Nc-Xu-Up (5'-CTT GGT GAT TCG CAA T AA ACA GCA TAC) and 5'-Nc-Xu-Lo (5'-CTT GGT GAT TCG CAA TAA ACA GCA TAC). The resulting 2,075-bp fragment was cloned into TOPO2.1 vector (Invitrogen, Carlsbad, CA) to generate plasmid pTOPKN1-17. Second, a portion of the 3' region was amplified with primers 3'-NcNox1 (5'-CTT TAG CGG CCG CAA TTT TAG TGT CGG GAA GG) and 3'-NcNox2 (5'-CTT TAG CGG CCG CAA TTT TAG TGT CGG GAA GG), which contained a NotI restriction site. The 1,814-bp PCR product cloned into TOPO2.1 vector was named pTOPKN1-2.

The noc-1 5' region derived from pTOPK1-7 was cloned into pKAD-10Nc, and the resulting plasmid was named pNCKA-6. Next, the noc-1 3' region derived from pTOPK1-7 was subcloned into pNCKA-6 to generate pNCKA-8, which was used to transform N. crassa and delete noc-1. For noc-2 deletion, the noc-2 3' region was amplified using primers NpP3394 (5'-TTT TCG GAG ACG TCG CAA GCC GAT GAT ACG ACC) and NOX-2N (5'-TTT TCG GAG ACG TCG CAA GCC GAT GAT ACG ACC). The PCR product (2,075 bp) was cloned into pDLAM89d to obtain plasmid pKAD28. The noc-2 genome obtained from pRATT25d was cloned into pKAD28 to generate pKAD-23.

Preparation of conidial spheroplasts and transformation of N. crassa. Conidia were harvested from culture slants grown for 3 days at 30°C in the dark and 2 days at room temperature in the light. For spheroplast preparation, conidia were harvested and treated as reported elsewhere (35). For transformation, we used 150 µl of spheroplasts and 7.42 µg of linear plasmid pNCKA-8 or 2 to 10 µg of linear plasmid pKAD9. The transformation mixture was mixed with warm selective top agar (Vogel's salts with 1 M sorbitol, 0.05% glucose, 0.05% fructose, 1% agar, and supplements) and plated onto a petri dish containing the same medium plus 250 µg/ml of hygromycin B (Sigma-Aldrich, St. Louis, MO) and 2% agar. Original transformants were purified three times on selective medium with 150 µg/ml of hygromycin B.

The amy1-51 strain (32) was also used for transformation. Fifty microliters of a suspension of conidia (1.25 x 10⁶) prepared in 1 M sorbitol was mixed with 40 µl of a solution containing 10 µg of the DNA fusion PCR product and incubated on ice for 5 min. Forty microliters of this mixture was transferred to 0.2-cm electroporation cuvettes and electroporated using 1.5 kV, 600 Ω, and 2.5 µF in Gene Pulser II and Pulse Controller II (Bio-Rad, Hercules, CA) instruments. After electroporation, 960 µl of 1 M cold sorbitol was added, mixed with 25 µl of recovery solution (Vogel's medium with 2% yeast extract), and incubated at 30°C for 2 h. This solution was mixed with 25 µl of regeneration agar (Vogel's medium with 1 M sorbitol, 2% yeast extract, and 1% agar), and plated immediately on solid Vogel medium with t-sorbos, fructose, and glucose plus 200 µg/ml of hygromycin B. Plates were incubated at 30°C for 5 days, and the resulting transformants were purified four times in selective medium with 200 µg/ml hygromycin B.

Southern blot analysis. For DNA genomic extraction, 1 x 10⁶ conidia were inoculated in liquid-supplemented Vogel's medium and grown for 48 h at 30°C without shaking. Mucilag was frozen in liquid nitrogen, lyophilized, and ground with a mortar and pestle under liquid nitrogen. DNA genomic DNA was extracted according to the method of Timberlake (42). Seven to 10 µg of DNA was digested with different restriction enzymes, fractionated in an agarose gel, transferred to Hybond N membranes (Amersham Biosciences, Piscataway, NJ), and hybridized with different radioactive probes.

RNA extraction and Northern blot analysis. Mycelial samples, frozen in liquid nitrogen and stored at −70°C until used, were ground with a mortar and pestle under liquid nitrogen. Total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Ten to 12 µg of RNA was separated in a 1% agarose gel containing formaldehyde, transferred to Hybond N membranes, and hybridized with noc-1 ORF- and noc-2 ORF-specific probes.

RESULTS

N. crassa noc-1 and noc-2 genes, encoding members of the fungal NoxA and NoxB subfamilies, are differentially expressed. The phylogenetic analysis of the NOX family indicates the presence of three subfamilies (NoxA to NoxC) within the fungi (1, 24). The analysis of the N. crassa genome sequence (16) indicated that this fungus contained two different noc genes, which we designated noc-1 and noc-2. These genes predicted hypothetical proteins NCU02110.3 (553 amino acids) and NCU10775.3 (581 amino acids), respectively. The alignment of NOX-1, NOX-2, and other NOX shows that these proteins contain all NOX family signature regions (see Fig. S1 in the supplemental material). Sharing an identity of only 36%, NOX-1 and NOX-2 belong to the NoxA and NoxB subfamilies, respectively. Like other NoxB members, NOX-2 contains an N-terminal extension of unknown function (see Fig. S1 in the supplemental material). To characterize NOX functions in N. crassa, we first analyzed the pattern of noc mRNA accumulation at different developmental stages. As shown in Fig. 1A, noc-1 mRNA was not detected in conidia, accumulated between 8 and 14 h of growth in liquid culture, and decreased between 20 and 24 h. In contrast, noc-2 mRNA was present in conidia, maintained similar levels between 8 and 14 h, and showed high accumulation levels between 20 and 24 h. During sexual development (Fig. 1B), noc-1 started to accumulate around 120 h, peaked at 144 h, and decreased at 192 h. Under these conditions, the highest noc-1 mRNA levels coincided with the formation of the female sexual structures or protoperithecia (shown in Fig. 2B; see also Fig. 6C), while noc-2...
mRNA was detected only before protoperithecal development. These results suggest that NOX-1 functions might be important during growth and sexual differentiation, while NOX-2 functions could be associated with conidial sexual structures and/or stationary-phase physiology.

NOX-1 is essential for sexual development. To determine *nox-1* function, we deleted most of its ORF by gene replacement, using the hygromycin resistance gene as a genetic marker (see Fig. S2 in the supplemental material). Although the resulting mutants showed several phenotypes (see below), we first analyzed them for sexual development defects. Results in Fig. 2 show that *Δnox-1* mutants, whether mating type *a* or *A*, were unable to cross with a wild-type strain and differentiate mature fruiting bodies or perithecia. However, this phenotype was observed only when *Δnox-1* mutants were used as the recipient or female sexual partner. Indeed, *Δnox-1* conidia were able to function as the donor or “male” partner and fertilize a wild-type strain, giving rise to perithecia (Fig. 2A, bottom) and viable ascospores (not shown). These results led us to ask whether *Δnox-1* mutants were able to differentiate the female sexual structures or protoperithecia. Consistent with their female sterility, we found that *Δnox-1* mutants were incapable of forming any protoperithecia (Fig. 2B). However, *Δnox-1* mutants were able to develop ascogonia (Fig. 2C, left), which is the first recognizable stage in protoperithecal differentiation (46). As the NOX are involved in superoxide (O$_2^·-$) production, we used a nitroblue tetrazolium (NBT) reduction assay (3) to detect superoxide production in intact ascogonia and protoperithecia. While ascogonia were not stained by NBT (not shown), a dark formazan precipitate, indicative of NBT reduction, was readily formed around developing protoperithecia (Fig. 2C, right). These results indicate that, while not required for initial formation of female sexual structures (ascogonia), NOX-1-generated ROS are essential for subsequent development and formation of mature and viable protoperithecia.

NOX-2 is required for sexual spore viability. We used a similar gene replacement strategy to delete most of the *nox-2* ORF (see Fig. S3 in the supplemental material). *Δnox-2* mutants did not show any obvious defect in cell growth or asexual or sexual development. However, when ascospores from heterozygous crosses were plated, *Δnox-2* colonies (hygromycin resistant) were recovered at a very low frequency (less than 5%). Nevertheless, we isolated *Δnox-2* strains from opposite mating types and performed different *Δnox-2* homozygous crosses. All the ascospores isolated from these crosses failed to germinate whether activated or not by heat shock, despite the fact that under the microscope, *Δnox-2* ascospores showed a wild-type appearance (Fig. 3). Incubation of *Δnox-2* ascospores with 1, 5, 10, or 100 mM H$_2$O$_2$ before, during, or after heat shock activation did not have any positive effects on ascospore germination (not shown). Likewise, the presence of 1 mM furfural, which promotes heat shock-independent germination of *N. crassa* ascospores (13), did not restore *Δnox-2* ascospore germination (not shown). Whether this phenotype is due to defective ascospore development or failure to germinate is not known. Nevertheless, our results show that NOX-2 is essential for sexual spore function in *N. crassa*.

NOR-1 is required for NOX-1 and NOX-2 function. Among microbial eukaryotes, one p67$^{phox}$ ortholog was identified in *D. discoideum* (25). Takemoto et al. (40) identified p67$^{phox}$ orthologs in several fungi and showed that *E. festucae* p67$^{phox}$ ortholog NoxR regulates NoxA but only during association of the fungus with its plant host. We asked whether the only NoxR ortholog present in *N. crassa* (NCU07850.3), which we have designated NOR-1 (NADPH oxidase regulator), was required for NOX-1 and/or NOX-2 functions. NCU07850.3 is incorrectly annotated as a protein of 471, instead of 571, amino acids (see Fig. S4 in the supplemental material). We deleted the nor-1 gene by transforming a *Δmus-51* mutant strain (32) with a PCR construct generated by double-joint PCR (48). In *mus* mutants most DNA integration events occur by homologous recombination, resulting in high gene targeting frequencies. Several *Δnor-1* mutants were identified after Southern blot analysis (see Fig. S5 in the supplemental material), all of which showed defects in asexual sporulation and radial growth, resembling those observed in *Δnox-1* mutants (see below). When *Δnor-1* mutants were analyzed for sexual development, we found a phenotype that was indistinguishable from that observed in *Δnox-1* mutants (see below). When *Δnor-1* mutants were analyzed for sexual development, we found a phenotype that was indistinguishable from that observed in *Δnox-1* mutants (see below). *Δnor-1* mutants failed to differentiate protoperithecia but were able to develop ascogonia (Fig. 4A). These results support the role of NOR-1 as a regulator of NOX-1 function during sexual development.

To explore NOR-1 roles in NOX-2 activity, we used *Δnor-1* conidia to fertilize protoperithecia from *Δnox-2* mutants. The ascospores from this cross that grew on nonepisensory medium were unable to grow on hygromycin-containing medium and therefore corresponded to strains carrying *nox-2* and *nor-1* wild-type alleles. The fact that no *Δnor-1* or *Δnox-2* ascospores were recovered from these crosses indicates that mutation of either *nor-1* or *nox-2* results in the same phenotype: the production of defective sexual spores (Fig. 4B). Our results support a model in which NOR-1 regulates NOX-1 activity, required for protoperithecal development, as well as the activity of NOX-2, required at a later stage of sexual development to produce functional ascospores.

NOX-1 and NOR-1 regulate asexual development and hyphal growth. The lack of the ROS-producing enzyme NOX-1 not only affected sexual development but also resulted in a
reduction in the amount of aerial mycelium compared to the wild type (Fig. 5A). In contrast, it has been reported that inactivation of the ROS-detoxifying enzyme CAT-3 leads to increased amounts of aerial mycelium and conidia (30). As these results support the notion that ROS levels regulate asexual development, we decided to examine the extent of asexual development in \( H9004 \) \( \text{nox-1} \), \( H9004 \) \( \text{nox-2} \), \( H9004 \) \( \text{nor-1} \), and \( H9004 \) \( \text{nox-1 cat-3 RIP} \) mutants. We found that, as reported, \( \text{cat-3 RIP} \) mutants produced denser aerial mycelium and greater conidiation than did the wild-type strain (Fig. 5A and B). In contrast, \( \text{nox-1} \) mutants not only formed shorter aerial mycelium but also produced conidiation yields lower than those of the wild type. Asexual development was not affected in the \( \text{nox-2} \) mutant.

The fact that a \( \text{nor-1} \) mutant shared \( \text{nox-1} \) phenotypes (Fig. 5A and B) further supports the essential role of NOR-1 in NOX-1 activation. Asexual development in the \( \text{nox-1 cat-3 RIP} \) double mutant was similar to that observed for the \( \text{nox-1} \) single mutant, indicating that NOX-1 activity is necessary for increased asexual development in \( \text{cat-3 RIP} \) mutants.

To determine whether ROS metabolism could have general
effects on mycelial growth, we determined the growth rate of \( \Delta \text{nox-1}, \Delta \text{nor-1}, \text{cat-3 RIP} \), and \( \Delta \text{nox-1 cat-3 RIP} \) mutants in race tubes. As shown in Fig. 5C, \( \Delta \text{nox-1}, \Delta \text{nor-1}, \) and \( \Delta \text{nox-1 cat-3 RIP} \) mutants showed clear and similar reductions in mycelial extension rates. In contrast, the growth rate of \( \text{cat-3 RIP} \) mutants was similar to that of the wild type. These results indicate that NOX-1 activity is necessary for proper hyphal growth and that lack of CAT-3 does not compensate for the lack of NOX-1.

Deletion of \( \text{pef-1} \) does not suppress the phenotypes caused by a lack of NOX-1 or NOX-2. NoxA and NoxB, the NOX-1 and NOX-2 orthologs in \( \text{D. discoideum} \), respectively, are required for cell aggregation and asexual sporulation. Remarkably, the elimination of ALG-2B restored normal development in both \( \text{noxA} \) and \( \text{noxB} \) null mutants (25). ALG-2B is one of two calcium-binding penta-EF hand proteins or peflins (28) present in this organism. Recently, it has been shown that Pef1p, the ALG-2 ortholog in \( \text{Saccharomyces cerevisiae} \), is involved in cell budding and polarization (47), but peflin function in filamentous fungi was unknown. In \( \text{N. crassa} \), we identified hypothetical protein NCU02738 as the only ALG-2 ortholog in this fungus and designated it as \( \text{PEF-1} \) (penta EF domain protein 1 or peflin 1). PEF-1 and its fungal orthologs are conserved at the C terminus, which includes the five putative calcium-binding EF domains, but show low conservation at the N terminus (see Fig. S6 in the supplemental material). We deleted the \( \text{pef-1} \) gene to evaluate its function in \( \text{N. crassa} \) and possible interactions with \( \text{nox-1} \). A \( \text{pef-1} \) deletion construct, based on hygromycin resistance, was generated by PCR and used to transform the \( \Delta \text{mus}-51 \) strain (32). Purified transformants were analyzed by Southern blotting using restriction enzyme PvuI (see Fig. S7 in the supplemental material). Strain \( \text{pef-1.9a} \) was selected out of four transformants with the correct \( \text{pef-1} \) deletion event and used in further experiments and sexual crosses. \( \text{pef-1} \) mutants were able to develop fertile protoperithecia (Fig. 6C) and viable ascospores in homozygous crosses (not shown) and showed wild-type growth rates in race tubes (Fig. 6D). In contrast, a \( \Delta \text{pef-1} \) mutant produced 65% more conidia than did the wild-type strain, despite the fact that the two strains formed similar amounts of aerial mycelia (Fig. 6A and B).

Next, we generated \( \Delta \text{pef-1} \Delta \text{nox-1} \) double mutants, which were confirmed by Southern blot analysis (not shown), to examine whether PEF-1 elimination could restore normal development in \( \text{nox-1} \)-null mutants. As shown in Fig. 6C, the \( \Delta \text{pef-1} \Delta \text{nox-1} \) mutant was not able to differentiate normal protoperi-
Indeed, when this mutant was fertilized with wild-type spores, no mature perithecia or ascospores were formed (not shown). The mutation of \textit{pef-1} also failed to restore normal conidiation (Fig. 6B) or hyphal growth rate (Fig. 6C) in a \textit{/H9004 nox-1} background. These results indicate that, in contrast to what occurs in \textit{D. discoideum}, mutation of the \textit{N. crassa} \textit{ALG-2} ortholog \textit{pef-1} did not restore any of the \textit{nox-1}-null mutant phenotypes.

**DISCUSSION**

\textit{NOX-1} is required for sexual and asexual development, and normal hyphal growth, while \textit{NOX-2} seems specifically involved in sexual spore function. Previous work indicates that \textit{NOX-1} orthologs play rather specific roles in fungal development. NoxA inactivation in \textit{A. nidulans} results in complete arrest of sexual development, without notably affecting growth (24). Likewise, lack of PaNox-1 in \textit{P. anserina} causes a major reduction in the number of protoperithecia and fruiting bodies but does not affect growth rate (29). \textit{noxA} mutants of the symbiotic fungus \textit{E. festucae} grow normally in culture but show unregulated and increased growth in its plant host (41). \textit{nox1} mutants of the plant pathogen \textit{M. grisea} show a slight increase in radial growth and fail to penetrate its plant host (12), while \textit{Claviceps purpurea} \textit{Cpxox1} mutants grow normally, despite showing decreased germination and defective colonization of plant ovarian tissue (17).

As we report here, \textit{N. crassa} \textit{NOX-1} is not only essential for protoperithecium development but also required for normal development of aerial hyphae and conidiation, as well as for vegetative growth. This suggests that all these processes require the production of ROS derived from \textit{NOX-1} activity. We detected superoxide production during differentiation of female organs or protoperithecia (Fig. 2C, right), while Hansberg et al. detected ROS at the three morphogenetic events that are characteristic of asexual sporulation in \textit{N. crassa} (21). Interestingly, the first peak of ROS detected during aggregation of hyphae, 30 min after the mycelium was exposed to air (21), coincided with increased \textit{nox-1} mRNA levels (not shown). ROS regulation of asexual development is further indicated because the elimination of ROS-decomposing (CAT-3) and ROS-generating (NOX-1) activities increases (30) and decreases (this work) aerial mycelium and conidiospore development, respectively. The fact that asexual development in the \textit{Delta nox-1 cat-3RIP} double mutant was similar to that observed for the \textit{Delta nox-1} single mutant suggests that \textit{NOX-1}-derived ROS are required for the increase in asexual development observed in \textit{cat-3} mutants. Although ROS are difficult to specifically detect at hyphal tips and have not yet been found to be involved in regulation of growth in \textit{N. crassa}, our results indicate that \textit{NOX-1}-derived ROS play a role in hyphal growth, perhaps regulating the rate of apical extension. ROS have also been detected during asexual spore germination (27).

Sexual spores from mutants lacking \textit{NOX-2} were not viable or able to germinate, suggesting that ROS might be required for spore germination. However, mutants lacking \textit{NOX-1} or \textit{NOX-2} did not show detectable defects in the germination rate of conidia (not shown).

**NOX redundancy and regulation.** NOX play partially redundant functions in some organisms. In \textit{D. discoideum}, \textit{noxA},

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**FIG. 4.** Mutants lacking putative NOX regulatory subunit NOR-1 share \textit{Delta nox-1} and \textit{Delta nox-2} sexual phenotypes. (A) Strains 74-OR8-1a (wild type [WT]) and Nc28nor-1 (\textit{Delta nor-1}) were induced to develop ascogonia and protoperithecia (arrowheads). (B) Ascospores from crosses 74-OR23-1A × 74-OR8-1a, 74-OR8-1a × Nc28nor-1 (\textit{Delta nor-1}), and CNCKA-Z (\textit{Delta nox-2}) × Nc28nor-1 (\textit{Delta nor-1}) were collected and plated as reported for Fig. 3. The few ascospores from the \textit{Delta nox-2} × \textit{Delta nor-1} cross that were able to germinate and generate colonies (arrowheads) were sensitive to hygromycin and therefore carried \textit{nox-2} and \textit{nor-1} wild-type alleles.
noxB, and noxC are sequentially expressed and elimination of any of these genes brings about the same phenotype (25). M. grisea Nox1 and Nox2 are independently required for pathogenicity, although inactivation of both NOX affects asexual development (12). In P. anserina, PaNox1 and PaNox2 play different roles during sexual development, but PaNox2 seems to partially replace PaNOX1 functions (29). Botrytis cinerea BcNoxA and BcNoxB are both required for sclerotium formation and pathogenicity, and double mutants show additive effects on these processes (36). In contrast, N. crassa NOX-1 and NOX-2 do not seem to play redundant functions. Although nox-2 is expressed during growth, its inactivation did not produce any of the phenotypes observed when nox-1 was eliminated. Furthermore, mutation of the p67phox ortholog NOR-1, required for NOX-1 and NOX-2 activity, did not enhance the phenotypes caused by the lack of NOX-1.

The fact that NOR-1 is required for NOX-1 and NOX-2 activity, despite the fact that these two NOX are required at different developmental stages, indicates that NOR-1 availability does not appear to be a limiting factor for NOX activity. In E. festucae the NOR-1 ortholog NoxR seems required for NoxA activity only during symbiosis, and it is not known if it is needed for NoxB activity (40). While this paper was in preparation, Segmüller et al. (36) reported that the NoxR ortholog BcNoxR is required for BcNoxA and BcNoxB functions in B. cinerea. However, these NOX do play partially redundant functions. Our results raise questions on what triggers NOX activation and suggest that the activity of all fungal A/B-type NOX is dependent on p67phox orthologs. As with many of the mammalian NOX, the Rac subunit seems essential for NOX activation in fungi (40). As occurs in plants, GDP dissociation inhibitors might in turn regulate Rac activity (8). Therefore, it is possible that events leading to Rac activation could be triggering NOX activation. In this context, it is interesting that GTPases Ras and Rac have been linked to ROS production in Colletotrichum trifoli (9).

As mitogen-activated protein kinase signaling has been involved in NOX regulation (24), it is interesting that N. crassa mutants lacking NOR-1 or the mitogen-activated protein kinase MAK-2 share several phenotypes, i.e., they show reduced growth rates, produce short aerial hyphae, fail to develop protoperithecia, and produce unviable ascospores (26). As mak-2 mutants are also defective for hyphal fusion (33), it will be interesting to assess this defect in the nox-1, nox-2, and nor-1 mutants, as well as to determine if MAK-2 is a positive upstream regulator of NOX function in this fungus.

Elimination of peflin PEF-1 results in increased conidiation but does not restore development in Δnox-1 mutants. In mammalian cells peflins such as ALG-2 have been shown to bind Ca²⁺ and regulate processes such as apoptosis and vesicle trafficking (28). Peflin Pef1p functions in cation-dependent budding and cell polarization in S. cerevisiae. We found that the lack of peflin PEF-1 did not have any evident impact on N.
crassa biology, except that Δpef-1 mutants showed higher conidiation, an effect that cannot be explained at this time.

As the mutation of PEF-1 ortholog ALG-2 bypassed the NoxA and NoxB requirement for asexual sporulation in D. discoideum (25), this supported the idea of cross talk between ROS and Ca\(^{2+}\) mobilization, as has been observed for plants (15). We found that the inactivation of PEF-1 did not restore the developmental defects of N. crassa nox-1-null mutants. However, this does not rule out a connection between ROS and Ca\(^{2+}\) signaling, and although we do not know how NOX-1 might regulate polar growth in N. crassa, it is interesting that polar growth in Arabidopsis thaliana root hair cells appears to be controlled by a positive feedback regulation between NOX-derived ROS and Ca\(^{2+}\) (39). Further research is needed to establish a connection between ROS and Ca\(^{2+}\) signaling in fungi.

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ADDENDUM IN PROOF

In additional experiments, the elimination of pef-1 also failed to restore ascospore germination in Δnox-2 mutants.

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