The NADPH oxidases NOX-1 and NOX-2 require the regulatory subunit NOR-1 to control cell differentiation and growth in *Neurospora crassa*.

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Abstract

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 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 contrary 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.
Introduction

A significant amount of recent research has established that reactive oxygen species (ROS), long considered as harmful byproducts, 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 conidia 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 protoperithecium and function as “female” or acceptor strain. A protoperithecium is fertilized through a specialized hyphae called the “trichogyne” which fuses with a cell, usually a conidia, 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 anti-oxidant enzymes should increase cell differentiation processes whereas the inactivation of pro-oxidant
enzymes should inhibit these processes. As NADPH oxidases produce ROS in a regulated manner, we sought to examine the occurrence of NADPH oxidase (NOX) enzymes in fungi and their possible roles in cell differentiation (1, 24).

Superoxide generation by the phagocyte NADPH oxidase involves the formation of an enzyme complex composed of the membrane-associated catalytic core gp91phox (Nox2) and p22phox subunits, as well as regulatory subunits p40phox, p47phox, 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 4, 31). Several Nox2 homologs (NOX1, 3, 4 and 5, DUOXA1 and DUOXA2) have been identified in mammalian cells, while two organizer (p47phox and NOXO1) and two activator (p67phox and NOXA1) subunits have been reported. Some of these NOX have been involved in cell proliferation and apoptosis, clearly indicating the importance of ROS in cell signalling (see 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-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 Panox1 gene
drastically reduces but does not eliminate the development of sexual fruiting bodies, while inactivation of the *Panox*-2 gene results in production of sexual spores that are non-viable 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 griseae* *NOX1* and *NOX2* mutants differentiate penetration structures called appressoria but are unable to penetrate the plant and therefore are non-pathogenic. In addition, *NOX1 NOX2* double mutants show a drastic reduction in the production of asexual spores or conidia (12). NOX genes have also been characterized or identified in the slime mold *Dictyostelium 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 is 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 which are their downstream effectors is still unclear. Ca^{2+} 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 peflin family (28). This suggests that ALG-2B inhibits a downstream effector of NOX signaling and indicates a cross talk between ROS and Ca^{2+} 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 (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 x 10^6 conidia and incubated for 6 days at 25°C in the light. Cultures were fertilized with ten 10 µl drops of a conidial suspension (2.5 x 10^6 conidia/drop) from the opposite mating type and incubation was continued. Between 12-14 days, the ascospores expelled from perithecia were harvested in sterile water and incubated overnight at room temperature. Ascospores were activated at 60°C during 30 min and plated on Vogel’s solid medium containing 2% L-sorbose, 0.05% fructose and 0.05% glucose and incubated for 24-48 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 during 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 only treated with H₂O₂.
Synchronous development of ascogonia and protoperithecia was induced according to Bistis (5). Briefly, water-agar plates were inoculated on the center with $1 \times 10^3$ conidia and incubated for 4-5 days at 25°C in the light. Next, 4 circles of solid crossing medium (4 mm in diameter) were transferred to the water-agar plate and placed equidistant and near the limits of the colony. Ascogonia and protoperithecia were observed 24 and 48 h later, respectively.

**Plasmid constructions.** nox-1 and nox-2 deletion plasmids were constructed based on the strategy published by Pratt and Aramayo (35). A nox-1 deletion construct was generated as follows: first, a 5' nox-1 region was amplified by PCR, using N. crassa genomic DNA as template and primers 5-NX-1-Up (5'-CCT TTT GCT GAG TTG TCG) and 5-NX1-Lo (5'-TGA ATC TAA TCT TGG). The resulting 2075 bp fragment was cloned into TOPO2.1 vector (Invitrogen, Carlsbad, CA) to generate plasmid pTOKNC1-7. Second, a portion of the nox-1 3' region was amplified with primers 3-NotNx1 (5'-GTT TAG CGG CCG CAA TTT TAG GTT CTG GAA GG) and 3-NxNot (5'-GTT TAG CGG CCG CTC GAA GAT GAT AAC CTG G), which contained a NotI restriction site. The 1814 bp PCR product cloned into TOPO2.1 vector was named pTOKNC-1. The nox-1 5' region derived from pTOKNC1-7 was cloned into pKAD-10Nc and the resulting plasmid was named pNCKA-6. Next, the nox-1 3' region derived form pTOKNC-1 was subcloned into pNCKA-6 to generate pNCKA-8, which was used to transform N. crassa and delete nox-1.
For nox-2 deletion, nox-2 3’ region was amplified using primers NotP3394 (5’-TTT AGC GGC CGC AAC GCC GAT AAG GAT ACC) and NOX-2Nt (5’-TTT AGC GGC CGC TTC ATC CAT TCC ACC ACC). The PCR product (2075 pb) was cloned into the pDLAM89d to obtain plasmid pKAD28. The mat-a1 gene obtained from pRATT25d was cloned into pKAD28 to generate pKAD-23. A nox-2 3.5 kb fragment containing the 5’ region and ORF was amplified with primers KpNOX-2 (5’-CGG GGT ACC TGA ACT GAG GCG ATA ACG) and nox2-2 (5’-TTA CCC AGG CTC ACT ATA C), using genomic DNA as template. This fragment was cloned into TOPO 2.1 vector to obtain pTOKAD-3.5. A 1.5 kb EcoRI fragment from pTOKAD-3.5 containing nox-2 5’ region was subcloned into pKAD23 to generate pKAD9, which was used to delete nox-2.

To delete nor-1, the 5’ and 3’ regions were amplified by PCR using genomic DNA as a template. The nor-1 5’ region was amplified with primers 5’GAPNOXR-UP (5’-GTA ACG CCA GGG TTT TCC CAG TCA CGA CGG CCT ATG TGA ACT CAC AAC C) and 3’GAPNOXR-LOW (5’-ATC CAC TTA ACG TTA CTG AAA TCT CCA ACG ACG ACT CGA GAT TAA CAG C). The 3’ primers were: 5’SIZENOXR-UP (5’-CTC CTT CAA TAT CAT CTT CTG TCT CCG ACC GAC AGA CCT GTA CTT TTG G) and 3’SIZENOXR-LOW (5’-GCG GAT AAC AAT TCC ACA CAG GAA ACA GCG CTG TCA TAG CAT AGC ATC C). The hygromycin resistance gene was amplified by PCR using plasmid pCSN43 as a template and primers hph F and hph R (10). The three PCR-products were mixed and used for fusion PCR with primers 5’GAPNOXR-UP and 3’SIZENOXR-LOW.

A pef-1 replacement construct was generated by double joint PCR, using genomic DNA as template. First, a 5’pef-1 fragment was amplified with primers 5’GAPALG-B (5’-GTA ACG CCA GGG TTT TCC
CAG TCA CGA CGG GAT ACT GTC CAT ACC TAC G) and 3’GAPALG-B
(5’-ATC CAC TTA ACG TTA CTG AAA TCT CCA ACG GAA AGG AGT AAA
GGA GTC G). Second, a 3’ pef-1 fragment was amplified with primers
5’ SIZE ALG-B (5’-CTC CTTCAA TAT CAT CTT CTG TCT CCG ACT CCA
CAG TAT GCT GTC TAC G) and 3’ SIZEALG-B (5’-GCG GAT AAC AAT
TTC ACA CAG GAA ACA GCG AGT AGC AAT GCA TGG AAG C). Third,
the hygromycin resistance gene was amplified by PCR using plasmid
pCSN43 as a template. The three PCR fragments were purified, mixed
and used in a fusion PCR with primers 5’GAPALG-B and 3’ Size ALG-B.

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 (35). For transformation, we used 150 µl of spheroplasts and
7.42 µg of linear plasmid pNCKA-8 or 2-10 µg of linear pKAD9. The
transformation mixture was mixed with warm selective top-agar
(Vogel’s salts with 1M sorbitol, 0.05% glucose, 0.05% fructose, 1.0%
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.

Strain ∆mus-51 (32) was also used for transformation. 50 µl of a
suspension of conidia (1.25 X 10⁸) 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. 40 µl of this mixture were transferred
to 0.2 cm electroporation cuvettes and electroporated using 1.5 kV,
600 Ohms and 2.5 µF in Gene Pulser II and Pulse Controller II (Bio-
Rad, Hercules, CA). After electroporation, 960 µl of 1M cold sorbitol were added, mixed with 25 ml of recovery solution (Vogel’s medium with 2% yeast extract) and incubated at 30°C for 2 h. This solution was mixed with 25 ml of regeneration agar (Vogel’s medium with 1M sorbitol 2% yeast extract and 1% agar), and plated immediately on solid Vogel with L-sorbose, fructose, 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 10^7 conidia were inoculated in liquid supplemented Vogel’s medium and grown for 48 h at 30°C without shaking. Mycelium was frozen in liquid nitrogen, lyophilized and ground with mortar and pestle under liquid nitrogen. Genomic DNA was extracted according to Timberlake et al., (42). 7-10 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 mortar and pestle under liquid nitrogen. Total RNA was isolated with TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer instructions. 10-12 µg of RNA were separated in a 1% agarose gel containing formaldehyde, transferred to Hybond-N membranes and hybridized with nox-1 ORF and nox-2 ORF specific probes.
Results

*N. crassa* nox-1 and nox-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-C) within the fungi (1, 24). The analysis of the *N. crassa* genome sequence (16) indicated that this fungus contained two different *nox* genes, which we designated nox-1 and nox-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 (Fig. S1). Sharing an identity of only 36%, NOX-1 and NOX-2 belong to the NoxA and NoxB subfamilies, respectively. As other NoxB members, NOX-2 contains a N-terminal extension of unknown function (Fig. S1). To characterize NOX functions in *N. crassa*, we first analyzed the pattern of *nox* mRNA accumulation at different developmental stages. As shown in Fig. 1A, *nox-1* mRNA was not detected in conidia, accumulated between 8-14 h of growth in liquid culture and decreased between 20-24 h. In contrast, *nox-2* mRNA was present in conidia, maintained similar levels between 8-14 h and showed high accumulation levels between 20-24 h. During sexual development (Fig. 1B), *nox-1* started to accumulate around 120 h, peaked at 144 h and decreased at 192 h. Under these conditions, the highest *nox-1* mRNA levels coincided with the formation of the female sexual structures or protoperithecia (shown in Figs. 2B and 6C), while *nox-2* mRNA was only detected before protoperithecia development. These results suggest that NOX-1 functions might be important during growth and sexual differentiation,
while NOX-2 functions could be associated to conidiospore 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 (Fig. S2). Although the resulting mutants showed several phenotypes (see below), we first analyzed them for sexual development defects. Results in Fig. 2 show that ∆nox-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 ∆nox-1 mutants were used as the recipient or female sexual partner. Indeed, ∆nox-1 conidia were able to function as 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 ∆nox-1 mutants were able to differentiate the female sexual structures or protoperithecia. Consistent with their female sterility, we found that ∆nox-1 mutants were incapable of forming any protoperithecia (Fig. 2B). However, ∆nox-1 mutants were able to develop ascogonia (Fig. 2C, left), which is the first recognizable stage in protoperithecia 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 nox-2 ORF (Fig. S3). Δnox-2 mutants did not show any obvious defect in cell growth, 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 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 recover 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 (Fig. S4). 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 (Fig. S5), 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 the one observed in Δnox-1 mutants. Indeed, Δnor-1 mutants failed to differentiate protoperithecia, but were able to develop ascogonia (Fig. 4A). These results supports NOR-1 role 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 non-selective 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 protoperithecia 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 when 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 that ROS levels regulate asexual development, we decided to examine the extent of asexual development in Δnox-1, Δnox-2, Δnor-1 and Δnox-1 cat-3RIP mutants. We found that, as reported, cat-3RIP mutants produced denser aerial mycelium and increased conidiation, as compared to the wild type strain (Fig. 5A-B). In contrast, Δnox-1 mutants not only formed shorter aerial mycelium but also produced lower than wild type conidiation yields. Asexual development was not affected in the Δnox-2 mutant. The fact that a Δnor-1 mutant shared Δnox-1 phenotypes (Fig. 5A-B) further supports the essential role of NOR-1 in NOX-1 activation. Asexual development in the Δnox-1 cat-3RIP double mutant was similar to the one observed for the Δnox-1 single mutant, indicating that NOX-1 activity is necessary for increased asexual development in cat-3RIP mutants.

To determine whether ROS metabolism could have general effects on mycelial growth, we determined the growth rate of Δnox-1, Δnor-1, cat-3RIP and Δnox-1 cat-3RIP mutants in race tubes. As shown in Fig. 5C, Δnox-1, Δnor-1 and Δnox-1 cat-3RIP mutants showed a clear and similar reduction in mycelial extension rate. In contrast, the growth
rate of cat-3<sup>RIP</sup> mutants was similar to 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 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 *D. discoideum*, respectively, are required for cell aggregation and asexual sporulation. Remarkably, the elimination of ALG-2B restored normal development in both noxA and 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 *Saccharomyces cerevisiae*, is involved in cell budding and polarization (47) but peflin function in filamentous fungi was unknown. In *N. crassa*, we identified hypothetical protein NCU02738, as the only ALG-2 ortholog in this fungus and designated it as 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 (Fig. S6). We deleted the *pef-1* gene, to evaluate its function in *N. crassa* and possible interactions with *nox-1*. A *pef-1* deletion construct, based on hygromycin resistance, was generated by PCR and used to transform strain ∆*mus-51* (32). Purified transformants were analyzed by Southern blot using restriction enzyme *PvuI* (Fig. S7). Strain pef-1.9a was selected out of four transformants with the correct *pef-1* deletion event, and used in further experiments and sexual crosses. ∆*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 ∆*pef-1* mutant produced 65%
more conidia than the wild type strain, despite that both strains formed similar amounts of aerial mycelium (Fig. 6A-B).

It has been shown that *S. cerevisiae* Pef1p binds calcium and Zinc *in vitro* and that *pef1* disruption causes defective growth in SDS or cation depleted medium (47). However, we plated Δ*pef-1* conidia on medium containing 0.005% SDS or 0, 10, 20 and 30 mM EGTA and found no significant differences in colony size after 48 h, as compared to the wild type strain (not shown). In summary, elimination of the only peflin present in *N. crassa* did not produce any clear phenotype, except for an increase in conidiation.

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

**Discussion**

**NOX-1** is required for sexual and asexual development, and normal hyphal growth, while **NOX-2** seems specifically involved in sexual spore function. Previous work indicates that NOX-1 orthologs play rather specific roles in fungal development. **NoxA** inactivation in *A. nidulans* results in complete arrest of sexual development, without notably affecting growth (24). Likewise, lack of PaNox-1 in *P. anserina* causes a major reduction in the number of protoperithecia and fruiting bodies but does not affect growth rate (29). **noxA** mutants in the symbiotic fungus *E. festucae* grow normally in culture, but show unregulated and increased growth in its plant host (41). **nox1** mutants in the plant pathogen *M. grisea* show a slight increase in radial growth and fail to penetrate its plant host (12), while *Claviceps purpurea* **Cpnox1** mutants grow normally, despite showing decreased germination and defective colonization of plant ovarian tissue (17).

As we report here, *N. crassa* **NOX-1** is not only essential for protoperithecia 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 **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 *N. crassa* (21). Interestingly, the first peak of ROS detected during aggregation of hyphae, 30 min after mycelia is exposed to air (21), coincided with increased **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 Δnox-1 cat-3\textsuperscript{RIP} double mutant was similar to the one observed for the Δnox-1 single mutant suggests that NOX-1 derived ROS are required for the increase in asexual development observed in cat-3 mutants. Although ROS are difficult to be specifically detected at hyphal tips and have not yet been involved in regulation of growth in N. crassa, our results indicate that 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 NOX-2 were not viable or able to germinate, suggesting that ROS might be required for spore germination. However, mutants lacking NOX-1 or 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 D. discoideum, noxA, 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 substitute PaNOX1 functions (29). Botrytis cinerea bcnoxA and bcnoxB are both required for sclerotia 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 manuscript
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, Rac subunit
seems essential for NOX activation in fungi (40). As it 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 MAP kinase signaling has been involved in NOX regulation (24), it is
interesting to note that N. crassa mutants lacking NOR-1 or the MAP
kinase MAK-2 share several phenotypes: i.e. show reduced growth
rate, produce short aerial hyphae, fail to develop protoperithecia and
produce unveivable 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 ∆nox-1 mutants. In mammalian
cells peflins such as ALG-2 have been shown to bind Ca\(^{2+}\) 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 ∆pef-1 mutants
showed higher conidiation, an effect that cannot be explained at this
time.

As mutation of PEF-1 ortholog ALG-2 bypassed NoxA and NoxB
requirement for asexual sporulation in D. discoideum (25), this
supported a cross talk between ROS and Ca\(^{2+}\) mobilization as it has
been observed in 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 don’t know how NOX-1 might
regulate polar growth in N. crassa, it is interesting to note that polar
growth in A. 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.
Acknowledgments

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Table 1. Neurospora crassa strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>74-OR23-1A</td>
<td>mat A</td>
<td>FGSC#987</td>
</tr>
<tr>
<td>74-OR8-1a</td>
<td>mat a</td>
<td>FGSC#988</td>
</tr>
<tr>
<td>∆mus-51</td>
<td>mus-51^Δ::bar^+ mat a</td>
<td>FGSC# 9718</td>
</tr>
<tr>
<td>RPNCR3A</td>
<td>mep^R^ his-3 In^A^ (SacI-BglII)^::A</td>
<td>Pratt and Aramayo (2002)</td>
</tr>
<tr>
<td>TKA1b</td>
<td>mep^R^ his-3 In^A^ (SacI-BglII)^::A nox-2^Δ::hph^+ mat A</td>
<td>This work, RPNCR3A transformed with pKAD9</td>
</tr>
<tr>
<td>TCNB2</td>
<td>mep^R^ his-3 In^A^ (SacI-BglII)^::A nox-1^Δ::hph^+ mat A</td>
<td>This work, RPNCR3A transformed with pNCKA8</td>
</tr>
<tr>
<td>TCNB10</td>
<td>mep^R^ his-3 In^A^ (SacI-BglII)^::A ∆nox-1^Δ::hph^+ mat A</td>
<td>This work, ∆mus-51 transformed with pNCKA8</td>
</tr>
<tr>
<td>H10.1</td>
<td>nox-1^Δ::hph^+ mat A</td>
<td>This work, progeny from TCNB10 X 74-OR8-1a</td>
</tr>
<tr>
<td>H10.17</td>
<td>nox-1^Δ::hph^+ mat A</td>
<td>This work, progeny from TCNB10 X 74-OR8-1a</td>
</tr>
<tr>
<td>CNCKA-Z</td>
<td>his-3::λA nox-2^Δ::hph^+ mat A</td>
<td>This work, progeny from TKA1b X 74-OR8-1a</td>
</tr>
<tr>
<td>CNCKA-AN1</td>
<td>In^A^ (SacI-BglII)^::A nox-2^Δ::hph^+ mat A</td>
<td>This work, progeny from TKA1b X 74-OR8-1a</td>
</tr>
<tr>
<td>Nc28nor-1</td>
<td>nor-1^Δ::hph^+ mus-51^Δ::bar^+ mat A</td>
<td>This work, ∆mus-51 transformed with nor-1 deletion PCR product</td>
</tr>
<tr>
<td>∆pef-1.9a</td>
<td>pef-1^Δ::hph^+ mus-51^Δ::bar^+ mat a</td>
<td>This work, ∆mus-51 transformed with pef-1 deletion PCR product</td>
</tr>
<tr>
<td>A9N1.10</td>
<td>pef-1^Δ::hph^+ nox-1^Δ::hph^+ mat a</td>
<td>This work. Progeny from ∆pef-1.9a X H10.17 (nox-1^Δ)</td>
</tr>
<tr>
<td>cat-3^RIP</td>
<td>cat-3^RIP mat a</td>
<td>Michán et al., (2003).</td>
</tr>
<tr>
<td>cat-3^RIP-A</td>
<td>cat-3^RIP mat A</td>
<td>This work, progeny from cat-3^RIP X 74-OR23-1A</td>
</tr>
<tr>
<td>C3H10.A10</td>
<td>cat-3^RIP nox-1^Δ::hph^+ mat a</td>
<td>This work, progeny from cat-3^RIP-A X H10.1</td>
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**Figure legends**

FIG. 1. The NADPH oxidase genes *nox-1* and *nox-2* are differentially expressed during growth and sexual development. (A) RNA was extracted from conidia, mycelia from wild type strain 74-ORS23-1A grown in liquid culture or (B) mycelia induced to undergo sexual development for the indicated time (hours), was hybridized with *nox-1* or *nox-2* specific probes. Lower panels show rRNA as loading reference. To induce sexual development, conidia were grown on plates with solid crossing medium covered with a cellophane membrane and samples were harvested at the indicated times.

FIG. 2. NOX-1 is essential for protoperithecia but not for ascogonia development. (A) Conidia from wild type strains 74-OR23-1A or 74-OR8-1a and Δnox-1 strains H10.1 (mat a) or H10.17 (mat A) were inoculated on crossing medium and grown for 6 days to be used as female partners, which were fertilized with WT or Δnox-1 strains from the opposite mating type. Pictures were taken 13 days after fertilization. The black round structures correspond to mature perithecia. (B) The same strains were grown under the identical conditions for 10 days and protoperithecia (arrowheads) development was observed under a stereoscopic microscope. (C) Ascogonia (arrows) and protoperithecia (arrowheads) development was induced according to Bistis (1983). Briefly, conidia from strains 74-OR23-1A and H10.1 were inoculated onto water-agar medium and after 4 days, 4 pieces of solid crossing medium were placed close to growing mycelia and incubation was continued for 24 h. NBT staining was carried out by flooding the plates with a water solution of 2.5 mM NBT during 30 min. Pictures were taken under a stereoscopic microscope.
FIG. 3. The ascospores from \( \Delta \text{nox-2} \) homozygous crosses are non-viable. Strains CNCKA-Z (\( \Delta \text{nox-2} \) A) and CNCKA-AN1 (\( \Delta \text{nox-2} \) a) were crossed and allowed to produce ascospores. Shot ascospores were collected as water suspensions, heat activated, plated on sorbose-containing medium and incubated for 3 days (top panels) or incubated in liquid minimal medium and observed under the microscope (lower panels; magnified 1250X). Only ascospores generated by the wild type strains were able to germinate in and generate colonies (arrowheads).

Fig. 4. Mutants lacking putative NOX regulatory subunit NOR-1 share \( \Delta \text{nox-1} \) and \( \Delta \text{nox-2} \) sexual phenotypes. (A) Strains 74-OR8-1a (WT) and Nc28nor-1 (\( \Delta \text{nor-1} \)) were induced to develop ascogonia and protoperithecia (arrowheads). (B) Ascospores from crosses 74-OR23-1A X 74-OR8-1a, 74-OR8-1a X Nc28nor-1 (\( \Delta \text{nor-1} \)) and CNCKA-Z (\( \Delta \text{nox-2} \)) X Nc28nor-1 (\( \Delta \text{nor-1} \)) were collected and plated as in Fig. 3. The few ascospores from the \( \Delta \text{nox-2} \) X \( \Delta \text{nor-1} \) cross that were able to germinate and generate colonies (arrowheads) were sensitive to hygromycin and therefore carried \( \text{nox-2} \) and \( \text{nor-1} \) wild type alleles.

FIG. 5. CAT-3, NOX-1 and NOR-1 regulate asexual development and/or hyphal growth. (A) The lack of CAT-3 or NOX-1/NOR-1 has opposite effects on the formation of aerial mycelium and conidiation (B). (C) \( \Delta \text{nox-1} \) and \( \Delta \text{nor-1} \) mutants show a drastic reduction in hyphal extension rate, which is not compensated by mutation of \( \text{cat-3} \). Strains 74-OR8-1a (WT), \( \text{cat-3}^{\text{RIP}} \), H10.1 (\( \Delta \text{nox-1} \)), CNCKA-AN1 (\( \Delta \text{nox-2} \) a), Nc28nor-1 (\( \Delta \text{nor-1} \)) and C3H10.1-10 (\( \text{cat-3}^{\text{RIP}} \) \( \Delta \text{nox-1} \)) were inoculated and grown at 30°C for 3 days in the dark, plus 2 days in the
light. Conidia were harvested as water suspensions and counted. Conidia numbers are mean values from four independent samples. Race tube assays were started with mycelial plugs and conducted under constant darkness. Growth was marked (vertical black lines) after 24, 48 and 72 h at 30°C.

FIG. 6. Inactivation of peflin PEF-1 does not suppress the developmental and growth defects of Δnox-1 mutants. (A) Strains 74-OR8-1a (WT), H10.1 (Δnox-1), Δpef-19a (Δpef-1) and A9N1.10 (Δpef-1 Δnox-1) were inoculated and conidia were counted (B) as indicated in Fig. 5. (C) Protoperithecia development was induced as indicated in Fig. 2 and samples were processed for scanning electron microscopy. (D) Race tube cultures were treated as in Fig. 5.


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