Nox Complex signal and MAPK cascade pathway are cross-linked and essential for pathogenicity and conidiation of mycoparasite *Coniothyrium minitans*

Wei Wei¹,²,†, Wenjun Zhu¹,², Jiasen Cheng², Jiatao Xie², Daohong Jiang¹,², Guoqing Li¹,², Weidong Chen³ & Yanping Fu²

The NADPH oxidase complex of a sclerotial mycoparasite *Coniothyrium minitans*, an important biocontrol agent against crop diseases caused by *Sclerotinia sclerotiorum*, was identified and its functions involved in conidiation and mycoparasitism were studied. Gene knock-out and complementary experiments indicated that *CmNox1*, but not *CmNox2*, is necessary for conidiation and parasitism, and its expression could be significantly induced by its host fungus. *CmNox1* is regulated by *CmRac1-CmNoxR* and interacts with *CmSlt2*, a homolog of *Saccharomyces cerevisiae* *Slt2* encoding cell wall integrity-related MAP kinase. In Δ*CmNox1*, *CmSlt2-GFP* fusion protein lost the ability to localize to the cell nucleus accurately. The defect of conidiation in Δ*CmRac1* could be partially restored by over-expressing *CmSlt2*, indicating that *CmSlt2* was a downstream regulatory factor of *CmNox1* and was involved in conidiation and parasitism. The expressions of mycoparasitism-related genes *CmPks1*, *Cmg1* and *CH1* were suppressed in the knock-out mutants of the genes in *CmNox1-CmSlt2* signal pathway when cultivated either on PDA. Therefore, our study infers that *CmRac1-CmNoxR* regulates *CmNox1-CmSlt2* pathway in regulating conidiation and pathogenicity of *C. minitans*.

*Sclerotinia sclerotiorum* (Lib.) de Bary is a significant necrotrophic pathogen that could infect more than 400 plant species worldwide and cause huge economic losses every year¹,². *Coniothyrium minitans* is an important mycoparasite of *Sclerotinia* spp.; it could parasitize and destroy both sclerotium and hypha of its hosts. It is an effective agent for controlling crop diseases caused by *S. sclerotiorum*³–⁵. Like its hosts, *C. minitans* also occurs widely⁶. It shares the same growing season as its hosts, and its growth and proliferation are tightly related to the hosts. Without host fungi, *C. minitans* stays dormant for seasons in soil⁷. *C. minitans* is one of the commercialized fungal agents for biological control of crop diseases⁸.

Understanding the parasitism and conidiation of *C. minitans* at molecular level could help us to utilize this biological control agent more efficiently. Studies indicated that the primary cell wall degrading enzymes secreted by *C. minitans* are β-1,3 glucanase (encoded by *Cmg1*) and chitinase (encoded by *CH1*). The expression level of *Cmg1* was enhanced when *C. minitans* parasitized *S. sclerotiorum*, indicating that β-1,3 glucanase played important roles in the parasitical process⁹. *C. minitans* also could secrete antifungal substances and degrade oxalic acid, an essential pathogenicity factor of *S. sclerotiorum*¹⁰,¹¹. The parasitism of *C. minitans* is likely very complicated¹², and shares some signal transduction pathways with conidiation. Fungal cell wall integrity-associated MAP kinase cascade, fatty acid beta-oxidation, reactive oxygen and nitrogen species, and possibly other unknown pathways in peroxisomes are required for both conidiation and mycoparasitism of *C. minitans*¹³,¹⁴. Recent research revealed that...

¹State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, Hubei Province, P R China. ²The Provincial Key Lab of Plant Pathology of Hubei Province, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, 430070, Hubei Province, P R China. ³United States Department of Agriculture, Agricultural Research Service, Washington State University, Pullman, WA, USA. ⁴Present address: Institute for Interdisciplinary Research, Jianghan University, Wuhan 430056, Hubei Province, P R China. Correspondence and requests for materials should be addressed to Y.F. (email: yanpingfu@mail.hzau.edu.cn)
C. minitans can regulate ambient pH by degrading oxalic acid to facilitate mycoparasitism of S. sclerotiorum\cite{15,16}. C. minitans requires a large amount of L-arginine during conidiation and L-arginine-derived nitric oxide was likely to be involved in conidiation with cyclic GMP functions as a second messenger\cite{17,18}. Qin et al. found that phosphoribosylamidotransferase is essential for conidiation of C. minitans via adenosine related molecules, and C. minitans is able to obtain adenosine or related components from its host during parasitization\cite{19}.

The NADPH oxidase complexes are conserved and play important roles in the life cycle of filamentous fungi\cite{20}. In rice blast pathogen Magnaporthe oryzae, NADPH oxidase-derived reactive oxygen species (ROS) is essential for pathogenicity\cite{21}, and further study found that NADPH oxidases are necessary for septin-mediated reorientation of the F-actin cytoskeleton to facilitate cuticle rupture and plant cell invasion\cite{22}. NADPH oxidases are involved in sclerotial formation and pathogenicity of necrotrophic fungal pathogen Botrytis cinerea and S. sclerotiorum\cite{23,24}, and fusion of conidial anastomosis tubes of B. cinerea\cite{25}. ROS is critical in maintaining a mutualistic interaction between Epichloë festucae and perennial ryegrass\cite{26–28}. In saprophytic fungi, such as Aspergillus spp., Neurospora crassa and Podospora anserina, NADPH oxidases are required for growth, cell differentiation, conidiation, and sexual reproduction\cite{29–31}. Trichoderma harzianum NADPH oxidases are involved in the antagonism against Pythium ultimum\cite{32}.

Besides NADPH oxidases mitogen-activated protein kinases (MAPKs) also play critical roles in pathogenicity and in fungal development\cite{33–35}. In endophytic fungi, a stress-activated mitogen-activated protein kinase (sakA) of E. festucae is essential to maintain mutualistic symbiosis with perennial ryegrass. Deletion of sakA converted this endophytic fungus to a pathogen of its host\cite{36}. MAP kinase cascade also is involved in the mycoparasitism and development of hyperparasitic fungi, such as C. minitans, Stachybotrys eogens and T. artoviride\cite{37,38}. The phenotype of ΔsakA mutants are very similar to that of mutants whose genes in the Nox complex were disrupted, suggesting that there is a possible link between ROS signal and MAPK signal pathway on the maintenance of mutualistic symbiosis\cite{39}. Similar phenomena were observed in tangerine pathogen Alternaria alternata\cite{40}; Medina-Castellanos et al. found that extracellular ATP could promote the Nox1-derived ROS and activate a MAPK pathway in T. artoviride\cite{41}. Recently, Jaimez-Arroyo et al. found that SrkA kinase could regulate stress responses and development in A. nidulans, and H2O2 could induce mitochondrial fragmentation and relocalize SrkA at the presence of SakA\cite{42}.

Previously, we investigated a fungal cell wall integrity-associated MAP kinase cascade in C. minitans and found that this cascade was required for conidiation and mycoparasitism\cite{43}. In this study, we analyzed the function of NADPH oxidases (Nox1/Nox2) complex of C. minitans, and found that CmNox1 played critical roles in conidiation and mycoparasitism, but not CmNox2. In C. minitans, CmNoxR interacts with CmRac1 to activate CmNox1, CmNox1 could also interact with CmSh2 and thus adjust its location to the cell nucleus, and deliver the signal of conidiation and mycoparasitism.

**Results**

**NADPH oxidases (Nox1/Nox2) in C. minitans.** Two NADPH oxidase genes, CmNox1 and CmNox2, were isolated from C. minitans. The deduced amino acid sequence of CmNox1 (GenBank Accession No: KJ596434) shows high similarity to Nox1 homologs from other filamentous fungi, including Alternaria alternata (AaNox1, BAK52327.1, 89% identity), Curvularia lunata (CINOX1, AHC53982.1, 89% identity), and Pyrenophora tritici-repentis (PrNOX1, XP_001935118.1, 88% identity). CmNox2 (GenBank Accession No: KJ596435) is highly similar to Nox2 homologs from other filamentous fungi. The multiple alignment analysis showed that both CmNox1 and CmNox2 contained NOX family signature regions. Phylogenetic analysis of NADPH oxidases in several fungi placed CmNox1 and CmNox2 homologs into two different clades based on the amino acid sequences (Supplementary Figure S1).

**CmNox1 is essential for ROS production and conidiation.** To study the function of CmNox1 in C. minitans, a replacement vector p3300neoCmNox1 (see Supplementary Figure S2a) was constructed and transformed into strain ZS-1 to disrupt CmNox1. Twenty transformants were obtained, and three of which, ΔCmNox1-1, ΔCmNox1-6, and ΔCmNox1-107 were selected randomly as candidates for further analyses. Furthermore, a complement vector pNox1 was transformed into mutant ΔCmNox1-6. The deletion and complementary events were confirmed by RT-PCR (see Supplementary Figure S2b) and Southern blot analysis (see Supplementary Figure S2c). Using the same methods, a replacement vector p3300neoCmNox2 was constructed (see Supplementary Figure S2a) and transformed into strain ZS-1 to disrupt CmNox2. Three deletion mutants ΔCmNox2-20, ΔCmNox2-323 and ΔCmNox2-347 were also confirmed by Southern blot analysis.

Colony staining with NBT solution showed that superoxide production was decreased significantly in ΔCmNox1-6, compared to ΔCmNox2-323 and the wild-type strain ZS-1 (Fig. 1a). The conidiation of the wild-type strain ZS-1, CmNox1 deletion mutants, CmNox1 complemented mutants, and CmNox2 deletion mutants were determined after incubating for 15 days on PDA (Fig. 1b). CmNox1 deletion mutants completely lost the ability to produce conidia (Table 1). In contrast, the wild-type strain ZS-1, CmNox1 complemented mutants and CmNox2 deletion mutants were normal in conidiation under the same condition (Table 1). Unlike strain ZS-1 and ΔCmNox2-323, which could form matured pycnidia and conidia, ΔCmNox1-6 could only form a few pycnidial primordia that could not further develop to mature pycnidia, and no conidium was produced (Fig. 1b). These data indicated that CmNox1, but not CmNox2, played significant roles in conidiation and production of superoxide. The experiments also suggested CmNox1 and CmNox2 are not essential for hyphal growth of C. minitans on PDA.

**CmNox1 is essential to parasitize sclerotia of S. sclerotiorum.** To determine whether CmNox1 is related to sclerotial parasitizing, the parasitic ability of CmNox1 mutants and the other strains to sclerotia of S. sclerotiorum were examined. The hyphae of ΔCmNox1-6 was inoculated on to sclerotia and incubated for 30
days at 20 °C, and no pycnidia and conidia were observed on either the surface or interior of the sclerotia (Fig. 2a, Table 1). The inoculated sclerotia were surface sterilized and then incubated on PDA containing 50 μg/ml hygromycin, and no *C. minitans* colony emerged from the sclerotia, suggesting that Δ*CmNox1-6* did not invade the inner of sclerotia (Fig. 2b, Table 1). Meanwhile, *CmNox1-C8*, Δ*CmNox2-323* and the wild-type strain ZS-1 could degrade sclerotia and produce mature pycnidia there. The results demonstrated *CmNox1* played a significant role in sclerotial mycoparasitism.

**Expression of CmNox1 is highly induced by S. sclerotiorum.** When dual cultured with *S. sclerotiorum*, the expression of *CmNox1* was highly induced. Compared to growing on PDA, the expression of *CmNox1* peaked at 12 hpi, and the high expression was maintained till 24 hpi, and then sharply declined to undetectable level at 36 hpi (Fig. 3a). This phenomenon suggested that *CmNox1* played an important role in parasitism. Expression of *CmNox2* also was up-regulated slightly by *S. sclerotiorum* at 12 hpi and 24 hpi, but compared to culture on PDA, the induction was much lower (Fig. 3b). The results showed that *S. sclerotiorum* could induce the expression of *CmNox1* and *CmNox2*, with stronger induction to *CmNox1*.

**CmRac1 interacts with CmNoxR together to regulate CmNox1 and to control conidiation in C. minitans.** It is reported that Bem1 and Cdc24 are components of the NADPH oxidase complex in filamentous fungi, and NoxR interacting with RacA in vitro together regulates ROS production and control hyphal branching and patterning in *E. festucae*7,29. Since *CmNox1* is involved in the production ROS in *C. minitans*, we identified *C. minitans* homologs of NoxR, GTPase Rac1 and Bem1, named *CmNoxR* (GenBank Accession No: KJ596436), *CmRac1* (GenBank Accession No: KJ596436) and *CmBem1*, respectively. The interaction between *CmRac1* and *CmNoxR*, *CmBem1* and *CmNoxR* were confirmed by Yeast two-hybrid (Fig. 3c,d).

To investigate the role of *CmNoxR*, *CmBem1* and *CmRac1* in *C. minitans*, replacement vector for *CmNoxR* (p3300neo*CmNoxR*), *CmRac1* (p3300neo*CmRac1*), and *CmBem1* (p3300neo*CmBem1*) were constructed and transformed into ZS-1. The *CmNoxR* deletion mutants (Δ*CmNoxR-5* and Δ*CmNoxR-107*), *CmBem1* deletion mutant (Δ*CmBem1-344*) and *CmRac1* deletion mutant (Δ*CmRac1-79*) were obtained and confirmed by RT-PCR. In contrast to the wild-type strain ZS-1, the *CmNoxR* deletion mutants and the *CmRac1* deletion mutant lost the ability to produce conidia and to parasitize *S. sclerotiorum*. In addition to the conidiation and parasitizing phenotype, growth rate also was reduced significantly in the *CmRac1* deletion mutant. The growth rate of Δ*CmRac1-79* was less than 0.6 mm/day on average on PDA, while it was about 3mm/day for strain ZS-1 under the same
matically reduced sclerotial mycoparasitism. The phenotype of ΔCmNox1 is essential for conidiation of C. minitans. ΔCmNox1-mediated conidiation and parasitism in C. minitans, were calculated from three replicates.

We expressed the CmSlt2-green fluorescent protein (GFP) fusion in the wild-type strain ZS-1 and ΔCmSlt2. CmSlt2-GFP was observed in the nucleus at any stages. The results suggested that CmSlt2 was a down-stream regulatory factor of CmNox1 and was involved in conidiation and parasitism.

| Strains          | Growth rate (mm/d) | Conidiation (x10^6 conidia/plate) | Rot index (%) |
|------------------|--------------------|-----------------------------------|---------------|
| ZS-1             | 2.9 ± 0.1^a        | 128 ± 7.2^a                       | 82 ± 3^a      |
| ΔCmNox1-1        | 2.8 ± 0.1^a        | 0^a                               | 0^a           |
| ΔCmNox1-6        | 2.8 ± 0.1^a        | 0^a                               | 0^a           |
| ΔCmNox1-107      | 2.9 ± 0.1^a        | 0^a                               | 0^a           |
| CmNox1-C1        | 2.9 ± 0.1^a        | 119 ± 4.8^a                       | 81 ± 2^a      |
| CmNox1-C8        | 2.8 ± 0.1^a        | 121 ± 4.8^a                       | 80 ± 2^a      |
| ΔCmNox2-20       | 2.9 ± 0.1^a        | 120 ± 5.4^a                       | 81 ± 3^a      |
| ΔCmNox2-323      | 2.8 ± 0.1^a        | 122 ± 5.4^a                       | 82 ± 3^a      |
| ΔCmNox2-347      | 2.9 ± 0.1^a        | 125 ± 5.4^a                       | 82 ± 3^a      |
| ΔCmRac1-79       | 0.5 ± 0.1^b        | 0^a                               | 0^a           |
| ΔCmRac1-95       | 0.5 ± 0.1^b        | 0^a                               | 0^a           |
| ΔCmRac1-102      | 0.4 ± 0.1^b        | 0^a                               | 0^a           |
| CmRac1-C1        | 2.9 ± 0.1^a        | 121 ± 9.8^a                       | 81 ± 4^a      |
| CmRac1-C4        | 2.9 ± 0.1^a        | 119 ± 9.8^a                       | 80 ± 4^a      |
| CmRac1-C7        | 2.8 ± 0.1^a        | 120 ± 9.8^a                       | 81 ± 4^a      |
| OVS-Rac1-1       | 0.6 ± 0.1^b        | 0.6 ± 5^a                         | 40 ± 5^a      |
| OVS-Rac1-3       | 0.5 ± 0.1^b        | 0.6 ± 5^a                         | 45 ± 5^a      |
| OVS-Rac1-4       | 0.6 ± 0.1^b        | 0.5 ± 5^a                         | 39 ± 5^a      |
| ΔCmNox1-R-5      | 2.9 ± 0.1^a        | 0^a                               | 0^a           |
| ΔCmNox1-R-107    | 2.8 ± 0.1^a        | 0^a                               | 0^a           |
| ΔCmNox1-R-160    | 2.8 ± 0.1^a        | 0^a                               | 0^a           |
| ΔCmBem1-344      | 2.9 ± 0.1^a        | 124 ± 14.2^a                      | 84 ± 2^a      |
| ΔCmBem1-359      | 2.9 ± 0.1^a        | 125 ± 14.2^a                      | 85 ± 2^a      |
| ΔCmBem1-370      | 2.9 ± 0.1^a        | 120 ± 14.2^a                      | 80 ± 2^a      |

Table 1. Comparison of hyphal growth rate, conidial production, and parasitic ability among mutants and the wild-type strain ZS-1 of C. minitans. Growth rate was detected by measuring the colony diameter of cultures incubated at 20 °C for 7 days. Conidia produced by 14-day-old cultures and counted with a haematocytometer. Rot index of sclerotia was calculated after infected by C. minitans for 30 days. Different letters in the same column indicated statistically significant differences (P = 0.05). Means and standard errors were calculated from three replicates.

CmSlt2 is CmNox1 effector required for conidiation and parasitism. To understand the mechanism of CmNox1-mediated conidiation and parasitism in C. minitans, we further investigated the functional relationship between CmNox1 and CmSlt2. We reported previously that CmShl2 (cell wall integrity-related MAP kinase) is essential for conidiation of C. minitans. CmSlt2 lost the ability to produce pycnidia and conidia, and had dramatically reduced sclerotial mycoparasitism. The phenotype of ΔCmSlt2 was similar to that of ΔCmNox1, thus CmSlt2 and CmNox1 might have somewhat interaction directly or indirectly. To identify whether CmSlt2 interact with CmNox1, full length gene of CmSlt2 and CmNox1 were cloned into yeast two-hybrid vector pGADT7 and pGBDT7 to test the possible interaction, and the results showed that CmSlt2 could interact with CmNox1 (Fig. 4a). Furthermore, we generated fusions of CmSlt2 and CmNox1 with N- and C-terminal domains of YFP and assessed their interaction in tobacco (Nicotiana benthamiana) using bimolecular fluorescence complementation (BiFC) (Fig. 4c). To confirm this interaction in C. minitans, a transformant with TrpC-CmNox1-Flag and TrpC-CmSlt2-GFP and a transformant with TrpC-CmSlt2-GFP and Vector-Flag in ZS-1 were constructed. In Western blot analysis with total protein, the anti-Flag and anti-GFP antibodies detected a 65-kDa and an 80-kDa band, respectively. In proteins eluted from anti-Flag agarose, the 80-kDa CmSlt2-GFP band was detected with an anti-GFP antibody in transformant with CmSlt2 and CmNox1, but not in transformant with CmSlt2 only (Fig. 4b). The results showed that CmSlt2 and CmNox1 interacted directly in C. minitans.

We expressed the CmSlt2-GFP fusion in the wild-type strain ZS-1 and ΔCmNox1, and found that over-expressing CmSlt2-GFP could not rescue the deficiency of conidiation and parasitism in ΔCmNox1 (Fig. 5a,b). In the wild-type strain ZS-1, CmSlt2 was located in the cytoplasm during the hyphal growth period (3-day-old hyphae), and was located in the nucleus in 40% of the hyphae at the later stage of conidiation (5-day-old hyphae) observed under a fluorescence microscope. While in ΔCmNox1, no fluorescence signal of CmSlt2-GFP was observed in the nucleus at any stages (Fig. 5c). The results suggested that CmSlt2 was a down-stream regulatory factor of CmNox1 and was involved in conidiation and parasitism.
Over expressing CmSlt2 in ΔCmRac1 could partially restore conidiation and parasitism. Since CmRac1 interacts with CmNoxR together in regulating CmNox1, and CmNox1 mediates the localization of CmSlt2 in nucleus, we suspected that CmRac1 is likely to regulate the expression of CmSlt2. qRT-PCR analysis showed that the expression of CmSlt2 was obviously decreased in ΔCmRac1 by 0.5 fold, compared to the wild-type strain (Fig. 6a). We fused CmSlt2 with a trpC promoter from Aspergillus and transformed into ΔCmRac1, and found that over-expressing of CmSlt2 could partially restore conidiation and parasitic ability of ΔCmRac1 (Fig. 6b, and Table 1), in spite of no significant improvement of the hyphal growth.

The expression of CmPks1 was suppressed in CmNox1 signal pathway mutants. C. minitans produces dark pigment and black pycnidia during the late stage of growth. However, all ΔCmRac1, ΔCmNoxR, ΔCmNox1, and ΔCmSlt2 produce little dark pigment, and colonies were whitish. It is likely that the expression of CmPks1, a melanin biosynthesis associated polyketide synthase-encoding gene, was suppressed in those mutants. The transcript profile of CmPks1 was monitored by qRT-PCR, and the result showed that the expression of CmPks1 peaked at 96 hpi in the wild type ZS-1 and was significantly suppressed in ΔCmNoxR, ΔCmRac1, ΔCmNox1 and ΔCmSlt2, whereas there was no obvious difference in the transcript level between ΔCmNox2 and ZS-1 (Fig. 7a). Further experiments proved that the expression of CmPks1 in ZS-1 was enhanced by 300–400 fold when dual cultured with S. sclerotiorum than cultured on PDA alone at 24 hpi and 36 hpi (Fig. 7b), while it could not be induced in ΔCmRac1 and ΔCmNox1 (Fig. 7c). These results indicated that the expression of CmPks1 could
be induced at the early stages of interaction with *S. sclerotiorum*, while could not in all of the CmNox1 signal pathway mutants either cultured on PDA or interacted with *S. sclerotiorum*. It suggested that the CmNox1 signal pathway is involved in regulating the expression of CmPks1.

The CmNox1 signal pathway is involved in regulating cell wall degrading enzyme genes. The parasitic ability of all mutants in CmNox1 signal pathways decreased obviously and it is also reported that the cell wall degrading enzyme Cmg1 (β-1,3 glucanase-encoding gene) and CH1 (chitinase gene) play important roles in the process of parasitizing sclerotia of *S. sclerotiorum*. Our results of qRT-PCR demonstrated that the transcript levels of both Cmg1 and CH1 in ZS-1 were enhanced significantly at 24 hpi and 36 hpi when dual cultured with *S. sclerotiorum* (Fig. 7e,h), while were suppressed in ΔCmNoxR, ΔCmRac1, ΔCmNox1 or ΔCmSlt2 when incubated on PDA compared to that in ZS-1 (Fig. 7d,f,g,i). The results indicated that Cmg1 and CH1 were involved in parasitism and could be regulated by the CmNox1 signal pathway.

**Discussion**

The mycoparasitism system of *C. minitans*/*S. sclerotiorum* is unique and important to probe fungi and fungi interaction. In this study, the role of NADPH oxidases complex of *C. minitans* on growth, conidiation and mycoparasitism were studied. We found that CmNox1, but not CmNox2, played essential roles on conidiation and mycoparasitism. Furthermore, we found that CmRac1 could interact with CmNoxR, and CmNox1 could interact with CmSlt2. Expression of CmSlt2 in ΔCmRac1 could partially restore the conidiation and parasitism. These
findings broadened and strengthened our knowledge of mycoparasitism, and supplied a possible link between
Nox complex signal and MAPK cascade signal in fungi.

Fungal Nox isoforms have different roles in fungi lifecycle. In *M. oryzae*, both Nox1 and Nox2 are essential for
pathogenicity21. Nox1 is required for penetration, hyphal elongation, and Nox2 is required for assembly of a toroi-
dal F-actin network during the penetration peg formation22; In *S. sclerotiorum*, Nox1 is required for virulence,
oxalic acid production and sclerotial development, while, Nox2 might be involved in sclerotial development24. In *P. anserine*, *PaNox1* mutants are impaired in the differentiation of fruiting bodies from their progenitor cells, and
deletion of the *PaNox2* specifically blocked ascospore germination33,54. New Nox proteins were also identified from
*P. anserine*, a Nox isoform Nox3 was found to play a minor role36. In *N. crassa*, Nox1 is required for female sterility,
and involved in asexual development and hyphal growth, while Nox2 might be involved in ascospore germination34.
In *E. festucae*, both Δ*NoxA* and Δ*NoxB* mutants could produce conidia, while NoxA, but not NoxB, is essen-
tial for hyphal polarized growth and hyphal fusion, NoxB did not affect conidiation29. In *Sordaria macrospora*,
Nox1 is required for fruiting body formation, normal hyphal growth, and hyphal fusion, while Nox2 is involved in
strict melanin-dependent ascospore germination35. In *C. purpurea*, CpNox1 is essential for infection, but CpNox2
is not essential for infection. Interestingly, Δcnox2 mutants converted endophytic lifestyle to pathogenic lifestyle,
and CpNox2 functioned in the infection process and moderates damage to the host56. In this study, the Nox1 of

Figure 4. Interaction of CmNox1 with CmSlt2. (a) Yeast two-hybrid assay of interaction between CmNox1
and CmSlt2. (b) Co-IP assays. Western blots of total proteins and proteins eluted from anti-Flag agarose from
transformant (*CmSlt2*-GFP and vector Flag) and transformant (*CmSlt2*-GFP and *CmNox1*-Flag) were detected
with anti-Flag or anti-GFP antibodies. (c) Bimolecular fluorescence complementation (BiFC) analysis of the
interaction between CmNox1 and CmSlt2 tagged with YFP in *Nicotiana benthamiana*. 
C. minitans is essential for mycoparasitism, conidiation and pigmentation, while deletion of Nox2 did not affect these bioprocesses. However, how Nox1 regulates these bioprocesses is necessary to be unraveled.

Rac1, a member of the Rho-family GTPases, widely exists in eukaryotes and in fungi as well. Sequence alignment of RAC1 homologs from fungi revealed significant conservation in amino acid composition and RAC1 from *M. grisea* could rescue the conidiation, parasitization and growth in Δ*Rac1* mutant of *C. minitans* (data not shown). Fungal Rac1 is crucial for the growth, virulence and development in many fungi, such as *M. grisea, U. maydis, A. fumigatus, Claviceps purpurea* and *F. graminearum*57–61. In *M. grisea*, MgRac1 is essential
for conidiogenesis, and contributes to the formation of appressorium and pathogenicity through activating its downstream factors: the PAK kinase Chm1 and NADPH oxidases\(^6\). In *Candida albicans*, Rac1 is an upstream regulatory factor of the MAP kinase Cek1 and Mkc1, but the control mechanism is still unclear\(^6\). In this study, the qRT-PCR assay demonstrated that the expression level of *CmSlt2* was obviously decreased in Δ*CmRac1*. When *CmSlt2* was over expressed in Δ*CmRac1*, the conidiation could be partially restored, further confirming that Rac1 was an upstream regulatory factor of MAPK cascade signal pathway.

The fact that the Nox complex mutants and the MAPK cascade pathway mutants of fungi sharing highly similar phenotypes suggests that these two signal pathways are cross-linked. This study provides further evidence supporting this cross-link since CmNox1 could interact with CmSlt2, and CmRac1 could interact with CmNoxR. Lalucque *et al.* reported that PaNox1 acted on the PaASK1/PaMKK1/PaMpk1 MAPK module by promoting nuclear translocation of the PaMpk1 MAP kinase in *P. anserina*\(^6\). More recently, H\(_2\)O\(_2\) was found to induce the relocalization of a putative MAPK-activated protein kinase SrkA to nuclei and mitochondria under the presence of SakA in *A. nidulans*\(^5\). We further found that CmNox1 could regulate the nucleus location of a cell wall integrity-associated MAP kinase (CmSlt2). In Δ*CmNox1*, CmSlt2 could not move into fungal nuclei, suggesting that Nox complex-derived ROS may function on the localization of CmSlt2.

**Figure 7.** Gene expression analysis in the wild-type strain ZS-1 and deletion mutants of *C. minitans*.
Relative transcript accumulations of *CmPks1* (a), *CH1* (d) and *Cmg1* (g) detected with qRT-PCR amplification in ZS-1, Δ*CmNox2*, Δ*CmSlt2*, Δ*CmNoxR*, Δ*CmRac1* and Δ*CmNox1* after growing on PDA for 96 hr. The expression of ZS-1 was set as level one. Relative transcript patterns of *CmPks1* (b), *CH1* (e) and *Cmg1* (h) in ZS-1 after contacting with *S. sclerotiorum* (red) or growing on PDA (black) for 0–48 hr. The gene expression of ZS-1 inoculated in plate at 0 hr was set as level one. Relative transcript patterns of *CmPks1* (c), *CH1* (f) and *Cmg1* (i) genes in ZS-1 (black), Δ*CmNox1* (red) and Δ*CmRac1* (blue) after growing on PDA for 0–48 hr. The gene expression of ZS-1 at 0 hr was set as level one. The relative level of transcript was calculated by the comparative Ct method. The level of *CmActin* transcript was used to normalize different samples. Bars represent means and standard deviations (three replications).
The Nox complex signal and MAPK cascade signal affect fungal gene expression globally to regulate complicated processes in physiology, pathogenicity and development. In *S. macrospora*, Nox1 affects the expression of genes involved in cytoskeleton remodeling, hyphal fusion, metabolism, and mitochondrial respiration. In *P. anserine*, the expressions of 15% genes were modified in ΔPaMpk1, ΔPaMpk2 and ΔPaNox1, and about 1000 genes were regulated similarly in these three mutants. We attempted to explain how the Nox complex signal and MAPK cascade signal regulate mycoparasitism by monitoring the expression levels of *CmPks1*, *CmG1* and *CH1* in *C. mimitans*. We found that expression of these genes was obviously enhanced when interacted with its host fungus *S. sclerotiorum*, while could not be induced in ΔCmNox1, ΔCmRac1 and ΔCmSlt2, indicating that these three genes were regulated by the Nox complex signal and MAPK cascade signal pathways, thus to affect parasitism. Mycoparasitism of *C. mimitans* is a very complicated process. There are many other genes that are also involved in the mycoparasitism and remain to be investigated.

Briefly, we analyzed the function of Nox complex on mycoparasitism and conidiation of *C. mimitans*, and we found that it is *CmNox1*, but not *CmNox2*, that is required for mycoparasitism and conidiation. We further found that *CmNox1* interacts with MAP kinase *CmSlt2* and affects the location of *CmSlt2*. Our finding suggests that Nox complex signal pathway and MAPK cascade signal pathway are cross-linked via *CmNox1*.

**Methods**

**Strains and cultural conditions.** The *C. mimitans* wild-types train ZS-1 (CCAM 041057) was isolated from garden soil at Zhushan County, Hubei Province, P R China. Strain Ep-1PNA367 was a virulent and virus-free strain of *S. sclerotiorum*, derived from a single ascospore of the hypovirulent strain Ep-1PNA50. The strains used in this research were maintained and cultured on PDA at 20–22 °C and stored in PDA slants at 4 °C. Cultures for genomic DNA and RNA isolation were conducted on PDA at 20–22 °C for 4 d. Conidia were prepared from 15-day-old cultures grown on PDA. The selective PDA was supplemented with 50 μg/ml of hygromycin B (Sigma) or 80 μg/ml of G418 (Sigma), depending on the selection marker in the plasmid vector.

**Analysis of colony morphology, growth rate, conidiation and parasitic ability.** Colony morphology was observed on PDA after incubating at 20 °C for 12 days. Observation of the pycnidal formation was performed by following the method described. To characterize the biological properties of the mutants, growth rate, conidial production and parasitic ability were examined as described. For all the transformants obtained, three individuals were examined (Table 1).

**ROS detection assay.** Production of superoxide was evaluated with NBT using the method modified from Chen et al. The wild-type ZS-1, mutants ΔCmNox1 and ΔCmSlt2 were grown on PDA for 7 days. Mycelia were incubated in 0.05 M sodium phosphate buffer, pH 7.5, containing 0.05% (w/v) NBT (Sigma–Aldrich). After 1 h of incubation, the culture was fixed in ethanol to stop the reaction. The stained sample was examined with a compound microscope at ×400 magnification.

**Isolation of *CmNox1*/*CmNox2.** A PCR primer pair 1F1/1R1 (Table S1) was designed based on the *C. mimitans* genome database. *CmNox1* was amplified from the ZS-1 genomic DNA by a 32-cycle PCR reaction (94 °C, 1 min; 58 °C, 1 min; 72 °C, 2 min), followed by a 10 min extension at 72 °C. The PCR product was cloned into the pMD18-T vector (TaKaRa) and confirmed by DNA sequencing. The cDNA of *CmNox1* was isolated by RT-PCR from total RNA of *C. mimitans* and direct DNA sequencing. The same method was used to clone *CmNox2* in *C. mimitans* with the primer pair 1F1 and 1R1, followed by cloning into the pMD18-T vector and direct DNA sequencing. The same method was used to clone *CmNox2* in *C. mimitans* with the primer pair 2F1/2R1 (Table S1).

**Vector construction and Agrobacterium-mediated transformation.** The *CmNoxR*, *CmRac1*, *CmNox1*, *CmNox2* and *CmBem1* replacement constructs were generated according to Qin et al. The *CmNox1* (primer pairs 9F1/9R1 and 10F1/10R1), *CmNox2* (primer pairs 11F1/11R1 and 12F1/12R1), *CmRac1* (primer pairs 13F1/13R1 and 14F1/14R1), *CmBem1* (primer pairs 15F1/15R1 and 16F1/16R1), and *CmSlt2* (primer pairs 17F1/17R1 and 18F1/18R1) deletion constructs were made by PCR amplification of the 5′- and 3′-flanks of the respective ORFs (0.8–1.2 kb) with genomic DNA of the wild-type strain ZS-1 as a template. The fragments were cloned upstream and downstream of *hph* cassette in pMD18 respectively, and then the structures were cloned into the corresponding sites of vector pneo-P3300III, which carries a neomycin resistance gene cassette (neo) as the second selection marker.

In order to construct the complementary vector of *CmNox1*, a 2.7 kb fragment containing the native promoter and ORF of *CmNox1* was amplified by PCR with primer pair 19F1/19R1 (Table S1) and cloned into vector pneoP3300. The complementary mutant *CmNox1*-C was generated by introducing the vector into ΔCmNox1-6, followed by screening for neomycin resistance and RT-PCR confirmation.

The cDNA of *CmSlt2* was amplified by RT-PCR with primer pair 20F/20R (Table S1) and cloned into the *HindIII/Smal* sites of pCIT vector, which contains the trpC promoter and terminator, and then the gene cassette was cloned into vector pneoP3300 to get the *CmSlt2* over-expression vector pOVSlt2. The over-expression mutant OVS-Rac1-1 was generated by introducing pOVSlt2 into ΔCmRac1-79, followed by screening for neomycin resistance and qRT-PCR confirmation.

To tag *CmSlt2* with the GFP, cDNA of *CmSlt2* without stop codon was amplified by RT-PCR with primer pair 20F/20R (Table S1) and cloned into the *HindIII/Smal* sites of pCIT vector. The GFP coding sequence was excised from plasmid pEGFP-1 with *SmaI/BamHI*, and the corresponding fragment was purified. Both the *CmSlt2* and GFP coding sequences were cloned into vector pneoP3300 digested with *XhoI* to yield plasmid pSGFP1. Mutants were generated by introducing of pSGFP1 into ΔCmNox1-6 and the wide-type strain ZS-1, followed by screening for neomycin resistance. GFP signal was observed under a Nikon Eclipse 80i fluorescent microscope (Nikon, Japan).
DNA extraction and Southern blot analysis. The genomic DNA of the wild-type strain ZS-1 and generated mutants in this study was extracted according to the standard protocols\(^\text{27}\). Southern blot analysis was performed according to Gong et al.\(^\text{27}\). Genomic DNA (ZS-1 and CmNox1 mutants) aliquots of 15 μg were digested with HincII, separated by electrophoresis on 0.8% agarose gel and transferred onto a Hybond N+ membrane (Amersham Pharmacia Biotech). Interior probe was amplified with the primer pair 30F and 30R (Fig. 2c, Table S1), while the exterior probe was amplified with the primer pair 31F and 31R (Fig. 2c, Table S1). CmNox2 mutants were also confirmed by Southern blot, with the probe amplified using primer pair 32F and 32R.

RNA manipulation and qRT-PCR analysis. The total RNA sample of fungal strain was isolated with TriZOL reagent (Invitrogen, USA) according to the manufacturer’s protocols and potential DNA contamination was removed by DNase I treatment (RNase Free) (TaKaRa, Dalian, China). The first-strand cDNA was synthesized with RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Lithuania) by following the manufacturer’s instructions. In quantitative Real-time PCR, CmPks1 (primer pair 21F/21R), CmRac1 (primer pair 22F/22R), CmNox1 (primer pair 23F/23R), CmNox2 (primer pair 24F/24R), CmNoxR (primer pair 25F/25R), Cmg1 (primer pair 26F/26R), CHI (primer pair 27F/27R) and CmSlt2 (primer pair 34F/34R) were amplified by using the respective primer pairs (Table S1). As an endogenous control, a 154-bp amplicon of actin was amplified with primer pair 28F and 28R (Table S1).

Yeast two-hybrid system. Yeast two-hybrid analysis was carried out using a GAL4-based yeast two-hybrid system-Matchmaker™ Gold Systems (Clontech, Palo Alto, CA). cDNA of CmNox1 (1F1/1R1), CmSlt2 (2F2/2R2), CmRac1 (3F/3R) and CmNoxR (4F/4R), were amplified with the respective primer pairs (Table S1) and inserted into the yeast vector pGADT7. To test the specificity of the interaction, the bait plasmid and the prey plasmids were co-transformed into yeast strain Y2HGold. The transformants were assayed on SD (synthetic drop-out)/-Trp-Leu-His-Ade plates and SD/-Trp-Leu-His-Ade plates with X-α-gal for β-galactosidase test.

Bimolecular fluorescence complementation experiments. For bimolecular fluorescence complementation experiments (BiFC), CmNox1 and CmSlt2 were tagged with separated halves of YFP, as previously reported, with the following modifications. To fuse YFP-N to CmSlt2, CmSlt2-cDNA without stop codon was amplified with primer pair 7F and 7R and cloned into the yeast vector pGADT7. To test the specificity of the interaction, the bait plasmid and the prey plasmids were co-transformed into yeast strain Y2HGold. The transformants were assayed on SD (synthetic drop-out)/-Trp-Leu-His-Ade plates and SD/-Trp-Leu-His-Ade plates with X-α-gal for β-galactosidase test.

In vivo Co-IP. Agrobacterium strain EHA105 carrying the TrpC-CmSlt2-GFP and TrpC-CmNox1-Flag expression vectors was co-infiltrated into C. minitans. The over-expression mutant was generated by screening for neomycin/hygromycin resistance. Mutant transformed with TrpC-CmSlt2-GFP and empty Vector-Flag was generated and used as a control. For in vivo Co-IP, 8-day-old mutant total protein was extracted in IP buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton, 1 mM EDTA, 1 mM MgCl₂, and protease inhibitor cocktail). A 15 μl aliquot of anti-Flag agarose (Beyotime) was added to the samples, and the mixtures were incubated for 4 h at 4 °C with shaking. The immunoprecipitated proteins were washed three times with IP buffer and eluted with SDS sample buffer. Eluted sample were loaded on the protein gels for immunoblot analysis using anti-GFP antibody or anti-Flag antibody (Sigma).

References
1. Bolton, M., Thomma, B. P. H. J. & Nelson, B. Sclerotinia sclerotiorum (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. Mol Plant Pathol 7, 1–16 (2006).
2. Amslem, J. et al. Genomic analysis of the necrotrophic fungal pathogens Sclerotinia sclerotiorum and Botrytis cinerea. PLoS Genet 7, e1002230 (2011).
3. Whipp, J. M. & Gerlagh, M. Biology of Coniothyrium minitans and its potential for use in disease biocontrol. Mycol Res 96, 897–907 (1992).
4. Li, G. et al. Biological control of Sclerotinia diseases of rapeseed by aerial applications of the mycoparasite Coniothyrium minitans. Eur J Plant Pathol 114, 345–355 (2006).
5. Whipp, J. M., Sreenivasapradas, S., Muthumeenksh, S., Rogers, C. W. & Challen, M. P. Use of Coniothyrium minitans as a biocontrol agent and some molecular aspects of sclerotial mycoparasitism. Eur J Plant Pathol 121, 323–330 (2008).
6. Sandsy-Winsch, C., Whipp, J. M., Gerlagh, M. & Kruse, M. World distribution of the sclerotial mycoparasite Coniothyrium minitans. Mycol Res 97, 1175–1178 (1993).
7. Yang, L. et al. Effects of soil temperature and moisture on survival of Coniothyrium minitans conidia in central China. Biol Control 55, 27–33 (2010).
8. Paulitz, T. C. & Belanger, R. R. Biological control in greenhouse systems. Annu Rev Phytopathol 39, 103–105 (2001).
9. Giczy, G., Kernyi, Z., Fulop, L. & Horvath, L. Expression of cmgl, an exo-β-glucanase gene from Coniothyrium minitans, increase during sclerotial parasitism. Appl Environ Microbiol 67, 865–870 (2001).
10. Yang, R., Han, Y., Li, G., Jiang, D. & Huang, H. Suppression of Sclerotinia sclerotiorum by antifungal substances produced by the mycoparasite Coniothyrium minitans. Eur J Plant Pathol 119, 411–420 (2008).
11. Ren, L., Li, G., Han, Y., Jiang, D. & Huang, H. Degradation of xylan by Coniothyrium minitans and its effects on production and activity of beta-1,3-glucanase of this mycoparasite. Biol Control 43, 1–11 (2007).
12. Muthumeenksh, S., Sreenivasapradas, S., Rogers, C. W., Challen, M. P. & Whipp, J. M. Analysis of cDNA transcripts from Coniothyriumminitans reveals a diverse array of genes involved in key processes during sclerotial mycoparasitism. Fungal Genet Biol 44, 1262–1284 (2008).
13. Zeng, F. Y. et al. A fungal cell wall integrity-associated MAP kinase cascade in Coniothyrium minitans is required for conidiation and mycoparasitism. Fungal Genet Biol 49, 347–357 (2012).
14. Wei, W. et al. CmPEX6, a gene involved in peroxisome biogenesis, is essential for parasitism and conidiation of sclerotial parasite Coniothyrium minitans. Appl Environ Microbiol 79, 3658–3666 (2013).
15. Zeng, L. M. et al. Degradation of oxalic acid by the mycoparasite Coniothyrium minitans plays an important role in interacting with Sclerotinia sclerotiorum. *Environ Microbiol* 16, 2591–2610 (2014).

16. Lou, Y. et al. CmpacC regulates mycoparasitism, oxalate degradation and antifungal activity in the mycoparasitic fungus Coniothyrium minitans. *Environ Microbiol* 17(11), 4711–4729 (2015).

17. Gong, X. Y. et al. L-arginine is essential for conidiation in the filamentous fungus Coniothyrium minitans. *Fungal Genet Biol* 44, 1368–1379 (2007).

18. Li, B. et al. Cyclic GMP as a second messenger in the nitric oxide–mediated conidiation of the mycoparasite Coniothyrium minitans. *Appl Environ Microbiol* 76, 2830–2836 (2010).

19. Qin, L. et al. Phosphoribosylamidotransferase, the first enzyme for purine de novo synthesis, is required for conidiation in the scerotic mycoparasite Coniothyrium minitans. *Fungal Genet Biol* 48, 956–965 (2011).

20. Scott, B. Conservation of fungal and animal nicotinamide adenine dinucleotide phosphate oxidase complexes. *Mol Microbiol* 95, 910–913 (2015).

21. Egan, M. J., Wang, Z. Y., Jones, M. A., Smirnoff, N. & Talbot, N. J. Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease. *Proc Natl Acad Sci USA* 104, 11772–11777 (2007).

22. Ryder, L. S. et al. NADPH oxidases regulate septin-mediated cytoskeletal remodeling during plant infection by the rice blast fungus. *Proc Natl Acad Sci USA* 110, 3179–3184 (2013).

23. Segmuller, N. et al. NADPH oxidases are involved in differentiation and pathogenicity in Botrytis cinerea. *Mol Plant Microbe Interact* 21, 808–819 (2008).

24. Kim, H. J., Chen, C. B., Kabbage, M. & Dickman, M. B. Identification and characterization of Sclerotinia sclerotiorum NADPH oxidases. *Appl Environ Microbiol* 77, 7721–7729 (2001).

25. Siegmund, U., Marschall, M. J., Takemoto, D., Park, P. & Scott, B. Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. *Plant Cell* 18, 1052–1066 (2006).

26. Turrà, D., Segorbe, D. & Di Pietro, A. Protein kinases in plant-pathogenic fungi: conserved regulators of infection. *Adv Bot Res* 77, 267–288 (2014).

27. Tanaka, A., Christensen, M. J., Takemoto, D., Park, P. & Scott, B. A p67 Phox-like regulator is recruited to control hyphal branching in a fungal-grass mutualistic symbiosis. *Plant Cell* 18, 2807–2821 (2006).

28. Eaton, C. J., Cox, M. P. & Scott, B. What triggers grass endophytes to switch from mutualism to pathogenism? *Plant Sci* 180, 190–195 (2011).

29. Takemoto, D. Conservation of fungal and animal nicotinamide adenine dinucleotide phosphate oxidase complexes. *Mol Microbiol* 95, 988–1005 (2015).

30. Roca, M. G. et al. Germing fusion via conidial anastomosis tubes in the grey mould Botrytis cinerea requires NADPH oxidase activity. *Fungal Biol* 116, 379–387 (2012).

31. Kayano, Y., Tanaka, A., Akano, F. & Scott, B. Differential roles of NADPH oxidases and associated regulators in polarized growth, conidiation and hyphal fusion in the symbiotic fungus Epichloe festucae. *Fungal Genet Biol* 56, 87–97 (2013).

32. Lara-Ortiz, T., Riveros-Rosas, H. & Aguirre, J. Reactive oxygen species generated by microbial NADPH oxidase NoxA regulate germination in the filamentous fungus *Sordaria macrospora*. *Current Genet* 52, 1540–1551 (2013).

33. Lou, Y., Chen, C. B., Kabbage, M. & Dickman, M. B. Regulation of apical dominance in *Aspergillus nidulans*. *Mol Microbiol* 70, 137–152 (2009).

34. Li, B. L-arginine is essential for conidiation in *Aspergillus nidulans*. *Environ Microb* 77, 3009–3016 (2011).

35. Semighini, C. P. & Harris, S. D. Regulation of cell wall integrity signaling during injury response in the fungus *Trichoderma atroviride*. *Comparative genomics of MAP kinase and calcium-calcineurin signalling components in plant and human pathogenic fungi*. *Fungal Genet Biol* 77, 46, 287–298 (2009).

36. Wang, C. F. et al. Functional analysis of the kinome of the wheat scab fungus *Fusarium graminearum*. *PLoS Pathog* 7, e1002460 (2011).

37. Hanel, L. P., Nicole, M. C., Duplessis, S. & Ellis, B. E. Mitogen-activated protein kinase signaling in plant-interacting fungi: distinct message from conserved messenger. *Plant Cell* 24, 1327–1351 (2012).

38. Teichert, I. et al. PRO40 is a scaffold protein of the cell wall integrity pathway, linking the MAP kinase module to the upstream activator protein kinase C. *PLoS Genet* 10, e1004582 (2014).

39. Turra, D., Segorbe, D. & Di Pietro, A. Protein kinases in plant-pathogenic fungi: conserved regulators of infection. *Annu Rev of Phytopathol* 52, 267–288 (2014).

40. Yen, Y. et al. The MAPKK FgMkk1 of *Fusarium graminearum* regulates vegetative differentiation, multiple stress response, and virulence via the cell wall integrity and high-osmolarity glycerol signaling pathways. *Environ Microbiol* 16, 2023–2037 (2014).

41. Tu, P. et al. Structure of the N. crassa Serk1, a novel protein kinase of the mitogen-activated protein kinase family. *Current Genet* 59, 43–54 (2013).

42. Gruber, S. & Zeilinger, S. The transcription factor Ste12 mediates the regulatory role of the Tmk1 MAP kinase in mycoparasitism and vegetative hyphal fusion in the filamentous fungus *Trichoderma atroviride*. *PLoS One* 9, e11636 (2014).

43. Zhu, L. & Zhang, K. R. Similar and distinct roles of NADPH oxidase components in the tangerine pathotype of *Alternaria alternata*. *Mol Plant Pathol* 14, 543–556 (2013).

44. Medina-Castellanos, E., Esquivel-Naranjo, E. U., Heil, M. & Herrera-Estrella, A. Extracellular ATP activates MARK and ROS signaling during injury response in the fungus *Trichoderma atroviride*. *Front in Plant Sci* 5, 659 (2014).

45. Jaimez-Arroyo, R. et al. The SrkA kinase is part of the SAKa mitogen-activated protein kinase interactome and regulates stress responses and development in *Aspergillus nidulans*. *Eukaryot Cell* 14, 495–510 (2015).

46. Lambou, K. et al. The crucial role of the Pdi tetraspan during ascosporogenesis in *Sordaria macrospora* provides an example of the convergent evolution of morphogenetic processes in fungal plant pathogens and saprobes. *Eukaryot Cell* 7, 1809–1818 (2008).

47. Dirshnabel, D. E. et al. New insights into the roles of NADPH oxidases in sexual development and ascospore germination in *Sordaria macrospora*. *Genetics* 196, 729–744 (2014).
56. Schurmann, J., Buttermann, D. & Herrmann, A. Molecular characterization of the NADPH oxidase complex in the ergot fungus *Claviceps purpurea*: CpNox2 and CpPls1 are important for a balanced host-pathogen interaction. *Mol Plant Microbe Interact* **26**, 1151–1164 (2013).
57. Mahlert, M., Levelek, L., Hlubek, A., Sandrock, B. & Bolker, M. Rac1 and Cdc42 regulate hyphal growth and cytokinesis in the dimorphic fungus *Ustilago maydis*. *Mol Microbiol* **59**, 567–578 (2006).
58. Chen, J. *et al.* Rac1 is required for pathogenicity and Chn1-dependent conidiogenesis in rice fungal pathogen *Magnaporthe grisea*. *PLoS Pathog* **4**, e1000202 (2008).
59. Rolke, Y. & Tudzynski, P. The small GTPase Rac and the p21-activated kinase Cla4 in *Claviceps purpurea*: interaction and impact on polarity, development and pathogenicity. *Mol Microbiol* **68**, 405–423 (2008).
60. Li, H. *et al.* The small GTPase RacA mediates intracellular reactive oxygen species production, polarized growth, and virulence in the human fungal pathogen *Aspergillus fumigatus*. *Eukaryot Cell* **10**, 174–186 (2011).
61. Zhang, C. *et al.* Functional characterization of Rho family small GTPases in *Fusarium graminearum*. *Fungal Genet Biol* **61**, 90–99 (2013).
62. Hope, H., Schmauch, C., Arkowitz, R. A. & Bassilana, M. The *Candida albicans* ELMO homologue functions together with Rac1 and Dck1, upstream of the MAP Kinase Cek1, in invasive filamentous growth. *Mol Microbiol* **76**, 1572–1590 (2010).
63. Lalucque, H., Malagnac, F., Brun, S., Kacka, S. & Silar, P. A Non-mendelian MAPK-generated hereditary unit controlled by a second MAPK pathway in *Podospora anserine*. *Genetics* **191**, 419–433 (2012).
64. Bidard, F., Coppin, E. & Silar, P. The transcriptional response to the inactivation of the PaMpk1 and PaMpk2 MAP kinase pathways in *Podospora anserine*. *Fungal Genet Biol* **49**, 643–652 (2012).
65. Cheng, J. S. *et al.* Production, survival and efficacy of *Coniothyrium minitans* conidia produced in shaken liquid culture. *FEMS Microbiol Lett* **227**, 127–131 (2003).
66. Xie, J. *et al.* Characterization of debilitation-associated mycovirus infecting the plant-pathogenic fungus *Sclerotinia sclerotiorum*. *J Gen Virol* **87**: 241–249 (2006).
67. Sambrook, J. & Russell, D. W. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. (2001).
68. Walter, M. *et al.* Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J* **40**, 428–438 (2004).

**Acknowledgements**

The research was financially supported by the National `973’ Basic Research Program of China (Grant No. 2012cb114000), the National Natural Science Foundation of China (grant 31371895), the Programme of Introducing Talents of Discipline to Universities in China (the 111 Project no. B14032), and the Program for Changjiang Scholars and Innovative Research Team in University of China (IRT1247).

**Author Contributions**

WW, DJ, WC and YF designed the research and wrote the paper; WW, WZ and JC executed the experiments. JC, JX, GL and YF performed the data and bioinformatics analyses. All authors read and approved the final manuscript.

**Additional Information**

**Supplementary information** accompanies this paper at http://www.nature.com/srep

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Wei, W. *et al.* Nox Complex signal and MAPK cascade pathway are cross-linked and essential for pathogenicity and conidiation of mycoparasite *Coniothyrium minitans*. *Sci. Rep.* **6**, 24325; doi: 10.1038/srep24325 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/