The NADPH oxidase-mediated production of hydrogen peroxide (H₂O₂) and resistance to oxidative stress in the necrotrophic pathogen *Alternaria alternata* of citrus

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SUMMARY

It has become increasingly apparent that the production of reactive oxygen species (ROS) by the NADPH oxidase (Nox) complex is vital for cellular differentiation and signalling in fungi. We cloned and characterized an *AaNoxA* gene of the necrotrophic fungus *Alternaria alternata*, which encodes a polypeptide analogous to mammalian gp91^{phox} and fungal Noxs implicated in the generation of ROS. Genetic analysis confirmed that *AaNoxA* is responsible for the production of ROS. Moreover, deletion of *AaNoxA* in *A. alternata* resulted in an elevated hypersensitivity to hydrogen peroxide (H₂O₂), menadione, potassium superoxide (KO₂), diamide and many ROS-generating compounds. The results implicate the involvement of *AaNoxA* in cellular resistance to ROS stress. The impaired phenotypes strongly resemble those previously seen for the *ap1* null mutant defective in a YAP1-like transcriptional regulator and for the *hog1* mutant defective in a HOG1-like mitogen-activated protein (MAP) kinase. The *noxA* null mutant was also hypersensitive to Nox inhibitors, nitric oxide (NO) donors and NO synthase inhibitors, implying a role of *AaNoxA* in the NO[•] signalling pathway. Expression of *AaNoxA* was activated by H₂O₂, menadione, KO₂, NO[•] donors and L-arginine (a substrate for NO[•] synthase). *AaNoxA* may be able to sense and respond to both ROS and nitric oxide. Moreover, *AaNoxA* is required for normal conidiation and full fungal virulence. *AaNoxA* promoted the expression of the *AaAP1* and *AaHOG1* genes in *A. alternata*. Inactivation of *AaNoxA* greatly reduced the transcriptional activation of *AaAP1* in response to ROS stress. Thus, we conclude that the regulatory functions of *AaNoxA* conferring ROS resistance are modulated partially through the activation of the YAP1- and HOG1 MAP kinase-mediated signalling pathways.

INTRODUCTION

Alternaria alternata is a common necrotrophic fungus. Many of these fungi produce unique host-selective toxins (HSTs) and cause

disease on different host plants (Kohmoto *et al.*, 1991). The tangerine pathotype of *A. alternata* causes brown spots on citrus leaves and fruit, primarily as a result of the production of a host-selective ACT toxin with a 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid chromophore (Kohmoto *et al.*, 1993). ACT toxin is extremely toxic, causing rapid electrolyte leakage from susceptible citrus cells. In addition to HSTs, recent studies have demonstrated that the mitigation of reactive oxygen species (ROS) produced by the host plants, operated by the fungal redox-responsive transcriptional regulator *AaAP1*, is also essential for *A. alternata* pathogenicity in citrus (Lin *et al.*, 2009, 2011; Yang *et al.*, 2009).

A synergistic interaction between *AaAP1*-controlled gene expression and *AaFUS3* mitogen-activated protein (MAP) kinase-mediated signalling pathways for multidrug resistance has also been discovered in *A. alternata* (Lin *et al.*, 2010). *AaAP1* has been shown to suppress *AaFUS3* phosphorylation. *AaFUS3* and *AaSLT2* MAP kinases are not involved in cellular resistance to oxidative stress, even though both *AaFUS3* and *AaSLT2* null mutants are defective in pathogenicity (Lin *et al.*, 2010; Yago *et al.*, 2011). A HOG1 MAP kinase homologue, *AaHOG1*, has been shown to be necessary for ROS resistance and pathogenicity. In contrast, a 'two-component' histidine kinase (*AaHSK1*) has no effect on ROS resistance or pathogenicity (Lin and Chung, 2010). Recent studies have discovered serendipitously that fungal strains disrupted at any of the *AaAP1*, *AaHSK1*, *AaFUS3*, *AaHOG1* and *AaSLT2* genes are hypersensitive to 2-chloro-5-hydroxypyridine (CHP) or 2,3,5-triiodobenzoic acid (TIBA). Moreover, two genes encoding putative major facilitator superfamily (MFS) transporters have been identified to be co-ordinately regulated by these signalling regulators. Pyridine, occurring ubiquitously in natural compounds, is a heteroaromatic compound composed of five carbons and one nitrogen atom. Pyridine has been shown to accelerate the production of superoxide and hydroxyl radicals in the presence of Cu²⁺ and hydrogen peroxide (H₂O₂) (Nerud *et al.*, 2001; Watanabe *et al.*, 1998). In biological systems, pyridine and its derivatives have important functions—as electron carriers, such as NADP/NADPH and flavin nucleotides (FAD/FADH), as constituents of RNA and DNA, and as energy storage molecules, such as ATP and GTP. TIBA is a plant growth regulator that is often used as a herbicide or as

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an inhibitor of indole-3-acetic acid (IAA) transportation (Lahey *et al.*, 2004; Tsurumi and Ohwaki, 1978).

The production of ROS, including superoxide ($O_2^{\cdot-}$), H_2O_2 and hydroxyl radicals ($OH^{\cdot}$), is an inevitable event in all organisms. ROS can serve diverse roles in cellular defence and in the signalling transduction pathways controlling cell differentiation or developmental modifications. The NADPH oxidase (Nox) complex transfers electrons from NADPH to oxygen molecules, leading to the production of superoxide, which is further metabolized to H_2O_2 by superoxide dismutase (SOD). H_2O_2 is less toxic, but stable, and able to pass freely through membranes (Branco *et al.*, 2004), often serving as a signalling stimulus. In humans, the phagocytic Nox complex, involved in the production of superoxide and immunity, contains two major catalytic components gp91^{phox} (also known as Nox2) and gp22^{phox}, and multiple regulatory subunits Rac (a small GTPase), p67^{phox}, p47^{phox} and p40^{phox} (Diekman *et al.*, 1994). Plants possess oxidases analogous to gp91^{phox}, which are vital for ROS generation in response to pathogen invasion (Torres *et al.*, 2002).

Some fungi also harbour Nox homologues, NoxA (Nox1), NoxB (Nox 2) and NoxC, which have been documented by genetic analysis to be required for cell differentiation, pathogenicity or cellulose degradation in diverse fungal species (Aguirre *et al.*, 2005; Brun *et al.*, 2009; Cano-Domínguez *et al.*, 2008; Egan *et al.*, 2007; Giesbert *et al.*, 2008; Lara-Ortiz *et al.*, 2003; Malagnac *et al.*, 2004; Scott and Eaton, 2008; Segmüller *et al.*, 2008; Semighini and Harris, 2008). Both NoxA and NoxB are analogous to the mammalian gp91^{phox}. The function of NoxA/B is tightly regulated by the regulatory subunit, NoxR (p67^{phox} homologue), and by the small GTPase (Rac homologue). The production of ROS mediated by the Nox complex has recently been shown to prevent excessive proliferation and hyphal branching of the fungal endophyte *Epichloë festucae* within its host plant (ryegrass, *Lolium perenne*), thus allowing the fungus to maintain mutually beneficial interactions (Scott *et al.*, 2007; Takemoto *et al.*, 2007). Functional disruption of a gene encoding the component of the Nox complex in *E. festucae* generated fungal strains defective in H_2O_2 generation, but highly pathogenic to the host plant (Takemoto *et al.*, 2006; Tanaka *et al.*, 2006). Yeast two-hybrid and pull-down analyses further indicated that Rac interacts with NoxR and both are essential for the functional activation of NoxA in *E. festucae* (Tanaka *et al.*, 2006, 2008).

The Nox complex is widely distributed in animals, plants and fungi, but appears to be completely absent in prokaryotes and most unicellular eukaryotes. No Nox gene homologues have been identified in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* or other species belonging to Saccharomycota, as well as *Ustilago maydis*, *Rhizopus oryzae* or *Cryptococcus neoformans* (Takemoto *et al.*, 2007). *Aspergillus* species have only one Nox homologue, whereas other fungi may have at least two Nox homologues (Aguirre *et al.*, 2005; Lara-Ortiz *et al.*, 2003; Malagnac *et al.*, 2004). In general, NoxA/B are thought to have evolved

in fungi for the formation of fruiting body or multicellular structures, or for ascospore germination (Takemoto *et al.*, 2007). The Nox complex may have very different roles in asexual fungi, such as *A. alternata*, which does not have known sexual stages. The maintenance of ROS homeostasis via the regulation of ROS formation by the Nox complex is critical for fungal cellular differentiation, development and pathogenesis. Previous studies with *A. alternata* have uncovered that AaAP1- and AaHOG1 MAP kinase-activated gene expression is essential for ROS resistance, and this ability is absolutely required for fungal pathogenicity on citrus (Lin and Chung, 2010; Lin *et al.*, 2009; Yang *et al.*, 2009). In order to further understand the mechanisms underlying oxidative stress resistance, we isolated and characterized the AaNoxA gene that encodes an orthologue of gp91^{phox} in the human phagocytic oxidase and fungal NoxA, as well as the developmental and physiological functions of this gene in *A. alternata*. We provided genetic evidence to indicate that the expression of the AaAP1 and AaHOG1 genes is regulated by AaNoxA in response to ROS and chemical stimuli. We also showed that AaNoxA is involved in sensing and responding to both ROS and $NO^{\cdot}$ in *A. alternata*.

RESULTS

Cloning of fungal NoxA gene homologue from *A. alternata*

A 668-kb fragment was amplified from genomic DNA of *A. alternata* with two degenerate primers NOXf1 and NOXr1. Sequence analysis of the amplicon revealed a strong amino acid similarity to fungal Noxs. The cloned gene was designated as *A. alternata* NoxA. The 5' and 3' flanking sequences were obtained from a genomic library. Several overlapping cDNA fragments were amplified and determined by sequencing. Analysis of the combined genomic and cDNA sequences identified an 1810-bp open reading frame (ORF) and the presence of three small introns (50, 52 and 55 bp). All three introns had typical 5'-xx/GT and xx/AG-3' splicing junctions commonly found in the genes of filamentous fungi. The AaNoxA gene product has 550 amino acids, displaying 72%–93% identity and 84%–96% similarity to a number of Noxs (NOXA or NOX1) or hypothetical proteins of fungi (Fig. S1, see Supporting Information). Analysis of the predicted translational product of the *A. alternata* NoxA gene identified a ferredoxin synthase-type FAD-binding domain, a NADPH-binding domain and six transmembrane domains, commonly found in the NoxA-like family.

Inactivation of the *A. alternata* NoxA gene

Two overlapping DNA fragments, 5'AaNoxA::PHY and 3'AaNoxA::YGT, were generated (Fig. S2A, see Supporting Information) and transformed directly into protoplasts of the wild-type

EV-MIL31 strain. Of five transformants recovered from a medium containing hygromycin, two (DN2 and DN6) exerted a reduced radial growth of 15% and were selected for further analyses. Polymerase chain reaction (PCR) diagnosis with the primers NoxA-atg and hyg3 amplified an expected 1.7-kb fragment from genomic DNA of the DN2 and DN6 strains; an expected 2.5-kb fragment was also obtained by PCR with the primers hyg4 and NoxA-taa (Fig. S2B). No fragments were amplified from genomic DNA of the wild-type with different primer pairs. Northern blot hybridization of fungal RNA to an *AaNoxA* probe identified a 1.8-kb transcript from the wild-type, but not from the DN2 and DN6 strains (Fig. S2C), confirming successful inactivation of the *AaNoxA* gene in *A. alternata*.

***A. alternata* NoxA is involved in resistance to oxidative and nitrosative stress**

Compared with the wild-type, the *A. alternata noxA* null mutants were hypersensitive to H₂O₂, *tert*-butyl-hydroxyperoxide (t-BHP), cumyl hydrogen peroxide and the superoxide-generating agents menadione (MND), potassium superoxide (KO₂) and diamide (Fig. 1A). The *A. alternata noxA* null mutants also exhibited an increased sensitivity to the singlet oxygen-generating compounds (haematoporphyrin and rose bengal), TIBA, CHP and SDS (Fig. 1B). All defect phenotypes strongly resembled those observed in the fungal strain impaired at AaAP1, a functional homologue of the yeast redox-responsive transcriptional regulator. Mutational inactivation of *AaNoxA* in an *A. alternata* strain resulted in less severe hypersensitivity to these compounds compared with the sensitivity seen in mutants defective at *AaAP1*. For example, H₂O₂ at 1.5 mM had no inhibitory effect on the fungal mutant impaired at *AaNoxA*, but completely blocked growth of the fungal mutant defect at *AaAP1*. Similarly, the *A. alternata ap1* mutant was highly sensitive to t-BHP, MND, KO₂, cumyl hydrogen peroxide, rose bengal and diethyl maleate, whereas the *AaNoxA* mutant was moderately sensitive. The *A. alternata noxA* mutant was more sensitive than the *ap1* mutant to diamide and SDS. Expression of a functional copy of *AaNoxA*, under the control of its own promoter, in the DN2 null strain resulted in fungal strains (NCp1, NCp7 and NCp16) that exhibited resistance to all test compounds (Fig. S3, see Supporting Information). This confirmed further that the *AaNoxA* disruption was indeed contributory to the deformed phenotypes.

Compared with the *ap1* mutant, the *A. alternata noxA* mutant displayed much greater sensitivity to diamide, a compound that is well known to stimulate the formation of superoxide anion (O₂^{•-}) and nitric oxide (NO). We tested whether the *A. alternata noxA* and *ap1* mutants were sensitive to diphenylene iodonium (DPI), apocynin (APC), sodium nitroprusside (SNP), hydroxylamine HCl (HDA), L-arginine (an NO[•] synthase substrate) and nitro-arginine methyl ester (nitro-Arg). Both DPI and APC are Nox inhibitors. SNP and HDA are capable of producing NO[•], whereas nitro-Arg is a NO[•]

synthase inhibitor. The *A. alternata noxA* mutant was moderately sensitive to DPI, APC, SNP, HDA, L-arginine and nitro-Arg (Fig. 1C). The *A. alternata ap1* mutant was also moderately sensitive to DPI, APC and SNP, but completely insensitive to HDA, L-arginine and nitro-Arg. The *noxA* mutant was apparently more sensitive than the *ap1* mutant to DPI.

Expression of *AaNoxA* in response to oxidative and nitrosative stress

Northern blot analyses were carried out to assess whether the expression of the *AaNoxA* gene responded to H₂O₂ and other compounds related to oxidative stress in axenic cultures. Accumulation of the 1.8-kb *AaNoxA* gene transcript was barely detectable when the fungus was grown on potato dextrose agar (PDA), but was elevated in the presence of H₂O₂, KO₂, MND, t-BHP and haematoporphyrin (Fig. 2A). Moreover, expression of the *AaNoxA* gene was up-regulated in the wild-type strain responding to L-arginine, SNP, HDA, DPI and APC (Fig. 2B). In contrast, the expression of *AaNoxA* was down-regulated by nitro-Arg (an NO[•] synthase inhibitor).

***AaNoxA* is required for conidiation and fungal virulence**

Although the *A. alternata noxA* null mutants displayed a slight growth reduction, both wild-type and mutant produced branching hyphae in a similar manner. Mutational inactivation of *AaNoxA* in *A. alternata* had little or no effect on conidial germination; more than 85% of conidia prepared from all test strains germinated as assayed on glass slides. The *A. alternata noxA* null mutants showed reduced conidiation and pigmentation compared with the wild-type in axenic cultures (Fig. 3A,B). Introduction and expression of a functional copy of *AaNoxA* in a null mutant fully restored conidiation and pigmentation. Light microscopy analysis revealed that the wild-type strain of *A. alternata* often formed multicellular, obpyriform conidia with both vertical and transverse septae and dark pigment on PDA. In contrast, the *A. alternata noxA* mutant generated less melanized conidia with less distinct septae (Fig. 3C). The complementation strain (NCp) produced dark-coloured conidia with both vertical and transverse septae, resembling those produced by the wild-type.

Pathogenicity assays using a point inoculation method indicated that the *A. alternata noxA* null mutants induced significantly smaller necrotic lesions than the wild-type on detached Minneola or calamondin leaves 3 days after inoculation (dai) (Fig. 4A). Statistical analyses using *t*-tests indicated that the mean number of lesions per leaf (*n* = 12) induced by the wild-type was significantly different from that induced by the *A. alternata noxA* mutant (*P* ≤ 0.05) (Fig. 4B). Pathogenicity assessed further on detached Minneola leaves sprayed uniformly with conidial

Fig. 1 Hypersensitivity of the wild-type (WT), *noxA* mutant and *ap1* mutant strains of *Alternaria alternata*. Fungal strains were grown on potato dextrose agar (PDA) or PDA amended with test compounds as indicated for 4–7 days. Radial growth was measured. Sensitivity (percentage growth reduction) was calculated as a cumulative percentage of the growth of the wild-type and the deletion mutants grown on the same plate. APC, apocynin; Arg, L-arginine; CHP, 2-chloro-5-hydroxypyridine; DEM, diethyl maleate; DPI, diphenylene iodonium; HDA, hydroxylamine HCl; HP, haematoporphyrin; MND, menadione; N-Arg, nitro-arginine methyl ester; RB, rose bengal; t-BHP, *tert*-butyl-hydroxyperoxide; TIBA, 2,3,5-triiodobenzoic acid; SDS, sodium dodecylsulphate; SNP, sodium nitroprusside. The data presented are the mean and standard deviation of two independent experiments with at least three replicates.

suspension revealed a reduction in necrotic lesions induced by the *A. alternata noxA* mutant relative to those induced by the wild-type (Fig. 4C). The mean number of diseased lesions per leaf ($n = 6$) induced by the wild-type was much larger than that induced by the *A. alternata noxA* mutant (Fig. 4D). Expression of a copy of *AaNoxA* in a *noxA* null mutant restored the ability for lesion induction. Inactivation of *AaNoxA* in *A. alternata* did not have an impact on ACT toxin production, as evidenced by thin layer chromatography analysis and a bioassay on detached calamondin leaves (Fig. S4, see Supporting Information).

AaNoxA is involved in the production of ROS

Nitroblue tetrazolium (NBT) can be reduced by superoxide to form a dark blue, water-insoluble formazan. The staining of fungal hyphae with NBT indicated that the *A. alternata noxA* mutant seemingly produced less superoxide anions than the wild-type. The wild-type mycelia were stained with NBT, forming dark blue precipitates around the colony (Fig. 5). The *noxA* mutant mycelia displayed less intensity, showing dense precipitates primarily along the surface of the colony. Microscopic examination revealed

Fig. 2 (A) RNA gel blotting. The wild-type strain of *Alternaria alternata* grown on cellophane overlaid onto potato dextrose agar (PDA) for 2 days was shifted to PDA containing H₂O₂ (3 mM), KO₂ (0.5 mg/mL), *tert*-butyl-hydroperoxide (t-BHP, 0.05%), menadione (MND, 1 mM) or haematoporphyrin (HP, 50 µM) and incubated for an additional 24 h. The mock treatment contained RNA obtained from fungal mycelia shifted to the nonamended PDA. Fungal RNA was purified, electrophoresed in a formaldehyde-containing denaturing gel, blotted and washed at high stringency after hybridization with an *AaNoxA* probe. (B) Image of RNA gel blotting. Fungal strains were shifted to PDA containing apocynin (APC, 200 µM), diphenylene iodonium (DPI, 20 µM), L-arginine (Arg, 100 mM), hydroxylamine HCl (HDA, 2 mM), sodium nitroprusside (SNP, 0.5 mM) or nitro-arginine methyl ester (N-Arg, 10 mM) and incubated for an additional 24 h. The intensity of the hybridizing bands was quantified using an ImageJ program (<http://rsb.info.nih.gov/ij/>).

that blue precipitates were observed in some of the young hyphal tips of the *noxA* null mutant. In contrast, nearly all hyphae at the proximal ends of the wild-type and the complementation strain were stained dark blue by NBT.

The staining of fungal hyphae with 3,3'-diaminobenzidine (DAB) resulted in a dark brown pigmentation—indicative of the formation of H₂O₂—in apical tips, also showing some differences between the wild-type and *noxA* null mutant (Fig. 6A). Quantitative analysis revealed a reduced accumulation of H₂O₂ inside the mutant cells relative to the wild-type (Fig. 6B). Acquisition and expression of *AaNoxA* restored the accumulation of H₂O₂ to the wild-type level. Similarly, the amount of H₂O₂ secreted into the medium by the *A. alternata noxA* null mutant was much smaller and slower than that produced by the wild-type (Fig. 6C). The genetically reverted strain accumulated extracellular H₂O₂ at a rate and magnitude similar to that of the wild-type.

To determine the production of other ROS, fungal mycelium was stained with 2,7-dichlorodihydrofluorescein diacetate (H₂DCFDA). H₂DCFDA is a fluorogenic probe commonly used to detect the cellular production of H₂O₂, hydroxyl radicals and nitric oxide. H₂DCFDA is not fluorescent until it is converted to H₂DCF when exposed to ROS. ROS production, based on fluorescent intensity, was apparently higher within incipient hyphae of the wild-type and the complementation strains (Fig. 7). The *noxA* null mutants emitted much fainter fluorescence which diffused along the hyphal cytoplasm. Fungal hyphae treated with water only did not emit fluorescence (data not shown). The results confirm further that NoxA plays an important role in ROS generation.

***AaNoxA* regulates a YAP1 transcription factor and a HOG1 MAP kinase**

Previous studies have revealed that the *A. alternata ap1* and *hog1* disruption mutants are hypersensitive to H₂O₂, ROS-generating compounds, TIBA and CHP. Northern blot analyses showed that the expression of the *AaAP1* gene was nearly abolished in the *noxA* mutants (Fig. 8A). The introduction of a copy of *AaNoxA* into a null mutant restored *AaAP1* gene expression, as exemplified in an NCp1 strain. Accumulation of the *AaHOG1* transcript was undetectable in the *noxA* mutants, yet was restored in the NCp1 strain expressing *AaNoxA* (Fig. 8B). There was no difference in expression of the *AaNoxA* gene between the wild-type, *A. alternata ap1* mutants and the genetically reverted strain (Fig. 8C). Similar to *AaAP1*, *AaHOG1* disruption apparently did not influence the accumulation of the *AaNoxA* transcript (Fig. 8D).

Expression of *AaAP1*

Expression of the *AaAP1* gene was investigated further in the wild-type and *noxA* mutant in response to TIBA and ROS-generating agents (Fig. 9). Assays by Northern blotting indicated that the accumulation of the *AaAP1* gene transcript was elevated in the *noxA* mutant in response to H₂O₂, KO₂ and other treatments (Fig. 9A). The increased level of the *AaAP1* gene transcript in the wild-type strain was much greater than that in the *noxA* mutant in

Fig. 3 The *Alternaria alternata* *noxA* mutants reduce pigmentation and conidiation. (A) Morphological appearance of the wild-type (WT) and two *noxA* mutants (DN2, DN6) grown on potato dextrose agar (PDA). (B) Quantitative analysis of conidial formation by *A. alternata* strains. The genetically reverted strain (NCp1) was derived from a mutant strain expressing a functional copy of *AaNoxA*. (C) Images of conidia. Note that the *noxA* null mutants produced light-coloured conidia with indistinct septae. Only representative replicates are shown.

response to either H_2O_2 or TIBA (Fig. 9B,C), further indicating that the activation of *AaAP1* gene expression was partially regulated by *AaNoxA*.

DISCUSSION

Successful pathogenesis by the necrotrophic pathogen *A. alternata* in citrus is highly dependent on the production of a host-specific toxin and the ability to detoxify ROS (Lin *et al.*, 2009). In this article, we have described the cloning and functional characterization of an *AaNoxA* gene, a homologue of gp91^{phox} (Nox2) in the human phagocytic oxidase, and have demonstrated pleiotropic roles in the tangerine pathotype of *A. alternata*. The cloned

AaNoxA gene, encoding a putative membrane-associated Nox, has been shown to promote ROS production, conidiation and fungal virulence. Impairment of the *AaNoxA* gene in *A. alternata* created a strain that was hypersensitive to H_2O_2 , superoxide-generating compounds (MND and KO_2), diamide, SDS, CHP, TIBA and potent singlet oxygen-generating compounds (haematoporphyrin and rose bengal). These deficiencies strongly resemble the phenotypes previously seen for the *A. alternata* *ap1* null mutant defective at a YAP1-like transcriptional regulator and for the *A. alternata* *hog1* mutant defective at a HOG1-like MAP kinase (Lin and Chung, 2010; Lin *et al.*, 2009). Diamide is a sulphhydryl-targeted compound that has been shown to facilitate the formation of superoxide and NO^- (de Lamirande *et al.*, 2009). In the

Fig. 4 Assays for the pathogenicity of *Alternaria alternata*. (A) Necrotic lesions induced by wild-type (WT) and two *nox4* null mutants (DN2 and DN6) on detached leaves of citrus cv. Minneola 3 days after inoculation. Conidial suspension (10^4 conidia/mL) was inoculated onto Minneola leaves by placing 5 μ L of suspension in each of the spots. The inoculated leaves were incubated in a mist chamber for lesion development. Some representative replicates are shown. (B) Quantification of the mean size of necrotic lesions induced by the *A. alternata* strains on detached calamondin leaves ($n = 12$) 2 or 3 days after inoculation (dai). The mock controls were treated with water only. The mean values that are significantly different from those of the wild-type, separated by Student's *t*-test ($P \leq 0.05$), are indicated by asterisks. The experiments were repeated twice with similar results. (C) Fungal pathogenicity assayed on detached Minneola leaves uniformly sprayed with conidial suspension (10^4 conidia/mL) of *A. alternata* with the inoculated leaves incubated in a mist chamber for 3 days. (D) Quantification of diseased lesions on detached Minneola leaves ($n = 6$) using an ImageJ program. Fungal strains NCp1, NCp7 and NCp16, derived from the transformation of *AaNOXA* in the DN2 mutant, exhibit the wild-type conidiation and pigmentation.

budding yeast *S. cerevisiae*, YAP1 senses peroxide stress and diamide stress using different mechanisms (Delaunay *et al.*, 2000).

Little is known about how the Nox complex is regulated in fungi. In mammalian systems, p38 MAP kinase (HOG1 homologue) has been shown to activate the Nox complex via the phosphorylation of p47^{phox} and p67^{phox} (NoxR homologue) (Brown *et al.*, 2009). Furthermore, a p21-activated kinase (Pak) is also known to regulate the mammalian Nox complex (Martyn *et al.*,

2005). Deletion of a Pak homologue in the ergot fungus *Claviceps purpurea* repressed accumulation of the *Nox1* gene transcript (Rolke and Tudzynski, 2008). In the filamentous fungus *Aspergillus nidulans*, expression of a *NoxA* homologue has been shown to be suppressed by SakA, a HOG1 MAP kinase homologue (Lara-Ortiz *et al.*, 2003). In the endophytic fungus *E. festucae*, inactivation of a *SakA* homologue did not alter the expression of *NoxA* or *NoxR*. It was speculated that the interaction might occur at the

Fig. 5 Accumulation and detection of superoxide anions within the hyphae of the wild-type, *noxA* null mutants ($\Delta noxA$) and the complementation strain (NCp16) of *Alternaria alternata*. Fungal strains were grown on potato dextrose agar (PDA) for 6 days and stained with nitroblue tetrazolium (NBT). Images of the edge of fungal colonies after staining with NBT (top panels). Microscopic images of fungal hyphal tips showing the deposition of water-insoluble formazan (indicated by an arrow) after staining with NBT. Bar, 20 μ m.

post-translational level (Eaton *et al.*, 2008). Studies with *Podospora anserina* have indicated that NoxA facilitates the nuclear localization of the PaMpk1 MAP kinase (Kicka *et al.*, 2006; Malagnac *et al.*, 2004).

Experimental evidence derived from the present study revealed a regulatory expression of the *AaAP1* and *AaHOG1* genes by *AaNoxA* in response to ROS and chemical stimuli, implying a close interaction between ROS production and resistance. The involvement of *AaNOXA* in ROS resistance is mediated through the regulation of *AaAP1* and *AaHOG1*. Both *AaAP1* and *AaHOG1* are absolutely required for cellular resistance to ROS and multidrug (Lin and Chung, 2010; Lin *et al.*, 2009). Deletion of *AaAP1* in the tangerine pathotype of *A. alternata* resulted in a severe reduction in catalase, peroxidase and SOD activities. It appears that *AaNoxA*, in conjunction with *AaAP1* and *AaHOG1*, is responsible for maintaining ROS homeostasis in *A. alternata*. Impairment of *AaNoxA* in *A. alternata* decreased the production and accumulation of superoxide and H_2O_2 . Thus, *AaNoxA* is one of the primary sources responsible for ROS production. Surprisingly, deletion of a *Nox1* homologue in *P. anserina* or *Magnaporthe grisea* increased ROS production (Egan *et al.*, 2007; Malagnac *et al.*, 2004).

In mammalian phagocytic cells, gp91^{phox} plays a central role in the high level of accumulation of superoxide in response to patho-

gen attacks (Diekman *et al.*, 1994). However, disruption of *AaNoxA* or *AaAP1* resulted in fungi showing an elevated sensitivity to NO^- donors, as well as NO^- synthase substrates and inhibitors, implying a possible involvement of the NOX system and *AaAP1* in the NO^- signalling pathways. The important role of *AaNoxA* in response to ROS and NO^- was further supported by observations that the expression of *AaNoxA* was activated by H_2O_2 , MND, KO_2 , haematoporphyrin, NO^- donors (SNP and HDA) and L-arginine (the prime substrate for NO^- synthase). Expression of *AaNoxA* was almost abolished in fungi treated with nitro-L-arginine, an NO^- synthase inhibitor. Hypersensitivity of the *A. alternata noxA* mutants to Nox inhibitors (DPI and APC) could be a result of the reduced expression of *AaAP1* and *AaHOG1*. In addition to ROS resistance, both *AaAP1* and *AaHOG1* are also required for multidrug resistance.

We have observed that *AaNoxA* inactivation does not alter hyphal branching (data not shown). Recent studies with *As. nidulans* revealed that the production and deposition of ROS, regulated by the Nox complex, are responsible for apical dominance and for the suppression of growth of secondary hyphae (Semighini and Harris, 2008). The endophytic fungus *E. festucae* apparently generates ROS by the Nox complex in order to restrict hyphal branching and extensive growth within its grass host, thus maintaining the mutualistic association with its host (Tanaka *et al.*, 2006). In

Fig. 6 The *AaNoxA* homologue is required for the production of H_2O_2 in *Alternaria alternata*. (A) Accumulation and detection of H_2O_2 within the hyphae after staining with 3,3'-diaminobenzidine (DAB). Bar, 20 μm . (B) Quantitative analysis and comparison of intracellular H_2O_2 among the wild-type (WT), two *noxA* null mutants (DN2 and DN6) and two genetically complemented strains (NCp1 and NCp7) using a xylenol orange assay. Asterisks indicate the values, separated by Student's *t*-test ($P \leq 0.05$), that are significantly different from those of the wild-type. (C) Detection of extracellular H_2O_2 in axenic culture by measuring the increase in absorbance at 240 nm. Complete medium was used as the mock control. The quantity of H_2O_2 was calculated by referring to a standard curve that was established using authentic H_2O_2 . Each column or point represents the mean number of $H_2O_2 \pm$ the standard deviation from two independent experiments with at least three replicates.

addition, the Nox homologues have been shown to be necessary for the formation of multicellular fruiting bodies in *Neurospora crassa*, *As. nidulans* and *P. anserina* (Cano-Domínguez *et al.*, 2008; Lara-Ortiz *et al.*, 2003; Malagnac *et al.*, 2004) and sclerotia in the phytopathogenic fungi *Botrytis cinerea* and *C. purpurea* (Giesbert *et al.*, 2008; Segmüller *et al.*, 2008).

AaNoxA has also been shown to be required for the formation of asexual spores (conidia) in *A. alternata*. Double mutations of

Nox1 and *Nox2* in *M. grisea* also reduced the production of conidia (Egan *et al.*, 2007). Similar reduction of conidia was observed in *N. crassa* disrupted at the *Nox1* locus (Cano-Domínguez *et al.*, 2008). These studies indicate an important function of the Nox complex in fungal conidial production. Previous studies have indicated that conidiation is partially regulated by the G protein-controlled cyclic adenosine monophosphate (cAMP) level in *A. alternata* (Wang *et al.*, 2010). Further investigations

Fig. 7 Detection of reactive oxygen species within *Alternaria alternata* hyphae staining with the oxidatively active fluorescent dye 2,7-dichlorodihydrofluorescein diacetate (H₂DCFDA, Molecular Probes). Fungal mycelium was collected from the wild-type (WT), the *nox4* null mutants (DN) and the genetically complemented strains (NCp) grown on potato dextrose agar for 6 days, stained with H₂DCFDA (2.5 µg/mL) dissolved in water for 15 min, and examined with a microscope equipped with a 450–490-nm excitation filter and a 520-nm barrier filter. Bar, 20 µm.

Fig. 8 Autoradiographic images of RNA gel blots. (A, B) Fungal RNA purified from the wild-type (WT), *noxA* null mutants (DN2 and DN6) and genetically complemented strain (NCp1) of *Alternaria alternata*, electrophoresed in a formaldehyde-containing gel, blotted and hybridized with an *AaAP1* (A) or *AaHOG1* (B) gene probe. (C) Fungal RNA purified from WT, two *ap1* null mutants (Y1 and Y2) and a strain expressing functional *AaAP1* (YCp1) was hybridized to an *AaNoxA* probe. (D) Fungal RNA purified from WT and two *hog1* null mutants (HG1 and HG2) was hybridized to an *AaNoxA* probe. Gels staining with ethidium bromide indicate relative loading of the RNA samples. The sizes of the hybridization bands are indicated in kilobase pairs (kb).

revealed that conidiation was also synergistically controlled by FUS3 and SLT2 MAPK-mediated signalling pathways because targeted disruption of each of the genes nearly abrogated the production of conidia in *A. alternata* (Lin *et al.*, 2010; Yago *et al.*, 2011). Expression of the Nox complex has recently been suggested to be regulated by Fus3/Kss1 (pheromone signalling) and Slr (cell integrity) MAP kinases in fungi (Cano-Domínguez *et al.*, 2008; Segmüller *et al.*, 2008). Our preliminary studies revealed that the expression of the *AaFUS3* gene and phosphorylation of AaFUS3 in *A. alternata* were negatively regulated by the G α subunit and cAMP-dependent protein kinase (K. R. Chung, unpublished data). Further tests are required to determine whether or not expression of the *AaFUS3* or *AaSLT2* gene is regulated by AaNoxA in the context of conidiation by *A. alternata*.

The Nox complex has important functions in phytopathogenic fungi. In the rice blast pathogen *M. grisea*, both Nox1 and Nox2 are crucial for appressorium-assisted penetration of rice (Egan *et al.*, 2007). In the necrotrophic fungus *B. cinerea*, Nox2 regulates the formation of an appressorium-like structure and Nox1 is required for *in planta* growth (Segmüller *et al.*, 2008). The *C. purpurea* NoxA homolog is required for full virulence in cereals (Giesbert *et al.*, 2008). It appears that AaNoxA is also an important virulence determinant in *A. alternata*, as the impaired mutant showed drastically decreased fungal virulence and rate of lesion formation on Minneola leaves. This pathological defi-

ciency may be primarily a result of the reduced expression of *AaAP1* and *AaHOG1*, because both genes have been demonstrated to be essential for pathogenicity in *A. alternata* (Lin *et al.*, 2009, 2011). In the present study, we showed that the expression of *AaAP1* and *AaHOG1* was controlled by AaNoxA. Similarly, the hypersensitivity of the *A. alternata noxA* null mutant to ROS, CHP, haematoporphyrin, TIBA, DPI and APC could also be attributed to the reduced expression of *AaAP1* and *AaHOG1*. The *A. alternata ap1* and *hog1* null mutants also displayed hypersensitivity to these compounds (Lin and Chung, 2010). It is tempting to speculate that AaNoxA may perceive environmental stimuli, producing low levels of superoxide and H₂O₂, which serve as secondary messages to activate the expression of various regulators, including *AaAP1*, *AaHOG1* and, perhaps, AaNoxA itself. The expression of *AaAP1* and *AaHOG1* might be up-regulated by other unidentified factors, rather than AaNoxA, because the phenotypes resulting from the inactivation of *AaNoxA* were not completely identical to the levels seen in the *ap1* and *hog1* null mutants. Through the coordinate regulation of ROS-detoxifying and sensing systems, *A. alternata* could effectively colonize host plants.

It remains uncertain whether the expression of AaNoxA is regulated by other Nox regulatory components, such as NoxR and Rac (small GTP-binding protein), as demonstrated in other filamentous fungi (Cano-Domínguez *et al.*, 2008; Segmüller *et al.*, 2008;

Expression of AaAP1

Fig. 9 Autoradiographic images of RNA gel blots. (A, B) The *Alternaria alternata* *noxA* null mutant (DN2) was grown on cellophane overlaid onto potato dextrose agar (PDA) for 2 days, shifted to PDA containing H_2O_2 (1 or 3 mM), KO_2 (0.5 mg/mL), *tert*-butyl-hydroperoxide (t-BHP, 0.05%), menadione (MND, 1 mM), haematoporphyrin (HP, 50 μ M) or 2,3,5-triiodobenzoic acid (TIBA, 2.5 or 5 mM), and incubated for an additional 24 h. Fungal RNA was hybridized with an *AaAP1* gene probe. (C) Total RNA was purified from the wild-type grown on H_2O_2 or TIBA, and hybridized with an *AaAP1* probe. Gels staining with ethidium bromide indicate relative loading of the RNA samples. The sizes of the hybridization bands are indicated in kilobase pairs (kb).

Semighini and Harris, 2008; Tanaka *et al.*, 2008). Our studies show that the expression of *AaAP1* and *AaHOG1*, whose functions have been defined by genetic analysis to be responsible for ROS resistance (Lin *et al.*, 2009), is partially regulated by *AaNoxA*. Indeed, the fungal mutants disrupted in *AaAP1*, *AaHOG1* and *AaNoxA* displayed several common phenotypes in which they were all sensitive to ROS-generating agents, CHP and TIBA, further supporting functional connections between *AaAP1*, *AaHOG1* and *AaNoxA*.

In summary, this study has demonstrated that *AaNoxA* acts as a key regulator for the production of ROS and for signalling transductions leading to appropriate conidiation, fungal virulence and cellular resistance to ROS and multidrugs in a necrotrophic pathogen of citrus. These regulatory functions of *AaNoxA* conferring ROS resistance are probably modulated partially through the activation of the *AaAP1* and *AaHOG1* genes, whose products are responsible for the activation of ROS-scavenging enzymes. Intriguingly, *AaNoxA* may be able to sense and respond to both ROS and NO , which have been intensively examined for their roles in cellular differentiation, defence and pathogenicity in diverse fungal species (Brown *et al.*, 2009). The results further highlight a fundamental complexity in maintaining the steady-state levels of ROS in fungal systems.

EXPERIMENTAL PROCEDURES

Fungal strains and culture conditions

The wild-type EV-MIL31 strain of *Alternaria alternata* (Fr.) Keissler, which causes brown spots on tangerines, was used throughout the study. Fungal mutants disrupted at the *AaAP1* gene, encoding an oxidative stress-responsive transcription activator, or the *AaHOG1* gene, encoding a HOG1 MAP kinase, were created in separate studies (Lin and Chung, 2010; Lin *et al.*, 2009). Fungi were cultured on PDA (Difco, Sparks, MD, USA), complete medium (CM) or minimal medium (MM) at 28 °C (Chung, 2003). Assays for chemical sensitivity were conducted by transferring hyphal segments with sterile toothpicks onto a medium containing the test chemical and incubating the plates in an incubator. Fungal growth, indicating the sensitivity of chemicals, was determined by measuring the radius of the colonies at 4–7 days, depending on the type of chemicals tested. Fungi were cultured in liquid CM for 3 days for protein purification. To purify DNA and RNA, fungal strains were grown on an agar medium with a layer of sterile cellophane.

Molecular cloning and sequence analysis

The *AaNoxA* gene fragment was amplified by PCR with the primers NOXf1 (5'-GAGACCTTYTGGTACTACTCAYC-3') and NOXr1 (5'-GATYTCGTTCTCRAAGACGTC-3') which are complementary to a *NoxA*-like gene of

A. brassicicola from the genome of *A. alternata*. The entire *AaNoxA* coding sequence and its 5' and 3' nontranslated regions were amplified by PCR from a library of *A. alternata*. The library was constructed from genomic DNA digested with *DraI*, *EcoRI*, *PvuI* and *StuI*, and ligated to adaptors using the Universal GenomeWalker kit (BD Biosciences, Palo Alto, CA, USA). PCR fragments were cloned into a pGEM-T easy vector (Promega, Madison, WI, USA) for sequencing from both directions at Eton Bioscience (San Diego, CA, USA). BLAST similarity searches were performed at the National Center for Biotechnology Information. ORF and exon/intron positions were deduced from comparisons of genomic and cDNA sequences.

Functional analyses through targeted gene disruption and complementation

The *AaNoxA* gene was disrupted in the EV-MIL31 strain using a split marker approach, as described previously (You *et al.*, 2009). Two truncated fragments, PHY and YGT, overlapping within the *HYG* gene, encoding a phosphotransferase under the control of the *As. nidulans trpC* promoter (P) and terminator (T), and conferring hygromycin resistance, were amplified by PCR with the primers M13R (5'-AGCGGATAACAATTCACACAGGA-3') and hyg3 (5'-GGATGCTCCGCTCGAAGTA-3') and the primers hyg4 (5'-CGTTGCAAGAACTGCCTGAA-3') and M13F (5'-CGCCAGGGTTTCC CAGTCACGAC-3'), respectively. The 5'*AaNoxA* fragment was amplified with the primers NoxA-pro2F (5'-TGAGACGCTCTTCACACACTAGCTG-3') and M13R:NoxA (5'-TCCTGTGTGAAATTGTTATCCGCTGGCGGACCTGCA CACGTTCAACA-3') and fused with the PHY fragment (Fig. S2A). The 3'*AaNoxA* fragment was obtained with the primers M13F:NoxA (5'-GTCTGACTGGGAAAACCTGGCGTCAAGTCGGTGACTTTACCCGC-3') and NoxA-taa (5'-TTAGAAATGCTCTCCAGAACTTGA-3') and fused with the YGT fragment. The italic sequences in the primers M13R:NoxA and M13F:NoxA are complementary to the primers M13R and M13F, respectively. Split marker fragments (10 µL each) were mixed and transformed into protoplasts prepared from the EV-MIL31 strain using CaCl₂ and polyethylene glycol, as described by Chung *et al.* (2002). Fungal transformants were recovered in a regeneration medium impregnated with 250 µg/mL hygromycin (Roche Applied Science, Indianapolis, IN, USA). Putative mutants disrupted at the *AaNoxA* locus were screened by PCR with the NoxA-atg (5'-ATGGGAGCAAGCGGAGGCAC-3') and hyg3 primers, or the NoxA-taa and hyg4 primers.

Manipulation of nucleic acids

A DNeasy Plant kit (Qiagen, Valencia, CA, USA) was used to isolate fungal genomic DNA. RNA was isolated with Trizol solution (Molecular Research Center, Cincinnati, OH, USA), treated with DNase and employed to synthesize the first strand of cDNA using the SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Double-stranded cDNA was amplified with gene-specific primers and analysed by sequencing. *Escherichia coli* DH5α cells were used for the propagation of plasmid DNA. Plasmid was purified using a Quickclean miniprep kit (GenScript, Piscataway, NJ, USA). Standard molecular procedures were followed for the digestion of DNA with endonucleases, electrophoresis, ligation, transformation of bacterial cells and Northern blot hybridization. DNA probes used for RNA or DNA hybridization were amplified by PCR and

simultaneously labelled with digoxigenin (DIG)-11-dUTP (Roche Applied Science) with gene-specific primers. A Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1]decan}-4-yl)phenyl phosphate (CSPD) chemofluorescent (Roche Applied Science) was used as a substrate for alkaline phosphatase in an immunological assay to detect the probe.

Detection and quantification of H₂O₂ and superoxide

Superoxide was detected by staining fungal hyphae with NBT, which is reduced by superoxide to form a dark blue, water-insoluble formazan (Shinogi *et al.*, 2003). Intracellular H₂O₂ of *A. alternata* was detected by staining fungal hyphae with DAB, showing a brown stain caused by the polymerization of DAB (Torres *et al.*, 2002). Peroxide was further determined on the basis of the oxidation of ferrous (Fe²⁺) to ferric (Fe³⁺) ions in the presence of xylenol orange in acidic conditions, producing a purple substance with an absorbance between 540 and 620 nm (Jiang *et al.*, 1990; Nourooz-Zadeh *et al.*, 1994). To detect and quantify intracellular H₂O₂, fungal isolates were grown on CM agar plates with a layer of cellophane for 3 days. Mycelia (1 g) were scraped off, ground with liquid nitrogen and soaked in 3 mL of 100 mM phosphate buffer (pH 7.0). Protein samples (50 µL) were added to a reaction mixture (500 µL) containing 250 µM ammonium ferrous(II) sulphate, 25 mM sulphuric acid, 100 mM sorbitol and 125 µM xylenol orange, and incubated at room temperature for 15 min. The amounts of H₂O₂ in the solution were determined spectrophotometrically by measuring the absorbance at 595 nm. Extracellular H₂O₂ was assayed by measuring an increase in absorbance at 240 nm (Aebi, 1984). Fungal isolates were cultured in liquid CM for 3 days and the amounts of H₂O₂ in the medium were determined spectrophotometrically over time. The concentration of H₂O₂ was calculated by referring to a standard curve that was established using authentic H₂O₂. ROS production was also evaluated using H₂DCFDA (Molecular Probes, Eugene, OR, USA). Fungal hyphae were stained with H₂DCFDA and examined with a Leitz Laborlux phase contrast microscope (Leica Microsystems, Deerfield, IL, USA).

Pathogenicity and toxin production

Assays for fungal pathogenicity were performed on detached Minneola (*Citrus paradisi* Macfad. × *C. reticulata* Blanco) or calamondin (*Citrus mitis* Blanco) leaves inoculated by placing 10 µL of conidial suspension on each spot or by uniformly spraying them with a minisprayer, as described previously (Lin *et al.*, 2009). The inoculated leaves were incubated in a mist box for 2–4 days for lesion development. ACT toxin was extracted from fungal cultures using Amberlite XAD-2 resin and ethyl acetate, and analysed by thin layer chromatography as described by Kohmoto *et al.* (1993). The production of ACT toxin was also assessed by applying 5 µL of ethyl acetate extracts onto detached calamondin leaves.

Nucleotide sequences

Sequence data reported in this article have been deposited in the GenBank/EMBL Data Libraries under Accession Nos. JN900389 (*AaNoxA*), FJ376607 (*AaAP1*) and GQ414509 (*AaHOG1*).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Alignment of amino acids among fungal NADPH oxidases NOXA and NOX1.

Fig. S2 Targeted disruption of *AaNoxA* in *Alternaria alternata* using a split marker approach. (A) Schematic depiction of the generation of truncated, but overlapping, hygromycin phosphotransferase gene (*HYG*) under the control of the *Aspergillus nidulans trpC* promoter, and gene disruption within *AaNoxA*. Oligonucleotide primers used to amplify each fragment are also indicated. (B) Image of DNA fragments amplified from genomic DNA prepared from wild-type (WT) and two putative disruptants (DN2 and DN6) with the primers Nox-atg and Hyg3, or the primers Nox-taa and hyg4, electrophoresed in agarose gel and stained with ethidium bromide. (C) RNA gel blotting. Fungal RNA was electrophoresed in a formaldehyde-containing denaturing gel, blotted and washed at high stringency after hybridization with an *AaNoxA* probe.

Fig. S3 Assays for sensitivity of the wild-type (WT), the *AaNoxA* mutant (ΔN) and the *AaAP1* mutant (ΔY) strains, and their com-

plementation strains (Cp) of *Alternaria alternata*. Fungal strains were grown on potato dextrose agar (PDA) or PDA amended with test compounds as indicated for 4–7 days. Radial growth was measured. Only certain representative replicates are shown. MND, menadione; t-BHP, *tert*-butyl-hydroxyperoxide.

Fig. S4 (A) Development of necrotic lesions on detached calamondin leaves after 3 days of treatment with ethyl acetate extracts prepared from cell-free culture filtrates of *Alternaria alternata* wild-type (WT), *noxA* deletion mutants DN2 and DN6, and *noxA* DN2/*AaNoxA* complementation strains (NCp1 and NCp7) grown in Richard's medium for 24 days. The mock controls were treated with ethyl acetate only. (B) Thin layer chromatography analysis of the ethyl acetate extract separated with benzene–ethyl acetate–acetic acid, indicating that the *noxA* null mutants are able to produce ACT toxin (indicated by an arrow) at levels similar to the wild-type.

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