Appressorium-localized NADPH oxidase B is essential for aggressiveness and pathogenicity in the host-specific, toxin-producing fungus *Alternaria alternata* Japanese pear pathotype

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SUMMARY

Black spot disease, *Alternaria alternata* Japanese pear pathotype, produces the host-specific toxin AK-toxin, an important pathogenicity factor. Previously, we have found that hydrogen peroxide is produced in the hyphal cell wall at the plant–pathogen interaction site, suggesting that the fungal reactive oxygen species (ROS) generation machinery is important for pathogenicity. In this study, we identified two NADPH oxidase (*NoxA* and *NoxB*) genes and produced *nox* disruption mutants. $\Delta noxA$ and $\Delta noxB$ disruption mutants showed increased hyphal branching and spore production per unit area. Surprisingly, only the $\Delta noxB$ disruption mutant compromised disease symptoms. A fluorescent protein reporter assay revealed that only NoxB localized at the appressoria during pear leaf infection. In contrast, both NoxA and NoxB were highly expressed on the cellulose membrane, and these Nox proteins were also localized at the appressoria. In the $\Delta noxB$ disruption mutant, we could not detect any necrotic lesions caused by AK-toxin. Moreover, the $\Delta noxB$ disruption mutant did not induce papilla formation on pear leaves. Ultrastructural analysis revealed that the $\Delta noxB$ disruption mutant also did not penetrate the cuticle layer. Moreover, ROS generation was not essential for penetration, suggesting that NoxB may have an unknown function in penetration. Taken together, our results suggest that NoxB is essential for aggressiveness and basal pathogenicity in *A. alternata*.

INTRODUCTION

Spore dispersal pathogens can easily spread their propagules. However, to manifest disease symptoms, the spores must pen-

etrate into the host cell. Therefore, these kinds of pathogens have developed infection machinery, such as the appressorium and infection peg, which facilitate penetration. The molecular components involved in appressorium formation have been well studied. Recently, the mitogen-activated protein kinase (MAPK) and cyclic adenosine monophosphate (cAMP) signalling cascades have been shown to be essential for appressorium formation (Wilson and Talbot, 2009). These signalling cascades are thought to be involved in glycogen metabolism to generate turgor pressure (Wilson and Talbot, 2009). However, the molecular mechanisms mediating the dramatic change in polarity required for penetration into the host cells remains to be determined. Mps1, a component of the MAPK cascade, appears to be involved in penetration in *Magnaporthe oryzae* (Xu *et al.*, 1998). Moreover, the disruption mutant $\Delta mps1$ has been shown to produce appressorium, but fails to allow penetration. These results suggest that the mechanisms involved in appressorium formation and penetration are distinct.

In the Japanese pear (*Pyrus pyrifolia*), *Alternaria alternata* Japanese pear pathotype has been shown to cause black spot disease. This fungal pathogen produces a host-specific (selective) toxin (HST), AK-toxin, which causes disease symptoms on specific cultivars, such as Nijisseiki, but is avirulent in the resistant cultivar Chojuro (Nishimura and Kohmoto, 1983). HST is considered to be the primary determinant of pathogenicity (Nishimura and Kohmoto, 1983; Scheffer and Livingston, 1984; Yoder, 1980). HST is known to kill host cells or to suppress host defence systems, subsequently allowing the fungal hyphae to penetrate easily into the host cells. Ultrastructural analysis has revealed that AK-toxin targets the plasmodesmata, causing electron ion leakage (Park *et al.*, 1987). Most researchers studying HST-producing fungi have mainly focused on the chemical characteristics, target sites and biosynthesis genes of HST. However, it is not clear how HST works on the host plant surface because the plant surface is covered with cuticle and it is difficult for HST to penetrate the host plant cells. However, HST production alone seems to be insufficient for

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pathogenicity. Nishimura *et al.* (1978) have proposed that aggressiveness (Gäuman, 1950) is a prerequisite for HST-producing phytopathogenic fungi. However, the evaluation of aggressiveness is challenging because no analytical measurements of aggressiveness have yet been defined. Indeed, most screening for pathogenic mutants in HST-producing phytopathogenic fungi has investigated mutants that are defective in HST biosynthesis, rather than broad changes in aggressiveness.

Fungal aggressiveness is characterized by a defined infection process. *Alternaria alternata* fungal spores germinate on pear leaves, extend their hyphae along the plant surface, form appressoria and then penetrate the host cells by a dramatic shift in cellular polarity at the apical hyphae, which is thought to be crucial for aggressiveness. In a previous study, transmission electron microscopy (TEM) analysis revealed that *A. alternata* penetrates the cuticle layers and extends into the pectin layer, suggesting a subcuticular intramural mode of infection (Suzuki *et al.*, 2002). Using the cerium chloride method in TEM (Shinogi *et al.*, 2003), we have shown that hydrogen peroxide is produced at the fungus–plant interaction site. Reactive oxygen species (ROS), including hydrogen peroxide, are generally produced by plants during disease resistance reactions. However, as we used an arborous plant as host (i.e. the Japanese pear), we did not observe any typical effector-induced disease resistance, such as hypersensitive reaction or oxidative burst (Kodama *et al.*, 1998). TEM analysis results have revealed cerium-positive signals on the fungal cell walls (Shinogi *et al.*, 2003), suggesting that ROS production plays an important role in the aggressiveness and pathogenicity of *A. alternata*. In addition, treatment with the NADPH oxidase (Nox) inhibitor diphenyleneiodonium (DPI) resulted in the disappearance of ROS at the fungal–plant interaction site and a loss of pathogenicity (Hyon *et al.*, 2010). Various Nox genes have been characterized in filamentous fungi. NoxA has been found to be involved in perithecia formation in *Aspergillus nidulans* and *Podospira anserina* (Lara-Ortiz *et al.*, 2003; Malagnac *et al.*, 2004). Moreover, Nox genes are also involved in fungal–plant interactions. In *M. oryzae*, both MoNoxA and MoNoxB mediate appressorium formation, making them essential for penetration (Egan *et al.*, 2007). Conversely, in *Botrytis cinerea*, NoxB is involved in penetration, whereas NoxA is involved in infectious growth *in planta* (Segmüller *et al.*, 2008). Nox1 of *Claviceps purpurea*, an orthologue of NoxA, is also involved in plant colonization (Giesbert *et al.*, 2008). Moreover, NoxA has been shown to be involved in the establishment of a symbiotic relationship between fungal pathogens and *Epichloë festucae* (Tanaka *et al.*, 2006). Thus, these filamentous fungi use fungal–plant interactions to facilitate infection; however, the differential expression patterns and localization of Nox proteins probably allow different strategies for pathogenicity or symbiosis.

In this study, we cloned the ROS-producing genes NoxA and NoxB in *A. alternata* Japanese pear pathotype and analysed the function, expression and localization of the resulting Nox proteins.

We also evaluated the importance of the ROS-producing machinery for aggressiveness and pathogenicity in HST-producing fungal pathogens.

RESULTS

Molecular cloning of Nox genes

To isolate Nox genes from *A. alternata* Japanese pear pathotype, we obtained partial polymerase chain reaction (PCR) fragments of NoxA from *A. alternata* using degenerate primers and heterologous PCR fragments of NoxB and NoxC obtained from *M. oryzae*. We used these PCR fragments as probes to screen for entire Nox genes from an *A. alternata* cosmid library. We selected cosmid clones from NoxA and NoxB probes, but did not obtain a positive signal from the NoxC probe, despite low-stringency conditions. The selected cosmid clones were subcloned. The subclone NoxA (pCoAaNoxAEI) encoded 0.49 kb of the 5′-untranslated region (5′-UTR), 1.81 kb of the coding region and 2.41 kb of the 3′-UTR, and subclone NoxB (pCoAaNoxBEI) encoded 2.44 kb of the 5′-UTR, 1.84 kb of the coding region and 0.24 kb of the 3′-UTR. NoxA and NoxB contained consensus motifs for a NADPH-binding domain, a FAD-binding domain and a haem-binding motif (Fig. S1, see Supporting Information). Phylogenetic analysis revealed that NoxA and NoxB had diverged before fungal speciation (Fig. 1).

Phenotypic analyses of nox gene disruption mutants

Next, we prepared $\Delta noxA$, $\Delta noxB$ and $\Delta noxA noxB$ disruption mutants (Fig. 2). In all mutants, mycelial growth rates were

Fig. 1 Phylogenetic relationship of human and fungal NADPH oxidase sequences. Amino acid sequence alignment and tree construction were performed with CLUSTALW software using MegAlign, a program in the Lasergene package (DNASTAR). GenBank or EMBL accession numbers are as follows: *Alternaria alternata* Japanese pear pathotype AaNoxA (AB646195) and AaNoxB (AB646288) (designated in bold type); *Botrytis cinerea* BcNoxA (CAP12516) and BcNoxB (CAP12517); *Epichloë festucae* EfNoxA (BAE72680) and EfNoxB (BAE72682); *Claviceps purpurea* CpNox1 (CAP12327) and CpNox2 (CAP12328); *Podospira anserina* PaNox1 (AAK50853) and PaNox2 (AAQ74977); *Magnaporthe oryzae* MoNox1 (ABS01490) and MoNox2 (ABS01491); *Homo sapiens* HsNox2 (NP_000388).

Fig. 2 Disruption of *NoxA* and *NoxB* in the Japanese pear pathotype of *Alternaria alternata*. (A) Physical map of the *A. alternata* Japanese pear pathotype *NoxA* and *NoxB* wild-type genomic regions (pAaCoNxA and pAaCoNxB) and linear inserts of replacement constructs (pDiAaNxA and pDiAaNxB) showing restriction enzyme sites for *EcoRV* (E). (B) Southern analysis of the wild-type strain No. 15A (WT), *noxA* disruption mutant strain A6 ($\Delta noxA$), *noxB* disruption mutant strain B11 ($\Delta noxB$), and *noxA* and *noxB* double-disruption mutant strain AB17 ($\Delta noxA\Delta noxB$). Genomic DNA was digested with *EcoRV* and hybridized with pAaCoNxA (probe A) or pAaCoNxB (probe B) fragments containing *Nox* regions digested with *EcoRV*. Molecular size markers in kilobases of *HindIII*-digested λ DNA markers are indicated.

reduced slightly (Fig. 3A,B), aerial hyphae became denser and the amount of sporulation per unit area increased (Fig. 3C). Morphologies of sporulation were the same among *nox* mutants, i.e. all isolates produced 'polo-type' spores (Fig. 3D). We also found that all *nox* mutants exhibited a hyperbranching phenotype (Fig. 4A,B). These phenotypic traits were more prominent in the $\Delta noxB$ mutant. $\Delta noxA$ and $\Delta noxB$ disruptions exhibited additive effects in the $\Delta noxA\Delta noxB$ double-disruption mutant (Fig. 4A,B).

An inoculation test revealed that the $\Delta noxA$ disruption mutant was still pathogenic, but the $\Delta noxB$ disruption mutant lost its pathogenicity (Fig. 5). The $\Delta noxA\Delta noxB$ disruption mutant also lost pathogenicity (data not shown). Next, we evaluated the ability of *nox* mutants to infect onion epidermal cells or cellulose membranes. Again, both $\Delta noxB$ and $\Delta noxA\Delta noxB$ disruption mutants were unable to infect these cells or tissue types (Fig. 4C). Electron microscopic observation revealed that the $\Delta noxB$ disruption mutant attempted to invade the host cell, but failed to penetrate (Fig. 6C,D). A subclone of the genomic *NoxB* gene containing a 0.88-kb upstream region complemented the penetration ability and lesion formation (Fig. 5). We also evaluated whether the $\Delta noxB$ mutant was defective in other aspects of the infection process by performing wound inoculation. In the $\Delta noxB$ inoculation on cultivar Nijisseiki, disease lesions were observed around the wounding site, although the lesions were smaller than those caused by inoculation with wild-type *A. alternata* and the $\Delta noxA$ mutant, suggesting that AK-toxin was effective around the wounding area (Fig. 5). By contrast, the inoculation of heat-shocked cv. Chojuro with the $\Delta noxB$ mutant did not cause any disease symptoms (Fig. 5), suggesting that the $\Delta noxB$ mutant was defective in both penetration ability and infectious growth *in planta*.

Hydrogen peroxide production in *nox* gene disruption mutants

Nox genes are thought to be involved in ROS production. Therefore, we evaluated hydrogen peroxide production in *nox* mutants during the infection process by light and electron microscopy. On pear leaf, 3,3'-diaminobenzidine (DAB) staining revealed that brown positive signals, indicating hydrogen peroxide formation, were detected at the appressoria in wild-type fungi and the $\Delta noxA$ mutant (Fig. 7A). In contrast, neither the $\Delta noxB$ nor the $\Delta noxA\Delta noxB$ mutant showed positive signals at the appressoria (Fig. 7A). TEM observation with cerium chloride treatment revealed that cerium deposits were diminished dramatically in $\Delta noxB$ and $\Delta noxA\Delta noxB$ mutants (Fig. 6C,D,F). Decreased hydrogen peroxide production was also observed in the $\Delta noxA$ mutant, albeit only slightly (Fig. 6F). We also found that the *noxB* disruption mutants ($\Delta noxB$ and $\Delta noxA\Delta noxB$) produced hydrogen peroxide ectopically, i.e. at locations other than the fungus-plant interaction site (Fig. 6C,E). Interestingly, in the wild-type and $\Delta noxA$ strains, hydrogen peroxide production was only minimally observed on onion epidermal sheaths and cellulose membranes by DAB staining, although the hyphae did penetrate into both substrates (Fig. 7B,C).

Expression of *Nox* genes

Next, we analysed the expression of *Nox* genes in the vegetative hyphae, cellulose membranes of germlings and Japanese pear leaves by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) of total RNA. Both *NoxA* and *NoxB* were expressed at relatively low levels during vegetative growth, but were induced during infection in both the cellulose membrane and leaves (Fig. 8A). Moreover, *NoxA* expression was generally higher

Fig. 3 Mycelial growth, sporulation and spore numbers in *Alternaria alternata* Japanese pear pathotype wild-type strain and *nox* mutants. (A) Colony morphologies on potato dextrose agar (PDA) medium. (B) Colony growth curves on PDA medium. (C) Spore numbers ($\times 10^3$) per square centimetre were calculated. Total spores were harvested from oatmeal agar, resuspended in 20 mL water and counted microscopically. Bars indicate standard errors (SEs), and different letters indicate significant differences (Tukey's test, $P < 0.05$). (D) Sporulation of *A. alternata* Japanese pear pathotype wild-type (WT), $\Delta noxA$, $\Delta noxB$ and $\Delta noxA noxB$ on oatmeal agar medium observed by scanning electron microscopy. All *nox* mutants produced spores (sp) of the 'polo' type. Bars, 50 μ m.

than *NoxB* expression, and *Nox* genes were induced most prominently on the cellulose membrane (Fig. 8A).

Localization of *Nox* gene products

Next, we evaluated the expression and localization of *Nox* proteins by in-frame translational fusion of fluorescent proteins at the C-terminus of *Nox* genes. In the case of *NoxA*, a subclone from the cosmid library contained 491 bp of the 5'-UTR that was considered to be insufficient as a natural promoter. Therefore, we obtained more of the upstream region by inverse PCR. The resulting construct was 1068 bp longer than the 5'-UTR of *NoxB*, suggesting that the

NoxA construct may have a sufficient promoter region (Fig. S2A, see Supporting Information). The *NoxA*- and *NoxB*-mCherry transformants complemented defects, e.g. reduced hyphal branching and recovered penetration ability (Fig. S2B,C). We could not see any mCherry-positive signal during vegetative growth (Fig. 8B). On the cellulose membrane, mCherry fluorescence was observed at appressoria in both the *NoxA*- and *NoxB*-mCherry transformants (Fig. 8C). Interestingly, in the case of pear leaf infection, mCherry fluorescence was observed at appressoria exclusively in the *NoxB*-mCherry transformants (Fig. 8D). The positive signals of *NoxB*-mCherry fluorescence were observed not only at appressoria, but also in infection hyphae (Fig. 8D).

Fig. 4 Morphological characteristics of *nox* gene mutants. (A) Spore germlings from *Alternaria alternata* Japanese pear pathotype, including wild-type (WT), $\Delta noxA$, $\Delta noxB$ and $\Delta noxAnoxB$, were inoculated on cellulose membranes at 25 °C for 24 h. Abbreviations: gt, germ tube; sp, spore. Bars, 25 μ m. (B) The frequencies of hyphal branching. Bars indicate standard errors (SEs), and different letters indicate significant differences (Tukey's test, $P < 0.05$). (C) The formation rate of infection hyphae was calculated from the total number of appressoria in the WT strain and *nox* mutants on onion epidermis at 25 °C. Diphenyleioidonium (DPI) was used at 1 μ M concentration. Averages and SEs from three independent experiments are shown. Bars indicate SEs. * $P < 0.05$ versus WT. ** $P < 0.01$ versus WT.

Enzymatic activity and HST production in *nox* mutants

nox mutants have been shown to affect the expression of numerous genes in *P. anserina* (Brun *et al.*, 2009). Therefore, we tested whether *Alternaria noxB* mutants affected the enzyme activities of cutinase and pectinase, which are thought to be important for the facilitation of plant infection, and HST production. Cutinase and pectinase activities were not affected in *nox* disruption mutants, when compared with the activities of these enzymes in wild-type fungi (Fig. S3, see Supporting Information). Moreover, using high-performance liquid chromatography (HPLC), AK-toxin was detected in all *nox* disruption mutants at levels similar to those found in the wild-type (Fig. S4, see Supporting Information).

Importance of aggressiveness for *Alternaria* infections

We found that $\Delta noxB$ and $\Delta noxAnoxB$ disruption mutants were avirulent in the susceptible cultivar Nijisseiki. This result suggests

that HST production alone is not sufficient to induce pathogenicity in $\Delta noxB$ and $\Delta noxAnoxB$ mutants. We also observed the infection process of the wild-type 15A strain on the resistant cultivar Chojuro and of the nonpathogenic O-94 strain on cultivar Nijisseiki. In both cases, infection pegs penetrated the cuticle layer and retarded the pectin layer (Fig. 9A). When we inoculated nonpathogenic O-94 fungi supplemented with HST (AK-toxin) on cultivar Nijisseiki (which is AK-toxin susceptible), black spot disease lesions were observed (N. Hosogi, unpublished data). We also tested the effects of wound inoculation on pathogenicity. In heat-shocked resistant pear leaves (cv. Chojuro), the $\Delta noxB$ mutant did not develop necrotic lesions. In susceptible (cv. Nijisseiki) pear leaves, the $\Delta noxB$ mutant caused black spots at the wounding sites, but these black spots did not spread throughout the plant (Fig. 5). Although wild-type and $\Delta noxA$ *A. alternata* isolates induced papilla formation in both susceptible (Nijisseiki) and resistant (Chojuro) cultivars, the $\Delta noxB$ mutant did not induce papilla formation in either cultivar (Fig. 9B,C).

Fig. 5 Inoculation of *nox* mutants on Japanese pear leaves. Disease symptoms of *Alternaria alternata* wild-type (WT) strain and *nox* mutants on Japanese pear cultivars. Fungal isolates from WT, $\Delta noxA$ and $\Delta noxB$ were inoculated on susceptible (Nijisseiki), heat-shocked resistant (Chojuro), wounded susceptible (Nijisseiki) and wounded heat-shocked resistant (Chojuro) pear leaves. Droplets of spore suspension (30 μ L; left, designated as '+') or distilled water (right, designated as '-') were inoculated on the detached leaves and incubated at 25 °C for 24 h. Arrows indicate the inoculation sites.

DISCUSSION

In this study, we found that NoxB, a ROS generator, was essential for penetration, which is a main pathogenicity factor determining the aggressiveness of plant pathogenic fungi. In *A. alternata*, two copies of the *Nox* gene were cloned, *NoxA* and *NoxB*. Despite low-stringency conditions using a heterologous *NoxC* fragment probe from *M. oryzae*, we were unable to obtain a clone of the *NoxC* gene, suggesting that the *A. alternata* *NoxC* gene may be a pseudogene, as in *Alternaria brassicicola* (Takemoto *et al.*, 2007), or may have been lost during speciation. From our data, we speculate that these distinct *Nox* genes may encode proteins with different roles.

In several fertile filamentous fungi, *NoxA* genes are essential for sexual reproduction (Lara-Ortiz *et al.*, 2003; Malagnac *et al.*, 2004), and ROS is thought to act as a secondary messenger to regulate the differentiation of sexual fruiting bodies. In contrast, NoxB is not involved directly in sexual reproduction. However, mutations in the *NoxB* gene result in a loss of the ability to undergo ascospore germination in *Neurospora crassa* and *P. anserina* (Cano-Domínguez *et al.*, 2008; Malagnac *et al.*, 2004), but not in *B. cinerea* (Segmüller *et al.*, 2008). In *A. alternata*, it is believed that the sexual stage was lost during speciation. Therefore, we could not evaluate the involvement of *Nox* genes in sexual reproduction in *A. alternata*, but instead evaluated vegetative growth traits.

In the current study, we found that the hyphae in both *nox* mutants exhibited slow growth and hyperbranching. The hyphae were dense, and the conidiation rate was increased per unit area. These traits were similar to those observed after mutation of *Nox* genes in *B. cinerea* (Segmüller *et al.*, 2008), but different from those observed in *M. oryzae*, in which the *MonoxA* mutant exhibited a faster growth rate than the wild-type strain, the *MonoxB* mutant exhibited the same growth rate as the wild-type strain and the double *MonoxA**noxB* mutant exhibited a dramatic reduction in conidiogenesis (Egan *et al.*, 2007). In other filamentous fungi, *P. anserina* Nox1 (*NoxA*) affects mycelial pigmentation (Malagnac *et al.*, 2004) and *N. crassa* Nox-1 (*NoxA*) affects aerial hyphae formation (Cano-Domínguez *et al.*, 2008). In contrast, *Nox* genes do not affect any vegetative growth phenotype in *As. nidulans* (Lara-Ortiz *et al.*, 2003). Based on these results, the functions of Nox proteins seem to be divergent during vegetative growth.

In several phytopathogenic fungi, *Nox* genes are involved in pathogenicity. In *M. oryzae*, both *NoxA* and *NoxB* are essential for penetration at the appressorium and invasive growth *in planta* (Egan *et al.*, 2007). In *B. cinerea*, *NoxA* is involved in the determination of the lesion diameter, whereas NoxB is involved in primary lesion formation, and NoxB is known to contribute more strongly than *NoxA* to pathogenicity (Segmüller *et al.*, 2008). In contrast, in *C. purpurea*, *NoxA* is involved in pathogenicity, and NoxB does not affect the colonization stage (Giesbert *et al.*, 2008; Schuermann

Fig. 6 Ultrastructural analysis of hydrogen peroxide generation in the appressoria of *Alternaria alternata* wild-type (WT) fungus and *nox* mutants on Japanese pear leaves. The isolates from WT strain No.15A (A), $\Delta noxA$ (B), $\Delta noxB$ (C) and $\Delta noxAnoxB$ (D) were inoculated on heat-shocked resistant pear (Chojuuro) leaves at 25 °C for 24 h. Arrowheads indicate cerium peroxide deposits indicative of hydrogen peroxide production. Abbreviations: ap, appressorium; ih, infection hypha; pc, plant cuticle; ue, upper epidermal cell. Bars, 0.5 μm . (E) Distribution of hydrogen peroxide-reactive product loci in the appressoria cell walls of the WT strain and *nox* mutants on the leaves. Type 1, cerium-positive signals were detected at the fungus–plant interaction site. Type 2, cerium-positive signals were detected at a site other than that of Type 1. Type 3, a mixture of Type 1 and Type 2. Type 4, no cerium-positive signals were detected. (F) Relative area ratio of hydrogen peroxide-reactive products in the appressorium cell wall (μm^2) to the area of the appressorium cell wall (μm^2), measured by image analysis. Bars indicate standard errors (SEs) of the mean ($n = 50$). Significant differences by *t*-test: * $P < 0.05$ and ** $P < 0.01$, both versus hydrogen peroxide production in WT.

Fig. 7 Detection of hydrogen peroxide by 3,3'-diaminobenzidine (DAB) staining during the infection process. (A) Fungal isolates of wild type, $\Delta noxA$, $\Delta noxB$ and $\Delta noxA noxB$ mutants were inoculated on the Japanese pear cultivar Chojuro (heat shocked) (A), onion epidermal sheaths (B) and cellulose membrane (C). Abbreviations: ap, appressorium; sp, spore. Bars, 25 μ m in 7A and 10 μ m in 7B, 7C.

et al., 2012). Moreover, in *E. festucae*, wild-type NoxA promotes mutualistic interactions with the host plant, whereas its deletion mutant is pathogenic; NoxB does not affect mutualism or pathogenicity in this organism (Tanaka *et al.*, 2006). Therefore, different *Nox* genes may contribute to pathogenicity in different ways for each phytopathogenic fungus.

In *A. alternata* Japanese pear pathotype, only NoxB was involved in pathogenicity. In another necrotrophic pathogen, *A. alternata* of citrus, it has been reported recently that *AaNoxA* is involved in ROS generation, resistance to oxidative stress and partially in fungal virulence, but the effect of *AaNoxB* was not examined (Yang and Chung, 2012). In our case, the *NoxA*-involved phenotype was modest compared with the citrus pathogen. Based on our cytological analyses, the $\Delta noxB$ disruption mutant lost its ability to penetrate the plant. Moreover, the $\Delta noxB$ disruption mutant did not develop lesions, even after wound inoculation of heat-treated resistant leaves (susceptible and AK-toxin-insensitive leaves). These traits were similar to those of *M. oryzae*. However, in *A. alternata*, only NoxB was involved in pathogenicity. To further explore these characteristics, we developed a reporter construct

consisting of a *Nox* protein fused with mCherry. On the cellulose substrate, mCherry signals were detected in both *NoxA* and *NoxB* fusion strains. This result could be explained by the fact that both *Nox* genes were highly expressed on the cellulose membrane, as determined by qRT-PCR. In contrast, when we inoculated these *Nox* reporter strains on Japanese pear leaves, only the *NoxB*-mCherry strain gave positive mCherry signals at the appressoria and infection hyphae. Moreover, *NoxB* localization and pathogenicity were correlated with ROS localization, as measured by the DAB staining method on pear leaves. This difference in the localization of *Nox* proteins may account for their differential contribution to pathogenicity. From qRT-PCR, we found that both *NoxA* and *NoxB* were induced at the early infection process on pear leaves, suggesting that differences in the localization of *Nox* proteins may be involved in post-translational processes.

Examples of the differential roles of *NoxA* and *NoxB* during vegetative growth have been reported in *P. anserina*. When *P. anserina* hyphae extend on a cellulose substrate, *Nox2* (*NoxB*) participates in hyphal reorientation towards cellophane and *Nox1* (*NoxA*) participates in the differentiation of needle-like hyphae

Fig. 8 Expression profile and localization of *NoxA* and *NoxB* in *Alternaria alternata* Japanese pear pathotype. (A) Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of *NoxA* and *NoxB* genes in *A. alternata* Japanese pear pathotype wild-type isolate. Total RNA was extracted from vegetative hyphae, inoculated on pear leaves [4–12 h post-inoculation (hpi)] and inoculated on cellulose membranes (4–12 hpi). Relative expression units were normalized to the expression of β -tubulin. (B–D) Localization of *NoxA*-mCherry (left) and *NoxB*-mCherry (right) fluorescent proteins during vegetative growth (B) and spore germination on cellulose membranes (C) and pear leaves (D). Scale bars, 10 μ m.

Fig. 9 The *noxB* mutant failed to penetrate the pectin layer and induce papilla formation in pear leaves. (A) Ultrastructure of appressoria on pear leaves. In the $\Delta noxB$ mutant, appressoria failed to penetrate the plant cuticle. In contrast, *A. alternata* Japanese pear pathotype (15A) and an *A. alternata* nonhost-specific (selective) toxin (HST) producer (O-94) penetrated the plant cuticle, but were stopped by the pectin layer on the resistant cultivar (Chojuro). Abbreviations: ap, appressorium; pa, papilla; pc, plant cuticle. Bars, 1 μm . (B) Papilla formation on susceptible (Nijisseiki) and resistant (Chojuro) cultivars inoculated with wild-type (15A), $\Delta noxA$ or $\Delta noxB$. Left, bright field; right, epifluorescent field (WU filters). Bars, 50 μm . (C) Papilla formation rate (%) on susceptible (Nijisseiki) and resistant (Chojuro) cultivars inoculated with wild-type (15A), $\Delta noxA$ or $\Delta noxB$. Significant difference by *t*-test: ***P* < 0.01, compared with the wild-type.

(Brun *et al.*, 2009). PaNox2 (PaNoxB) is important for the establishment of the bulges that contact the surface of the substrate, which are similar to appressoria in phytopathogenic fungi (Brun *et al.*, 2009). *Podospira anserina* is unable to penetrate living material, however, and Nox machineries may have evolved to overcome a degrading cellulose substrate and acquire nutrients at appropriate target sites. Thus, it is thought that NoxB-mediated penetration mechanisms are important in the pathogenic processes of phytopathogenic fungi.

The impact of Nox-mediated ROS generation and its derivative signalling cascades is still unknown. In onion epidermal sheaths or cellulose membranes, wild-type *A. alternata* penetrated into both substrates without hydrogen peroxide production at appressoria. This evidence suggests that ROS production at the appressorium is not essential for penetration and that some pear leaf components are required for hydrogen peroxide production. Future studies are needed in order to elucidate the components responsible for ROS

generation in pear leaves. In addition, Nox proteins may have undiscovered functions. In *P. anserina*, Nox2 (NoxB) participates in reorientation of the cellulose degradation machinery (Brun *et al.*, 2009). In *A. alternata*, NoxB may regulate reorientation of the substrate penetration machinery. Indeed, when the $\Delta noxB$ disruption mutant was inoculated on the Japanese pear leaf, ROS was detected at regions different from the plant–pathogen interaction site.

Black spot disease, caused by *A. alternata* Japanese pear pathotype, produces AK-toxin, which is an important disease determinant factor. Our study showed that the $\Delta noxB$ deletion mutant could not produce black spot lesions, despite its ability to produce AK-toxin. Nishimura and Kohmoto (1983) have reported that HST production alone is not sufficient for full pathogenicity, whereas aggressiveness (Gäuman, 1950) of phytopathogenic pathogens is essential in necrotrophic pathogens. In this study, we found that NoxB was involved in the determination of the aggressiveness of

A. alternata. It is possible that cell wall-degrading enzymes are involved in penetration; however, the $\Delta noxB$ deletion mutant did not affect cell wall-degrading enzymes, including cutinase and pectinase. In our ultrastructural analysis, the $\Delta noxB$ disruption mutant did not penetrate into the cuticle layer. In contrast, *A. alternata* O-94, a non-toxin producer, could penetrate into the cuticle layer. Moreover, the $\Delta noxB$ disruption mutant lost the ability to form papilla, suggesting that the $\Delta noxB$ disruption mutant could not penetrate and subsequently could not be sensed by host plant cells. Again, these data indicate that NoxB is important for penetration. This trait may already be present for the fundamental aggressiveness of *A. alternata*, regardless of the strain (i.e. pathogenic or nonpathogenic strain). Considering the evolution of *Alternaria* phytopathogenic fungi, we know that the HST biosynthesis gene cluster confers phytopathogenicity to *Alternaria* species. Future studies are needed to elucidate the details of NoxB protein function in the fungal penetration of the Japanese pear leaf.

EXPERIMENTAL PROCEDURES

Fungal strains

Alternaria alternata Japanese pear pathotype no. 15A and nonpathogenic O-94 (non-HST producer) were used in this study (Tanabe *et al.*, 1988). The *nox* mutants created in this study are listed in Table S1 (see Supporting Information). We maintained the isolates on potato dextrose agar (PDA; Becton Dickinson, Franklin Lakes, NJ, USA) medium. To promote sporulation, mycelial plugs were inoculated on oatmeal agar medium (40 g oatmeal, 20 g agar and 5 g sucrose in 1 L water) at 25 °C for 4 days, and the aerial mycelia were then rubbed and incubated for an additional 3 days at 25 °C under near-UV light (360 nm, 40 W). Spores were harvested with distilled water and filtered using Kimwipe paper (Crecia Corp., Tokyo, Japan). The fluids were centrifuged once at $1200 \times g$ for 5 min. We collected the spores, suspended them in distilled water and adjusted the spore concentration to 5×10^5 spores/mL for lesion observation and 3×10^6 spores/mL for electron microscopy observation.

Cloning of NADPH oxidase genes from *A. alternata*

Genomic DNA was extracted as described previously (Ikeda *et al.*, 2011). First, we designed degenerate primers from the consensus regions of *NoxA*, *NoxB* and *NoxC*. The PCR conditions were as follows: 20- μ L reaction mixture containing 1 U rTaq DNA Polymerase (Toyobo, Osaka, Japan), $1 \times$ PCR buffer (with the addition of 2.5 mM $MgCl_2$), 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.5 μ M each primer and 2 ng template DNA in a Mastercycler (Eppendorf Japan, Tokyo, Japan) programmed for 10 min at 95 °C, 35 cycles of 1 min at 94 °C, 30 s at 55 °C and 1 min at 72 °C, and a final extension for 10 min at 72 °C. We only obtained PCR fragments from *NoxA* primers (*NoxA*-F and *NoxA*-R; the primers used in this study are listed in Table S2, see Supporting Information). For the cloning of *NoxB* and *NoxC*, we obtained partial fragments of *MoNoxB* (MGG_06559.6) and *MoNoxC* (MGG_08299.6) from the genomic DNA template of *M. oryzae*

Guy11. We constructed the following primers: *MoNoxB*-Fw, *MoNoxB*-Rv, *MoNoxC*-Fw1, *MoNoxC*-Rv1, *MoNoxC*-Fw2 and *MoNoxC*-Rv2.

To obtain entire genomic sequences of *Nox* genes from *A. alternata*, we screened a cosmid library for these genes (Kimura and Tsuge, 1993) using colony replica hybridization probes with partial sequences of *NoxA* from *A. alternata* and *NoxB* and *NoxC* (two fragments from four primer combinations) from *M. oryzae*. Positive signals were obtained only from *MoNoxA* and *MoNoxB* probes. The screened cosmid clones (pCoAaNoxA and pCoAaNoxB) were digested with *EcoRI* and subcloned into pBluescriptII SK+ vector, e.g. pCoAaNoxAEI containing 4.7 kb of *NoxA* and pCoAaNoxBEI containing 4.5 kb of *NoxB*. The *NoxA* and *NoxB* nucleotide sequences containing flanking regions were determined using an ABI3100 (Applied Biosystems, Tokyo, Japan) and deposited in GenBank as accession numbers AB646195 and AB646288, respectively.

Disruption of *Nox* genes by homologous recombination

To obtain disruption mutants of *Nox* genes, we performed homologous recombination using the disruption vectors pSP72-HPH and pSP72-NPT. *Bam*HI-*Sal*I fragments of pDH25 and pII99 (Kimura and Tsuge, 1993), containing PtpC-hygromycin B phosphotransferase (*hph*) and PtpC-neomycin phosphotransferase (*nptII*), respectively, were ligated with *Bam*HI-*Sal*I-digested pSP72 (Promega, Madison, WI, USA), resulting in pSP72-HPH and pSP72-NPT, respectively. PCR products targeting the upstream and downstream regions of *NoxA* were ligated into pSP72-HPH, and those targeting the upstream and downstream regions of *NoxB* were ligated into pSP72-NPT, resulting in pDiAaNoxA and pDiAaNoxB, respectively.

Reporter gene construction

To evaluate the expression and localization of *Nox* genes, we constructed reporter genes that were fused with mCherry in-frame at the C-terminus using an In Fusion HD Cloning Kit (Clontech, Mountain View, CA, USA). In the case of *NoxA* reporter genes, because the 5'-end was too short (491 bp), we performed inverse PCR. *Pst*I-digested genomic DNA was ligated and amplified with AaNoxA13 and AaNoxA11 primers. The amplified fragments were sequenced and ligated into pCoAaNoxAEI using the In Fusion HD Cloning Kit, resulting in pCoAaNoxAEI', containing the 1.0-kb upstream region of *NoxA*. mCherry protein genes were obtained from pmCherry (Clontech). The forward primer was designed at the start codon and the reverse primer was designed at the stop codon of each fluorescent gene. The In Fusion HD Cloning Kit was used following the manufacturer's instructions. Each primer for the mCherry gene added a 15-bp nucleotide sequence compatible with the terminal sequence of each *Nox* gene and the 3'-flanking region of each *Nox* gene (see Fig. S2A and Table S2). pCoAaNoxAEI' and pCoAaNoxBEI were amplified with primers (see Fig. S2A and Table S2) which were designed to split just before the stop codon. The resulting reporter genes were designated as pReAaNoxA-mCherry and pReAaNoxB-mCherry, respectively.

Transformation and molecular techniques

Protoplast preparation and the transformation of *A. alternata* were performed using previously described methods (Tsuge *et al.*, 1990). Colonies

that appeared 5–10 days after plating on the selective regeneration medium (Tsuge *et al.*, 1990) were transferred to PDA containing hygromycin B at 100 µg/mL, and transformants were selected after incubation at 25 °C for 5 days. The integration of the construct plasmid in each mutant was analysed by PCR and Southern blotting. Colony replica hybridization, Southern blotting and other molecular techniques were performed as described by Sambrook and Russell (2001). For the detection of Southern blotting signals, we used the ECL Direct Nucleic Acid Labelling and Detection System (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

RNA isolation and qRT-PCR

Total RNA was isolated from frozen mycelial powder using Sepasol RNA I Super (Nacalai Tesque, Kyoto, Japan). One microgram of total RNA was treated with DNase I (Takara, Ohtsu, Japan) and reverse transcribed into first-strand cDNA using a PrimeScript RT Reagent Kit (Takara). qRT-PCR was carried out using Thunderbird SYBR qPCR Mix (Toyobo) according to the manufacturer's instructions with specific primers for our targets (AanoxA-qPCR-Fw, AanoxA-qPCR-Rv, AanoxB-qPCR-Fw and AanoxB-qPCR-Rv) and an internal control gene (β -tubulin; Aa- β -tub1 and Aa- β -tub2). Fluorescence from the DNA-SYBER Green complex was monitored in a Thermal Cycler Dice Realtime System (Takara) throughout the reaction. The expression of target mRNA was normalized to the expression of the housekeeping gene β -tubulin.

Inoculation

We used four inoculation materials: cellulose membrane 27/32 (Viskase Companies Inc., Darien, IL, USA), onion epidermal sheaths and two cultivars of Japanese pear [*Pyrus pyrifolia* var. *culta*: cv. Nijisseiki (susceptible) and cv. Chojuro (resistant)]. Young leaves of the Japanese pear were detached from terminal shoots that were grown in the orchard.

Drops of spore suspension (20 µL) were applied onto the surfaces of the inoculation materials described above. We also used heat-shocked resistant (induced susceptible) leaves to exclude the effect of AK-toxin, as *A. alternata* Japanese pathotype is known to produce AK-toxin (Tanaka, 1933). Heat-shocked resistant shoots were prepared by the method described by Shinogi *et al.* (2003) and Hyon *et al.* (2010). Spore-inoculated shoots were grown at 25 °C during a 12-h photoperiod. After inoculation, the spore-inoculated leaves were treated with a mixture of ethanol and acetic acid (96:4, v/v) at 25 °C overnight to remove chlorophyll. The specimens were stained with 0.25% Coomassie Brilliant Blue (Nacalai Tesque) in methanol, acetic acid and distilled water (50:5:45, v/v/v). We observed the infection behaviour of the fungal pathogen with a light microscope (BX51: Olympus, Tokyo, Japan). To detect callose deposition (papilla formation), aniline blue staining was examined (Sherwood and Vance, 1976).

Detection of hydrogen peroxide by light microscopy, TEM and scanning electron microscopy

To detect hydrogen peroxide by light microscopy and TEM, we performed DAB staining and cerium chloride staining, respectively. The specimen preparation, observation and image analysis have been described

previously (Hyon *et al.*, 2010; Shinogi *et al.*, 2003). Pieces of inoculated leaves were vacuum infiltrated in 1 mg/mL of DAB solution (Nacalai Tesque) and 5 mM CeCl_3 solution buffered with 50 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS; pH 7.2) at room temperature for 1 h. The pieces were prefixed in 2.5% glutaraldehyde, buffered with 0.1 M cacodylate buffer (pH 7.2) at 4 °C overnight, washed with the same buffer three times for 10 min each and post-fixed with 1% buffered osmium tetroxide at 4 °C for 1 h. The pieces were then dehydrated in a series of ethanol (50%, 70%, 90% and 100%) and embedded in Spurr's resin mixture (Nissin EM, Tokyo, Japan). Sections with a thickness of 90–120 nm were cut from resin blocks with an ultramicrotome (MT-1; Sorval, Norwalk, CT, USA) using a diamond knife (Diatome, Bienne, Switzerland). Unstained sections were observed with an H-7100 electron microscope (Hitachi, Hitachinaka, Japan). For image analysis, electron microscopic negatives were printed on the same printing paper at the same magnification (5000 $\times$). Each of the micrographs was scanned at 600 dots per inch (dpi) and saved as a JPEG file. We analysed the volume of cerium-reactive products on the electron micrographs using ImageJ software (<http://rsb.info.nih.gov/ij/index.html>). We then measured the areas of cerium-reactive products at appressoria cell walls. The relative ratio of the product area per unit area (μm^2) of the appressoria cell wall was determined as the ratio of the area of cerium peroxide deposit (μm^2) to the area of the appressoria cell wall (μm^2). We measured three different blocks from each of the infected leaves. For scanning electron microscopy analysis, freeze-dried mycelia were exposed to osmium vapour, sputtered with an osmium plasma coater (Meiwafosis Co. Ltd., Tokyo, Japan) and observed with an S-4800 scanning electron microscope (Hitachi).

Confocal microscopy observation

Image acquisition was performed using an FV1000 (Olympus) confocal microscope system. mCherry was excited by a 559-nm laser (diode-pumped solid state laser), and its emission was detected using an mCherry filter set.

Assay for AK-toxin production

We examined whether AK-toxin I was produced from germinating spores using the method described by Hyon *et al.* (2010). We uniformly sprayed 100-mL spore suspensions (5×10^5 spores/mL) onto paper towels and incubated them in a moist chamber for 24 h at 25 °C. The spore germination fluids were harvested by squeezing the paper towels and filtering the liquid through a filter paper to remove the spores. The filtrates were adjusted to pH 5.5 with 1 M KH_2PO_4 , and the toxin was extracted with diethyl ether. After removal of the solvent by vacuum evaporation, the residues were dissolved in 1 mL of methanol, and the samples were subjected to HPLC (SCL-10A, SPD-10A, LC-10AS; Shimadzu, Kyoto, Japan). The HPLC stainless steel column ($4.6 \times 250 \text{ mm}^2$) was packed with reverse-phase Shim-pack CLC-ODS M (Shimadzu). HPLC-grade solvents were used for analysis. Elutions were made with a mixture of acetonitrile, acetic acid and water (50:1:49, v/v/v) at a flow rate of 1 mL/min, and detection of the toxin was carried out by measuring the absorbance at 290 nm. Crystalline AK-toxins I and II, which were used as the authentic toxin in this study, were provided by Professor H. Miyagawa (Graduate School of Agriculture, Kyoto University, Kyoto, Japan).

Enzyme activities

Mycelial colonies were grown on cellulose membranes, ground in liquid nitrogen and extracted in protein extraction buffer. The dissolved solutions were centrifuged at 10 000 g, and the resulting supernatants were collected. Total esterase activity was measured using a Lipase Kit S (DS Pharma Biomedical Co., Ltd., Osaka, Japan) without phenylmethanesulphonyl fluoride (PMSF) supplementation. Pectinase activity was analysed using the method of Bach and Schollmeyer (1992).

ACKNOWLEDGEMENTS

We thank Professor Takashi Tsuge, Graduate School of Bioagricultural Sciences of Nagoya University, Nagoya, Japan, for providing the *A. alternata* 15A genomic cosmid library. This research was supported by Grants-in-Aid for Scientific Research B (No. 18380033) and Grants-in-Aid for Young Scientists A (No. 23688006) from the Japan Society for the Promotion of Science.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Alignment of the predicted amino acid sequences of NoxA and NoxB in *Alternaria alternata* Japanese pear pathotype with other phytopathological fungi. The amino acid sequences of NoxA and NoxB were aligned with *Botrytis cinerea* BcNoxA (CAP12516) and BcNoxB (CAP12517), *Epichloë festucae* EfNoxA (BAE72680) and EfNoxB (BAE72682), *Claviceps purpurea* CpNox1 (CAP12327) and CpNox2 (CAP12328), *Podospora anserina* PaNox1 (AAK50853) and PaNox2 (AAQ74977), and *Magnaporthe oryzae* MoNox1 (ABS01490) and MoNox2 (ABS01491), deduced from genomic sequence assembly and direct DNA sequencing. Amino acids conserved in all proteins are shaded. Consensus motifs of the NADPH-binding domain and FAD-binding domain are indicated. Haem-binding motifs are indicated by asterisks.

Fig. S2 Construction of fluorescent protein reporter genes and complementation tests of the defects of each *nox* disruption mutant. (A) Genetic maps of pReAaNxA-mCherry and pReAaNxB-mCherry constructs. (B) Number of branched hyphae in wild-type, $\Delta noxA$ and $\Delta noxA::noxA$ -mCherry strains. (C) Frequencies of infection hyphae formation in wild-type, $\Delta noxB$, $\Delta noxB::noxB$ -EGFP and $\Delta noxB::noxB$ -mCherry strains.

Fig. S3 Effects of plant cell wall-modifying enzymes in *nox* mutants. Cutinase and pectinase activities (relative activities) were evaluated.

Fig. S4 High-performance liquid chromatography (HPLC) analysis of the spore germination fluid of wild-type and *nox* mutants of

Alternaria alternata Japanese pear pathotype. Germination fluids from wild-type strain No. 15A (WT), *noxA* disruption mutant strain A6 ($\Delta noxA$), *noxB* disruption mutant strain B11 ($\Delta noxB$) and *noxA noxB* double-disruption mutant strain AB17 ($\Delta noxA\Delta noxB$) were harvested after incubation for 24 h and subjected to HPLC analysis to detect AK-toxin I. Arrowheads indicate the peaks of AK-toxin I production corresponding to authentic AK-toxin I. The amount of AK-toxin I (in femtomoles) released from a single germinated spore was calculated (average and standard errors).

Table S1 Fungal strains used in this study.

Table S2 Primers used in this study.