THE NADPH OXIDASE-MEDIATED OXIDATIVE STRESS SIGNALING, RESISTANCE, PATHOGENICITY AND DEVELOPMENT IN *Alternaria alternata* OF CITRUS

By

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To my parents

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$CaCl_2$	Calcium chloride
$Ca(NO_3)_2 \cdot 4H_2O$	Calcium nitrate 4-hydrate
CFW	Calcofluor white
CR	Congo red
DAB	3,3’-diaminobenzidine
DNA	Deoxyribonucleic acid
dpi	day post-inoculation
FAD	Flavin adenine dinucleotide
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HDA	Hydroxylamine hydrochloride
HP	Hematoporphyrin
HST	Host-selective toxin
HYG	Hygromycin phosphotransferase gene
INA	2,6-dichloroisonicotinic acid
KH_2PO_4	Monopotassium phosphate
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate
MAPK	Mitogen-activated protein kinase
MND	Menadione
min	minute
NaCl	Sodium chloride
Na_2HPO_4	Sodium phosphate dibasic
NADPH	Nicotinamide adenine dinucleotide phosphate
Nox	NADPH oxidase

N-Arg	Nitro-arginine methyl ester
ORF	Open reading frame
PDA	Potato dextrose agar
PEG	Polyethylene glycol 3350
RB	Rose Bengal
RMM	Regeneration medium
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
Sur	Sulfonylurea-resistance gene
tBH	*tert*-butyl hydroperoxide

Abstract of Dissertation Presented to the Graduate School
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The necrotrophic fungus *Alternaria alternata* causes brown spot disease in many citrus cultivars. This pathogen produces a host-selective toxin that kills host cells prior to colonization and acquires nutrients from dead cells. *A. alternata* infection of citrus leaves induces rapid lipid peroxidation and accumulation of hydrogen peroxide (H_2O_2). *A. alternata* has evolved an effective reactive oxygen species (ROS) detoxification system to successfully colonize within the oxidative environment of necrotic tissues. Fungal NADPH oxidase complex (Nox), which has been implicated in the production of low-level ROS, contains mainly NoxA, NoxB (gp91^{phox} homologs) and NoxR (p67^{phox} homolog). This dissertation research determines the developmental and pathological functions of Nox in the tangerine pathotype of *A. alternata*. Each of the *AaNoxA*, *AaNoxB* and *AaNoxR* genes was cloned and disrupted independently or in combination, revealing that all three Nox components are required for the accumulation of cellular ROS. Nox mutants also displayed increased sensitivity to H_2O_2 and various oxidants as well as reduced fungal virulence, implicating a critical role of AaNox in oxidative stress tolerance during host invasion. A fungal strain carrying a *NoxA NoxB* or a *NoxA NoxR* double mutation was more sensitive to the test compounds than the strain mutated at a

single gene. The expression of both *YAP1* and *HOG1* genes, whose products are involved in resistance to ROS, was down-regulated in fungi carrying defective *NoxA*, *NoxB* or *NoxR*. The requirement of Nox in ROS resistance was demonstrated further by characterizing an *AaGPx* gene encoding a putative glutathione peroxidase. *AaGPx* plays a role in oxidative stress resistance and virulence and its expression is coordinately regulated by NOX, YAP1 and HOG1. This research also demonstrates that Nox, likely interconnecting with the FUS3 MAP kinase- and the cAMP-dependent protein kinase A (PKA)-mediated pathways, plays a central role in vegetative growth and conidia formation. Interestingly, only *AaNoxB*, but not *AaNoxA*, has a role in the accumulation of chitin, the maintenance of cell wall integrity and the sensitivity to fungicides. These novel phenotypes have not been identified in any fungus. Overall, this study reveals that AaNox is one of the key regulators for ROS production and for signaling transductions leading to proper fungal development, as well as fungal virulence and cellular resistance to ROS; and that the ability of ROS-detoxification mediated by interconnected pathways is absolutely required for *A. alternata* survival and pathogenesis.

CHAPTER 1
LITERATURE REVIEW

Introduction of *Alternaria alternata* (Fr.) Keissler

Taxonomy and Geographical Distribution

Alternaria brown spot of citrus is caused by the necrotrophic fungus *Alternaria alternata* (Fr.) Keissler. The disease was first described on Emperor Mandarin in Australia in 1903 (Cobb, 1903). The causal pathogen was identified as *Alternaria citri* Ellis & Pierce (Kiely, 1964; Pegg, 1966) and later reclassified to *A. alternata* (Simmons, 1967; Kohmoto et al., 1979). *A. alternata* is classified in the Kingdom Fungi, Phylum Ascomycota, Class Dothideomycetes, Subclass Pleosporomycetidae, Order Pleosporales, and Family Pleosporaceae. Citrus brown spot was first reported in Florida on Dancy tangerine in 1976 (Whiteside, 1976). The disease is also known in many citrus-growing areas of South Africa, Israel, Turkey, Colombia, Spain, Italy, Brazil, Argentina, and Peru (Timmer et al., 2003; Peres and Timmer, 2006).

Host Range and Disease Symptoms

On citrus, *Alternaria* species cause four distinct diseases — Alternaria black rot of fruit, Alternaria brown spot of tangerine, Alternaria leaf spot of rough lemon and *Mancha foliar* on Mexican lime. Alternaria black rot is caused by *Alternaria citri* Ellis & Pierce. This disease causing internal decay is a common post-harvest disease worldwide. *A. citri* does not produce host-selective toxin (HST). This pathogen infects at the stylar or stem end of the fruit and causes an internal discoloration of the fruit core. *A. citri* infects citrus fruit through wounds or natural crack and endopolygalacturonase produced by *A. citri* is essential for symptom development (Isshiki et al., 2001; Katoh et al., 2006). *Mancha foliar* is caused by *A. limicola* (Palm and Civerolo, 1994). *A. limicola*

does not produce HST and primarily affects Mexican lime [*Citrus aurantifolia* (Christm. et Panz) Swingle]. It produces small, reddish brown lesions on leaves that are surrounded by chlorotic halos. Small raised lesions are produced on fruit, but the symptoms disappear as the fruit grows (Akimitsu et al., 2003).

Alternaria brown spot of tangerine and leaf spot of rough lemon are caused by two different pathotypes of *A. alternata* that produce distinct HST (Kohmoto et al., 1979; Kohmoto et al., 1991). On young leaves, lesions first appear as brown to black spots surrounded by yellow halos within 24 hours after infection (Fig. 1-1). Lesions usually continue to expand with circular or irregular chlorosis due to cell death caused by the toxin (Kohmoto et al., 1993). Chlorosis and necrosis often occur along the veins as the toxin is translocated through the vascular system. Young shoots are also infected and produce brown lesions. Shoot infection and abscission of affected leaves could result in twig dieback. Young fruit is susceptible to *A. alternata* infection soon after petal fall. Severely infected fruit sometimes abscise, resulting in yield loss (Timmer et al., 2000). Symptoms on mature fruit vary from small, dark specks to large, sunken lesions. The rough lemon pathotype affects rough lemon (*Citrus jambhiri* Lush) and Rangpur lime (*Citrus* x *limonia Osbeck*) (Timmer et al., 2003). Symptoms on fruit induced by the rough lemon pathotype are relatively smaller than those produced by the tangerine pathotype (Timmer et al., 2003). Both tangerine and rough lemon pathotypes of *A. alternata* are similar in morphology, but can be differentiated by host preference, toxin production and genetic analysis (Peever et al., 1999; Akimitsu et al., 2003).

Host-Selective Toxins

Host-selective toxins produced by *A. alternata* are essential for fungal pathogenesis (Gardner et al., 1986; Kohmoto et al., 1993) and are important

determinants of host range (Kohmoto et al., 1991; Walton, 1996). There are seven known pathogenic variants (pathotypes) of *A. alternata.* Each pathotype produces a unique HST that is selectively toxic to limited plant species or cultivars within a species (Table 1-1) (Nishimura and Kohmoto, 1983; Kohmoto et al., 1991; Ito et al., 2004). The tangerine pathotype produces ACT (*A. citri* toxin) that is toxic to tangerine (*C. reticulata* Blanco) and grapefruit (*C. paradisi* Macfad.), as well as hybrids from grapefruit and tangerine or tangerine and sweet orange (*C. sinensis* (L.) Osbeck). ACT containing a core 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid structure primarily disrupts plasma membrane and causes rapid electrolyte leakage (Kohmoto et al., 1993). The rough lemon pathotype produces ACRL toxin that is toxic to rough lemon (*C. jambhiri* Lush) and Rangpur lime (*C.* x *limonia* Osbeck). ACRL affects the functions of mitochondria, causing metabolite leakage, uncoupling of oxidative phosphorylation and changes in membrane potentials (Akimitsu et al., 1989; Otani et al., 1995). ACRL is not toxic to tangerines, grapefruit and their hybrids.

Life Cycle

A. alternata has no known sexual stage. Sporulation is affected by environmental stimuli (e.g. light, oxygen, nutrition and stress) (Rotem, 1994). Formation of conidia (asexual spore) is essential for dispersing fungal inoculum and for initiating Alternaria diseases on citrus. Conidia have thick and melanized cell walls and can tolerate unfavorable environmental conditions. Conidia produced on affected citrus leaves and fruit are dispersed by rain or wind. Under favorable conditions (e.g. high humidity, optimum temperature 25-30°C), *A. alternata* germinates in 1-3 hour to form penetration hyphae on susceptible cultivars (Akimitsu et al., 2003). *A. alternata* could penetrate

through stomata or natural wounds without the formation of appressoria. Fungal hyphae proliferate in the substomatal cavities (Rotem, 1994).

The Biology of Reactive Oxygen Species

All aerobic organisms generate reactive oxygen species (ROS), including superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$), as inevitable byproducts of normal metabolism (Apel and Hirt, 2004). ROS, partially reduced oxygen species, are highly toxic to cells and indiscriminately damage DNA, proteins, lipids, carbohydrates and other cell components (Heller and Tudzynski, 2011). ROS are harmful, but cells are able to mitigate by producing ROS scavenging enzymes, such as superoxide dismutases (SOD), catalases and peroxidases. ROS not only play a vital role in cellular defense against pathogen attack, but also serve as signaling molecules for cellular development and differentiation (Orozco-Cardenas et al., 2001; Brown and Griendling, 2009). ROS also play an important role in the plant-microbe interactions. Recognition of a non-compatible pathogen by the plant cells triggers an ‘oxidative burst’, defined by a rapid increase of ROS production and other defense responses (Auh and Murphy, 1995). However, the production of ROS by plants may have different effects towards pathogens with different lifestyles (biotrophs vs. necrotrophs). To successfully colonize a host plant and survive in harsh environments, necrotrophic fungi have evolved sophisticated mechanisms for cellular protection against the toxicity of ROS.

Generation of Reactive Oxygen Species

Ground state oxygen can be converted to toxic ROS either by energy transfer or by electron transfer reactions (Fig. 1-2). Formation of singlet oxygen ($^1O_2$) results from energy transfer, whereas sequential electron transfers between reducing agents lead to

the production of the highly reactive $O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$. $O_2^{\cdot-}$ and H_2O_2 may react with each other and generate highly reactive hydroxyl radicals and hydroxyl anions (OH^-) via the Haber-Weiss reaction (Kehrer, 2000) or with transition metals (e.g. iron and copper) via the Fenton reaction (Halliwell and Gutteridge, 1992). ROS are often produced as by-products of various metabolic pathways in mitochondria, peroxisomes and chloroplasts of plants. ROS can be generated by enzymatic and non-enzymatic systems. In the presence of reducing agents, $O_2^{\cdot-}$ can be generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Bedard and Krause, 2007), peroxidase (Bolwell et al., 2002; Bindschedler et al., 2006), xanthine oxidase (Harrison, 2002), oxalate oxidase (Hu et al., 2003), superoxide dismutase (SOD) (Auh and Murphy, 1995), or amine oxidase (Cona et al., 2006). In addition, many photo-activated perylenequinone pigments produced by many plant-associated fungi are capable of producing toxic ROS upon exposure to light (Daub et al., 2013).

ROS Scavenging

All cells have evolved effective mechanisms, both enzymatic and non-enzymatic, to cope with the toxicity of ROS. SOD, catalases, peroxiredoxins and peroxidases, such as glutathione peroxidase (GPx) have long been known to have catalytic ROS scavenging abilities (Fig. 1-3). SOD are able to dismutate $O_2^{\cdot-}$ into H_2O_2 and O_2, acting as the first line of ROS detoxification. Catalases, peroxiredoxins and peroxidases detoxify H_2O_2. Non-enzymatic antioxidants, such as glutathione, ascorbic acid, flavonoids, alkaloids and carotenoids are capable of detoxifying ROS (Jamieson, 1998). Glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine) containing a redox-active sulfhydryl group is the predominant non-protein thiol compound present in nearly all living organisms (Meister and Anderson, 1983). GSH is required for maintaining cellular redox

state, which could affect the protein thiol/disulfide equilibrium (Grant et al., 1996a; Grant et al., 1996b; Emri et al., 1997; Dickinson and Forman, 2002). GPx reduces peroxides by oxidising two reduced GSH to form an oxidized glutathione disulfide (GSSG) (Fig. 1-3). Oxidized GSSG can be recycled to GSH by glutathione reductase (GR) coupling with NADPH as an electron donor (Blokhina et al., 2003). Cellular redox status is maintained by the GSH/GSSG ratio (Schafer and Buettner, 2001). When the GSH/GSSG ratio decreases, cells are under oxidative stress. In addition, GSH is capable of reducing disulfide bonds in proteins and detoxifying xenobiotics by serving as an electron donor (Apel and Hirt, 2004; Wu et al., 2004).

In plant-microbe interactions, the roles of ROS may vary depending on the lifestyle of the pathogen and the physiological conditions of the host (Heller and Tudzynski, 2011). The YAP1 redox-responsive transcription factor homologous to mammalian AP-1 (activating protein 1) is one of the most important determinants of oxidative stress response in the budding yeast *Saccharomyces cerevisiae* (Toone et al., 1998; Delaunay et al., 2002; Toledano et al., 2004). In the biotrophic fungus *Ustilago maydis*, YAP1 regulates a number of genes involved in ROS detoxification and is required for full virulence (Molina and Kahmann, 2007). Similarly, YAP1 of the hemibiotrophic fungus *Magnaporthe oryzae* is involved in oxidative stress responses and is critical for pathogenicity (Guo et al., 2011). However, YAP1-mediated ROS detoxification is not essential for virulence in the necrotrophic fungi *Botrytis cinerea* and *Cochliobolus heterostrophus* (Lev et al., 2005; Temme and Tudzynski, 2009). In the tangerine pathotype of *A. alternata*, the YAP1 transcriptional regulator, AaAP1, a HOG1 mitogen-activated protein kinase (MAPK) homolog, AaHOG1, and a SKN7 response

regulator have been shown to be required for ROS resistance and pathogenicity/virulence (Lin et al., 2009; Lin and Chung, 2010; Chen et al., 2012). In addition, *A. alternata* NPS6, a fungal nonribosomal peptide synthetase analog is involved in siderophore-mediated iron acquisition and ROS resistance and is a virulence factor (Chen et al., 2013). Those studies implicate the importance of effective ROS detoxification in the successful pathogenesis of *A. alternata* (Chung, 2012).

ROS in Cellular Development

ROS also play an important role in cell differentiation (Wojtaszek, 1997; Bartosz, 2009). Under normal growth conditions, cells maintain low-level ROS by balancing between their generation and scavenging. Cells initiate differentiation in response to a transient increase of ROS (Moye-Rowley, 2003). Assays by spontaneous oxygen-dependent chemiluminescence indicate that the levels of ROS are elevated during conidia development in *Neurospora crassa* (Hansberg et al., 1993). The social amoeba *Dictyostelium discoideum* produces and secretes $O_2^{\cdot-}$ during the transition from single cell to multicellular form in response to a heat-labile factor (Bloomfield and Pears, 2003). In response to heat stress, the multicellular green algae *Volvox carteri* initiates sexual reproduction due to an increased cellular ROS level (Nedelcu et al., 2004). To avoid damages, cells must equip ROS scavenging systems during development. The genes encoding antioxidant enzymes are often up-regulated during developmental processes in fungi (Aguirre et al., 2005). In *Colletotrichum graminicola*, a manganese SOD coding gene is up-regulated during spore development (Fang et al., 2002). Copper-zinc SOD activity is elevated during transition from yeast to hyphae in *C. albicans* (Martchenko et al., 2004). *Aspergillus nidulans* and *N*. *crassa* have several catalase-coding genes that are differentially expressed in different cellular compartments or cell types (Kawasaki

and Aguirre, 2001; Michan et al., 2002). *As*. *nidulans* and *N*. *crassa* catalase-coding genes are induced by oxidative stress and up-regulated at the initiation of conidiation. Exogenous application of water-soluble antioxidants reduces cellular ROS level and suppresses conidia formation in *N*. *crassa* (Hansberg et al., 1993). Moreover, an *N*. *crassa* catalase-3 null mutant shows increased sensitivity to oxidative stress, accompanied by increased protein oxidation, carotene synthesis, hyphal adhesion and conidiation (Michan et al., 2003). Mutational inactivation of the *N*. *crassa* copper-zinc SOD coding gene (*sod-1*) increases carotene levels and affects the polarity of perithecia (Yoshida and Hasunuma, 2004).

Fungal NADPH Oxidase System

The NADPH oxidase (Nox) transfers electrons from NADPH to oxygen molecules via two electron carriers, flavin adenine dinucleotide (FAD) and two independent hemes, resulting in the production of $O_2^{\cdot -}$ (Vignais, 2002). Nox is widely distributed in animals, plants and fungi. NOX is required for oxidative burst defense response in mammalian phagocytes (mainly neutrophils and macrophages) (Babior, 1999; Cross and Segal, 2004). The phagocyte Nox is a multiunit enzyme composed of a catalytic subunit $gp91^{phox}$ (also known as Nox2), an adaptor protein $p22^{phox}$, three cytosolic regulatory subunits $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$ and the small GTPase Rac2. (Diebold and Bokoch, 2001; Lambeth, 2004). In resting cells, $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$ form a complex in the cytosol. $gp91^{phox}$ and $p22^{phox}$ form a heterodimeric flavohemoprotein, known as a cytochrome b_{558} and are localized in the cell membrane. All $gp91^{phox}$ homologs analyzed to date have the six transmembrane domains and putative FAD and NADPH binding domains in the cytoplasmic C-terminus (Takemoto et al., 2007).

Plants contain NADPH oxidases homologous to gp91^{phox}, designated respiratory burst oxidase homologs (Rboh). Rbohs are required for ROS accumulation in response to pathogen invasion (Torres et al., 2002; Yoshioka et al., 2003; Torres et al., 2005). Rboh is involved in the regulation of root hair growth through activation of Ca^{2+} channels (Foreman et al., 2003). In addition, Rboh interplaying with abscisic acid-mediated signaling pathways is involved in the regulation of stomatal closure, seed germination and root elongation (Kwak et al., 2003).

Some fungi and algae also possess gp91^{phox} homologs (Herve et al., 2006; Takemoto et al., 2007). However, *Nox* gene homologs are not found in *Schizosaccharomyces pombe*, *Ustilago maydis*, and *Rhizopus oryzae*. Many filamentous fungi such as *Aspergillus* spp. have only one copy of *Nox* homolog; others, including *Podospora anserina* and *Fusarium solani*, have multiple *Nox* homologs (Takemoto et al., 2007). A Nox ortholog, Yno1p/Aim14p, was recently characterized to be required for extramitochondrial generation of ROS, apoptosis and formation of actin cables in *S*. *cerevisiae* (Rinnerthaler et al., 2012).

Filamentous fungi have three NADPH oxidase subfamilies – NoxA, NoxB and NoxC. Both NoxA and NoxB are ortholog to the gp91^{phox}. NoxB is structurally similar to NoxA but contains an additional 40 amino acids in the N-terminus. NoxC has an additional 170–250 amino acids in the N-terminus. NoxC contains a putative calcium-binding EF-hand motif, commonly found in human Nox5 and the plant Rboh (Takemoto et al., 2007). Fungal Nox complexes are involved in a wide range of physiological and developmental functions (Table 1-2). NoxA (Nox1) homologs play a critical role in the development of sexual fruiting bodies in *As. nidulans* (Lara-Ortiz et al., 2003), *P*.

anserina (Malagnac et al., 2004) and *N. crassa* (Cano-Dominguez et al., 2008). NoxB (Nox2) homologs are required for ascospore germination in *P. anserina* and *N. crassa* (Malagnac et al., 2004; Cano-Dominguez et al., 2008). The fungal endophyte, *Epichloë festucae*, lacking *NoxA* but not *NoxB* is highly pathogenic, producing excessive hyphal branching and showing unregulated growth within its plant host (ryegrass, *Lolium perenne*) (Tanaka et al., 2006). Nox system is required for pathogenicity in many phytopathogenic fungi. Both *NoxA* and *NoxB* are required for sclerotial differentiation and pathogenicity in the necrotrophic fungus *B. cinerea*. A mutant impaired for *BcnoxA* has full penetration ability compared to wild-type but colonizes host tissue slowly. The *BcnoxB* mutant shows impaired function of appressoria and delayed formation of primary lesions. The *BcnoxR* mutant forms wild-type appressoria but fails to penetrate host tissue. However, a *B. cinerea* mutant lacking *NoxA* or *NoxB* does not reduce ROS accumulation, suggesting the presence of an alternative source of ROS production during plant infection (Segmuller et al., 2008). Deletion of *Nox1* or *Nox2* in *M. oryzae* yielded fungal strains that are unable to form appressorium and are non-pathogenic to rice (Egan et al., 2007). In *Sclerotinia sclerotiorum*, *Ssnox1* silenced mutant shows significant reduction in virulence and sclerotia development, whereas *Ssnox2* silenced mutant remains fully pathogenic even though the mutant fails to form sclerotia (Kim et al., 2011).

Little is known about how the Nox complex is regulated in fungi. Fungal NoxR is analogous with the mammalian $p67^{phox}$. The *E. festucae NoxR* is required for the expression of *NoxA* within the host plant. Inactivation of *NoxR* in *E. festucae* yielded fungal strains with reduced H_2O_2 production becoming highly pathogenic to its host

plant (Takemoto et al., 2006; Tanaka et al., 2006). Yeast two-hybrid and co-immunoprecipitation assays have demonstrated that RacA (mammalian Rac2 homolog) and two polarity proteins, Bem1 and Cdc24, physically interact with NoxR (Takemoto et al., 2006; Takemoto et al., 2011). *NoxR* homolog is also required for activation of both *BcnoxA* and *BcnoxB* in *B. cinerea* (Segmuller et al., 2008). Similarly, the *N. crassa* NOR-1 (NoxR homolog) is required for the expression of both *Nox-1* and *Nox-2* during discrete developmental stages (Cano-Dominguez et al., 2008).

MAPK signaling also impacts ROS production in fungi. In *As. nidulans*, SakA (a HOG1 homolog) suppresses the expression of the *NoxA* gene (Lara-Ortiz et al., 2003). In *B. cinerea*, BcSak1 has no effect on expression of *BcnoxA* and *BcnoxB*. However, MAPK Bmp3, a homolog of yeast Slt2 involved in the cell wall integrity pathway suppresses *BcnoxB* expression but up-regulates *BcnoxA* (Segmuller et al., 2008). In *P. anserina*, nuclear localization of PaMpk1 (Slt2 homolog) is dependent on PaNox1 (Kicka et al., 2006).

Oxidative Stress Signaling

ROS play an important signaling role in development and adaptation processes in animals, plants and fungi (Thon et al., 2007; Van Norman et al., 2011; Kennedy et al., 2012). Among ROS, H_2O_2 is relatively less toxic, more stable and able to pass freely through the plasma membrane. In *S. cerevisiae*, YAP1, a leucine zipper (bZIP)-containing protein is responsible for transcriptional activation of genes involved in ROS detoxification as well as drug and heavy metal resistance. YAP1 has two highly conserved Cys-rich domains (CRDs), which are critical for resistance to oxidative stress and for appropriate nuclear localization (Kuge et al., 1997; Coleman et al., 1999; Delaunay et al., 2000). YAP1 is localized in the cytoplasm under non-stress conditions.

Low-level H_2O_2 induces oxidation of a glutathione peroxidase-like protein (Gpx3/HYR1) that subsequently oxidizes the Cys-residues, resulting in the formation of disulfide bonds and a conformational change of YAP1. The modified YAP1 is no longer recognized by the Crm1 nuclear exporter, thus resulting in nuclear localization of YAP1 (Kuge et al., 1997; Kuge et al., 1998; Delaunay et al., 2000).

In the tangerine pathotype of *A. alternata*, the expression of *AaAP1* is responsive to H_2O_2, menadione and *tert*-butyl hydroperoxide. Upon exposure to H_2O_2, AaAP1::sGFP is localized in the nucleus. Inactivation of the *A. alternata AP1* gene resulted in fungal mutants that are hypersensitive to several oxidants and are non-pathogenic. Moreover, *AaAP1* mutants show reduced antioxidant activities, including catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase (Lin et al., 2011). Co-application of *AaAP1* mutants with the NADPH oxidase inhibitor apocynin or diphenylene iodonium partially restored pathogenicity of the mutants. These results suggest that the AaAP1-mediated effective detoxification of ROS is required for successful colonization of citrus by *A. alternata*.

Research Overview

The major goal of this research is to determine the biological functions of the Nox in *A. alternata*. This dissertation research has three major objectives: (1) to characterize and determine the function of Nox components (NoxA, NoxB and NoxR) in ROS generation; (2) to investigate the molecular mechanism underlying Nox-mediated oxidative stress signaling, ROS detoxification and virulence; and (3) to understand the roles of Nox in developmental and physiological processes.

To achieve these objectives, reverse genetics approaches were applied in the tangerine pathotype of *A. alternata*. Fungal strains impaired for each of the *Nox* genes

were created by targeted gene disruption and analyzed by histochemical staining and biochemical assays (Chapter 2). Nox mutants are hypersensitive to various oxidants and display reduced virulence on detached citrus leaves, confirming further the critical role of ROS detoxification for the pathogenicity of *A. alternata* on citrus (Chapter 3). Nox was found to be required for the expression of the *YAP1* and *HOG1* genes, establishing a direct connection among Nox, YAP1 and HOG1 MAPK. Moreover, Nox plays a central role in vegetative growth, conidia formation and cell wall integrity (Chapter 4). Lastly, a *GPx* gene encoding a putative glutathione peroxidase was inactivated in the tangerine pathotype of *A. alternata*. GPx was found to be required for ROS resistance and fungal virulence (Chapter 5). Northern blot analysis revealed that the expression of *GPx* is regulated by YAP1, HOG1, and NOX, indicating that the glutathione system also plays an important role in the regulation of redox homeostasis and oxidative resistance in *A. alternata*.

Table 1-1. Host-selective toxins in *Alternaria alternata*

Pathotype of *A. alternata*	Host	Disease	Toxin	Target site	Reference
Apple pathotype	Apple	Alternaria blotch of apple	AM-toxin	Chloroplast and plasma membrane	(Okuno et al., 1974; Kohmoto et al., 1976)
Japanese pear pathotype	Japanese pear	Black spot of Japanese pear	AK-toxin	Plasma membrane	(Nakashima et al., 1982)
Strawberry pathotype	Strawberry	Alternaria black spot of strawberry	AF-toxin	Plasma membrane	(Nakatsuka et al., 1986)
Tomato pathotype	Tomato	Stem canker of tomato	AL-toxin	Mitochondria	(Gilchrist and Grogan, 1976)
Tobacco pathotype	Tobacco	Brown spot of tobacco	AT-toxin	Mitochondria	(Mikami et al., 1971)
Tangerine pathotype	Tangerine and their hybrids	Brown spot of tangerines	ACT toxin	Plasma membrane	(Kohmoto et al., 1979)
Rough lemon pathotype	Rough lemon and Rangpur lime	Leaf spot of rough lemon	ACRL toxin	Mitochondria	(Gardner et al., 1986)

Table 1-2. Functions of fungal Nox complexes

Nutritional mode	Fungal species	Nox components	Functions	Reference
Saprophyte	*Aspergillus nidulans*	NoxA	Sexual fruiting body (Cleistothecia) development, localization of ROS at hyphal tips	(Lara-Ortiz et al., 2003; Semighini and Harris, 2008)
Saprophyte	*Podospora anserina*	Nox1	Sexual fruiting body (Perithecia) formation, cellulose degradation, hyphal interference	(Malagnac et al., 2004; Silar, 2005; Brun et al., 2009)
		Nox2	Ascospore germination, cellulose degradation	
		Nox3	No obvious function (play minor role, if any)	
		NoxR	Regulation of Nox1 and Nox2	
Saprophyte	*Neurospora crassa*	Nox1	Perithecia formation, asexual development, normal hyphal growth	(Cano-Dominguez et al., 2008)
		Nox2	Ascospore germination	
		NOR1	Regulation of Nox1 and Nox2	
Saprophyte	*Saccharomyces cerevisiae*	Yno1	Extramitochondrial ROS generation, apoptosis, regulation of the actin cytoskeleton	(Rinnerthaler et al., 2012)
Hemi-biotroph	*Magnaporthe oryzae*	Nox1	Pathogenicity, appressoria formation/penetration	(Egan et al., 2007; Tudzynski et al., 2012)
		Nox2	Pathogenicity, appressoria formation/penetration	
		Nox3	No obvious function	
Necrotroph	*Botrytis cinerea*	NoxA	Virulence (lesion development), sclerotia formation, conidial anastomosis tubes formation	(Segmuller et al., 2008; Roca et al., 2012; Siegmund et al., 2013)
		NoxB	Penetration, virulence, sclerotia formation	
		NoxR	Regulation of NoxA and NoxB	
Necrotroph	*Sclerotinia sclerotiorum*	Nox1	Virulence, sclerotia development, oxalate secretion	(Kim et al., 2011)
		Nox2	Sclerotia development	
Biotroph	*Claviceps purpurea*	Nox1	Virulence, conidia germination, sclerotia formation	(Giesbert et al., 2008; Tudzynski et al., 2012)
		Nox2	Balanced infection, mutant is hypervirulent	
Endophyte	*Epichloë festucae*	NoxA	Maintain mutualistic interaction with ryegrass host	(Takemoto et al., 2006; Tanaka et al., 2006; Tanaka et al., 2008)
		NoxB	No obvious function	
		NoxR	Regulation of NoxA	

Figure 1-1. Alternaria brown spot caused by *A. alternata* on tangerine leaves and fruits.

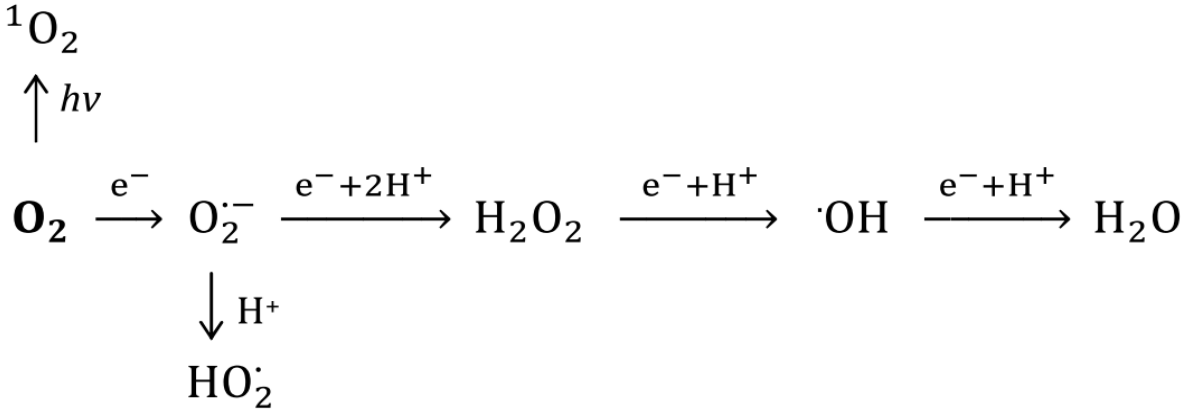
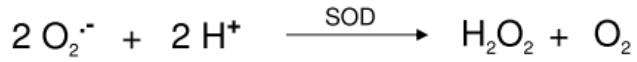
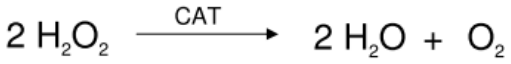


Figure 1-2. Generation of ROS from ground state oxygen (O_2) by energy transfer or by sequential electron transfer.

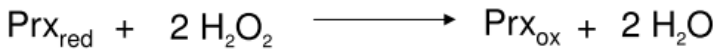
Superoxide dismutase (SOD)



Catalase (CAT)



Peroxiredoxin (Prx)



Glutathione peroxidase (GPx)

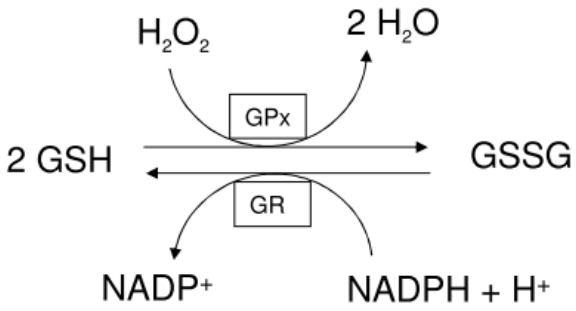


Figure 1-3. ROS detoxification mechanisms. Superoxide is dismutated by superoxide dismutase (SOD). Hydrogen peroxide is decomposed by catalases (CAT), peroxiredoxins (Prx) and glutathione peroxidase (GPx). Glutathione reductase (GR) recycles the oxidized glutathione (GSSG) back to reduced glutathione (GSH) at the expense of NADPH.

CHAPTER 2
GENERATION OF REACTIVE OXYGEN SPECIES BY NADPH OXIDASES IN *Alternaria alternata* OF CITRUS

Introduction

The tangerine pathotype of *A. alternata* causes brown spots on citrus leaves and fruit. This necrotrophic fungal pathogen produces a unique host-selective ACT toxin to kill host cells prior to invasion and acquires nutrients solely from dead tissues (Akimitsu et al., 2003). In addition to ACT toxin, the effective scavenging or detoxification of H_2O_2 and other ROS also is critical for fungal survival in the host plant. The colonization of *A. alternata* in the leaves of citrus induces rapid lipid peroxidation, increased accumulation of H_2O_2 and cell death (Lin et al., 2009, 2011). *A. alternata* may have evolved sophisticated mechanisms for cellular protection against the toxicity of plant-derived ROS. Apart from ACT toxin, recent studies have demonstrated that the ability to alleviate ROS via the YAP1 transcription regulator, the HOG1 mitogen-activated protein kinase, and the SKN7 regulator is required for *A. alternata* pathogenicity/virulence in citrus (Chen et al., 2012; Lin et al., 2009, 2011; Lin and Chung, 2010). However, the cellular mechanisms by which YAP1, SKN7 or HOG1 is responsible for H_2O_2 tolerance and fungal pathogenesis remain largely unresolved.

Both the beneficial and detrimental effects of ROS have been known in animals, plants and fungi (Apel and Hirt, 2004; Aguirre et al., 2005). In mammalian phagocytes, NADPH oxidase (Nox) produces ROS, which are involved in defense responses and cellular differentiation (Babior et al., 2002; Lambeth, 2004). In phagocytic cells, Nox transfers electron from NADPH to an oxygen molecule, leading to the generation of superoxide ($O_2^{\bullet-}$). $O_2^{\bullet-}$ is then converted to H_2O_2 by SOD, resulting in the ‘oxidative

burst' to kill pathogens (Babior et al., 2002). Nox is a multi-subunit oxidase, composed of two catalytic subunits gp91^{phox} and p22^{phox}, and multiple regulatory components, Rac, p67^{phox}, p47^{phox} and p40^{phox}. Both gp91^{phox} and p22^{phox} are localized in the membrane, whereas all regulatory components are localized in the cytosol. In addition to a primary contribution to defense responses, the Nox complex plays a role in cell proliferation, apoptosis and hormone responses in animals (Lambeth, 2004; Lardy et al., 2005). Plants also contain Nox enzymes, designated respiratory burst oxidase homologs (Rboh), which are involved in a wide range of physiological processes (Simon-Plas et al., 2002; Foreman et al., 2003) and in the ROS generation in response to pathogen attack (Keller et al., 1998; Torres et al., 2002).

Some fungi also contain Nox homologs (NoxA, NoxB and NoxC). NoxA and NoxB are analogous to the mammalian gp91^{phox}, whereas NoxC, containing a calcium-binding EF-hand motif, is similar to the mammalian Nox5 and the plant Rboh enzymes (Takemoto et al., 2007). NoxA and NoxB are structurally similar. Both oxidases contain an FAD-binding domain, an NADPH-binding domain and six transmembrane domains. NoxB has an extension of approximately 40 amino acids in the N terminus. NoxA and NoxB are commonly found in fungi, whereas NoxC is present only in few fungal species (Takemoto et al., 2007). The regulatory subunit p67^{phox} homolog, designated NoxR, is also found in fungi (Takemoto et al., 2006). The roles of NoxA (Nox1), NoxB (Nox2) and NoxC in cell differentiation, pathogenicity/virulence or cellulose degradation have been determined by genetic analysis in diverse fungal species (Lara-Ortiz et al., 2003; Malagnac et al., 2004; Tanaka et al., 2006; Egan et al., 2007; Cano-Dominguez et al., 2008; Giesbert et al., 2008; Scott and Eaton, 2008; Segmuller et al., 2008; Semighini

and Harris, 2008). However, inactivation of NoxC in *Podospora anserina* and *Magnaporthe oryzae* did not show discernible phenotypes (Brun et al., 2009; Tudzynski et al., 2012). Gene inactivation of fungal Nox homologs often leads to the reduced production of H_2O_2 in many fungi. Nox enzymes are conserved but their actual roles in physiology and development may vary among fungal species. In order to understand the biological roles of Nox for the generation and detoxification of H_2O_2 in *A. alternata*, I isolated and characterized *NoxA*, *NoxB* and *NoxR* genes. Analyses of targeted gene disruption mutants reveal that all three Nox components are required for ROS production.

Materials and Methods

Fungal Strains and Cultures Conditions

The wild-type EV-MIL31 strain of *A. alternata* (Fr.) Keissler (Table A-1) used in this study was single-spore isolated from diseased leaves of Minneola tangelo, a hybrid between Duncan grapefruit (*C. paradisi* Macfad.) and Dancy tangerine (*C. reticulata* Blanco) in Florida. Fungal strains were cultured on potato dextrose agar (PDA; Difco, Sparks, MD, USA) at 28 ºC. For DNA and RNA purification, fungal strains were grown on agar medium covered with a layer of sterile cellophane. Conidia were harvested by flooding with sterile water followed by low speed centrifugation (3,000 × *g*) from fungal cultures grown on PDA under constant fluorescent light for 3 to 4 days. Unless otherwise indicated, all chemicals used in this study were purchased from Sigma-Aldrich-Fluka (St. Louis, MO, USA).

Molecular Cloning and Sequence Analysis

All oligonucleotide primers used in this study are compiled in Table B-1. A 0.7-kb *NoxA* gene fragment was amplified from genomic DNA of *A. alternata* by PCR with two

degenerate primers NOXf1 and NOXr1 as reported by Giesbert and colleagues (Giesbert et al., 2008). The entire *NoxA* coding sequence and its 5′ and 3′ nontranslated regions were amplified by PCR from a library of *A. alternata*. The chromosome library was constructed from genomic DNA cleaved with four different restriction enzymes (*Dra*I, *Eco*RI, *Pvu*I and *Stu*I) and ligated to adaptors using the Universal GenomeWalker kit (BD Biosciences, San Jose, CA, USA). A 2.8-kb DNA fragment containing the entire *NoxB* ORF and its 5' and 3' untranslated regions was amplified by PCR with the primers NoxB-pro1F and NoxB-TAG from genomic DNA of *A. alternata*. An *A. alternata NoxR* gene fragment (0.4-kb) was amplified by PCR with two degenerate primers p67f1 and p67r1 as reported by Takemoto and colleagues (Takemoto et al., 2006). The full-length *NoxR* gene was obtained by PCR from the genome walking library. Similarity searches were performed at the National Center for Biotechnology Information using the BLAST network service. ORF and exon/intron positions were deduced from comparisons of genomic and cDNA sequences or predicted using Softberry gene-finding software (http://www.softberry.com). Amino acid sequences were aligned by BioEdit using CLUSTALW (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

Construction of Split Maker Fragments for Targeted Gene Disruption

NoxA gene was disrupted in the EV-MIL31 strain using a split marker approach as previously described (You et al., 2009; Yang and Chung, 2012). A *HYG* gene encoding a phosphotransferase under control of the *Aspergillus nidulans* trpC promoter (P) and terminator (T) and conferring hygromycin resistance was used as a dominant selectable marker. Two truncated fragments, PHY and YGT, overlapping within the *HYG* gene, were amplified by PCR with the primers M13R and hyg3 and the primers hyg4 and M13F, respectively. The 5' *NoxA* fragment was amplified with the primers

NoxA-pro2F and NoxA:M13R and fused with the PHY fragment (Fig. 2-4). The 3' *NoxA* fragment was amplified with the primers M13F:NoxA and NoxA-TAA and fused with the YGT fragment. The underlined sequence in the primers NoxA:M13R and M13F:NoxA (Table B-1) represents the oligonucleotides that are completely complementary to the sequence of the primers M13R and M13F, respectively.

Similar approaches were performed to disrupt *NoxB* gene (Fig. 2-5). A 2.1-kb 5'NoxB::PHY fusion fragment (0.9 + 1.2-kb) was amplified by two-round PCR with the primers NoxB-3F, NoxB:M13R, M13R and hyg3. A 2.5-kb 3'NoxB::YGT fusion fragment (0.7 + 1.8-kb) was amplified with the primers hyg4, M13F, M13F:NoxB and NoxB-TAG. Two split marker fragments were generated to disrupt *NoxR* gene (Fig. 2-6). A 5' NoxR::PHY fusion fragment (0.7+1.2-kb) was amplified by primers NoxR-pro2F, NoxR:M13R, M13R and hyg3. A 3' NoxR::YGT fragment (1.0 + 1.8-kb) was amplified with the primers hyg4, M13F, M13F:NoxR and NoxR-tail.

Genetic complementation was performed by co-transforming a functional *Nox* gene under control of its endogenous promoter with the pCB1532 plasmid, carrying the *Magnaporthe grisea* acetolactate synthase gene (*Sur*) cassette, which confers resistance to sulfonylurea (Sweigard et al., 1997). A functional *NoxA* gene cassette was amplified by PCR with the primers NoxA-pro1F and NoxA-TAA. A *NoxB* cassette was amplified with the primers NoxB-2F and NoxB-TAG. A *NoxR* cassette was amplified with the primers NoxR-pro2F and NoxR-tail.

Preparations of Protoplasts and Fungal Transformation

For protoplast isolation, fungal isolates were grown in 50 mL potato dextrose broth (PDB, Difco, Sparks, MD, USA) for 4 days, ground in a sterile blender, mixed with 200 mL fresh PDB, and grown for additional 12-18 hours. Fungal mycelia were collected

by centrifugation at 6,500 × *g* at 4 ºC and washed twice with a solution containing 1 M NaCl and 10 mM $CaCl_2$. The resulting fungal mycelia were resuspended in osmotic buffer (10 mM Na_2HPO_4, pH 5.8, 20 mM $CaCl_2$, 1.2 M NaCl) containing a mixture of cell wall-degrading enzymes [5 mg/mL driselase (InterSpex, San Mateo, CA), 8 mg/mL β-D-glucanase (InterSpex), 850 units/mL β-glucuronidase type H2, 81.25 units/mL lyticase] with gentle shake (<100 rpm) for 2 hours. Fungal protoplasts were harvested by centrifugation at 3,000 × *g* at 4 ºC and washed by sterile STC solution (1.5 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5) (Chung et al., 2002). Protoplasts were dissolved in four parts of STC and one part 50% polyethylene glycol 3350 (PEG) and used directly for fungal transformation or stored at -80 ºC. Fungal transformation was performed by a $CaCl_2$ and PEG-mediated method to introduce split marker fragments (10 µL each) into protoplasts (1×10^6/mL) prepared from the wild-type or other recipient strains as appropriate. Fungal transformants were regenerated and selected at 28 ºC on regeneration medium (RMM) containing 1 mg/mL $Ca(NO_3)_2 \cdot 4H_2O$, 0.2 mg/mL KH_2PO_4, 0.25 mg/mL $MgSO_4 \cdot 7H_2O$, 0.15 mg/mL NaCl, 1% glucose and 1 M sucrose, amended with 250 µg/mL hygromycin (Calbiochem, La Jolla, CA, USA) or 5 µg/mL sulfonylurea (Chem Service, West Chester, PA, USA). Successful integration of *HYG* within the targeted gene was examined by PCR with multiple sets of primers and by Northern-blot hybridization with a *NOX* gene specific probe.

Manipulation of Nucleic Acids

Fungal DNA was isolated using a DNeasy Plant kit (Qiagen, Valencia, CA, USA). RNA was purified with Trizol reagent (Molecular Research Center, Cincinnati, OH), treated with DNase, and used to synthesize the first strand of cDNA using the

SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Double-stranded cDNA was amplified by PCR with gene-specific primers and analyzed by sequencing. PCR amplicons were sequenced at Eton Bioscience (Research Triangle Park, NC, USA). *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 electrophoresis and Northern blot hybridization (Sambrook and Russell, 2001). RNA was blotted onto a nylon membrane and hybridized to a digoxigenin (DIG)-11-dUTP (Roche Applied Science, Indianapolis, IN, USA)-labeled DNA probe. Probes were amplified and labeled by PCR with gene-specific primers. A CSPD chemifluorescent (Roche Applied Science, Indianapolis, IN, USA) was used as a substrate for alkaline phosphatase in an immunological assay to detect the probe.

Detection and Quantification of ROS

The content of intracellular H_2O_2 of *A. alternata* hyphae was detected by a 3,3'-diaminobenzidine (DAB) staining assay (Torres et al., 2002). Fungal strains were grown on PDA for 5 days, stained with 5 mM DAB for 12-18 hours, and examined with a Leitz Laborlux phase contrast microscope (Leica Microsystems, Exton, PA, USA). The reaction of DAB with H_2O_2 produced a deep brown polymerization product. Peroxides were further quantified on the basis of the oxidation of ferrous (Fe^{2+}) to ferric (Fe^{3+}) ions, which reacted with xylenol orange under acidic condition, producing a purple substance with an absorbance between 540 nm and 620 nm (Jiang et al., 1990; Nourooz-Zadeh et al., 1994). To quantify intracellular H_2O_2, fungal isolates were grown on a layer of cellophane overlaid on CM agar plates for 3 days. Mycelia 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 mM ammonium ferrous (II) sulphate, 25 mM sulphuric acid, 100 mM sorbitol and 125 mM xylenol orange, and incubated at room temperature for 15 min. The amounts of H_2O_2 in the solution were determined spectrophotometrically by measuring the absorbance at 595 nm. The extracellular H_2O_2 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 H_2O_2 in the medium were measured spectrophotometrically over time. The concentration of H_2O_2 was calculated by referring to a standard curve that was established using authentic H_2O_2 (Fisher Scientific, Pittsburgh, PA, USA). Superoxide was detected by staining the fungal hyphae with nitroblue tetrazolium (NBT) (Shinogi et al., 2003). Accumulation of intracellular ROS was also evaluated by the 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) fluorescent probe (Molecular probes, Eugene, OR, USA). H_2DCFDA is commonly used to detect the cellular production of H_2O_2, hydroxyl radicals and nitric oxide (Possel et al., 1997). This cell-permeable ROS indicator is non-fluorescent until acetate groups are removed (H_2DCF) when exposed to ROS. Fungal hyphae were stained with 2.5 µg/mL H_2DCFDA for 20 min and examined with a Leitz Laborlux phase contrast microscope.

Nucleotide Sequences

Sequence data reported in this study have been deposited in the GenBank/EMBL Data Libraries under Accession No.: JN900389 (NoxA), JX136700 (NoxB) and JX207117 (NoxR).

Results

Characterization of *NoxA*, *NoxB* and *NoxR* homologs from *A. alternata*

The *A. alternata NoxA* gene (*AaNoxA*) contains an 1810-bp ORF interrupted by three small introns (50, 52 and 55 bp). The *AaNoxA* gene product has 550 amino acids, displaying 72%-93% identity and 84%-96% similarity to a number of Nox homologs (NoxA or Nox1) or hypothetical proteins of fungi (Fig. 2-1).

The *A. alternata NoxB* gene (*AaNoxB*) gene has an 1840-bp ORF interrupted by three introns (48, 47 and 59 bp). The AaNoxB polypeptide contains 561 amino acids, showing strong similarity to many NADPH oxidases of fungi. AaNoxB is most similar (97% similarity and 94% identity) to the NADPH oxidase 5 of *Pyrenophora tritici-repentis* (accession number XP_001936616). Alignment and comparison of the deduced amino acid sequences revealed that AaNoxA bears some resemblances to AaNoxB showing 34.9% identity and 52.4% similarity (Fig. 2-2). Both AaNoxA and AaNoxB have an FAD-binding domain, a NADPH-binding domain and six transmembrane domains.

The *A. alternata* NoxR gene (*AaNoxR*) has an 1811-bp ORF, interrupted by three introns (62, 54 and 53 bp), which encodes 546 amino acids. The deduced amino acid sequence of AaNoxR displays strong similarity to the *Epichloë festucae* EfNoxR, the *Botrytis cinerea* BcNoxR and the human Hsp67^{phox} (Fig. 2-3). AaNoxR has an N-terminal tetratricopeptide repeat (TPR) domain and a putative Nox activation domain, commonly found in the mammalian p67^{phox} and fungal NoxR-like family.

Targeted Disruption of *NoxA*, *NoxB* and *NoxR* in *A. alternata*

The *AaNoxA* gene was interrupted by transforming two DNA fragments (5'AaNoxA::PHY and 3'AaNoxA::YGT) (Fig. 2-4A), into protoplasts of the wild-type strain (EV-MIL31) of *A. alternata*. Of five fungal transformants selected from medium

containing hygromycin, two (DN2 and DN6) displayed reduced growth by 12% in relation to the EV-MIL31 strain and were examined further by PCR with different primer sets (Fig. 2-4B). A 1.7-kb fragment was amplified from genomic DNA of DN2 and DN6 strains using the primers NoxA-ATG and Hyg3. An expected 2.5-kb fragment was also obtained by PCR with the primers Hyg4 and NoxA-TAA. No DNA fragments were amplified from genomic DNA of the wild-type strain with these primer sets. Northern blot analysis (Fig. 2-4C) indicated that hybridization of total RNA prepared from the wild-type strain with an *AaNoxA*-specific probe identified an expected 1.8-kb transcript. The 1.8-kb *AaNoxA* transcript was absent in both DN2 and DN6 strains, confirming the successful inactivation of *AaNoxA* in *A. alternata*.

Two overlapping DNA fragments (5′NoxB::5′HYG and 3′NoxB::3′HYG) were generated (Fig. 2-5A) and transformed directly into protoplasts of the EV-MIL31 strain to disrupt *AaNoxB*. Of 30 transformants recovered from medium containing hygromycin, two strains (DB5 and DB6) displayed reduced growth by 11% and increased sensitivity to H_2O_2. PCR diagnosis (Fig. 2-5B) with the primers NoxB-1F and NoxB-tag identified an expected 1.0-kb fragment from wild-type strain. In contrast, an expected 3.4-kb fragment was amplified from DNA purified from DB5 and DB6. Using the primers NoxB-pro2F and Hyg3, a 2.9-kb fragment was amplified from genomic DNA of DB5 and DB6. Likewise, an expected 2.5-kb fragment was amplified from DNA of DB5 and DB6 with the primers Hyg4 and NoxB-tag. No fragment was amplified from the wild-type strain using these primer sets. Northern blot hybridization of fungal RNA to an *AaNoxB*-specific probe identified a 1.8-kb transcript from the wild-type, but not from DB5 and DB6 strains (Fig. 2-5C). Truncated transcripts (<1 kb) were detected in RNA purified

from the putative mutant strains, confirming the successful disruption of *AaNoxB* in *A. alternata*.

The *AaNoxR* gene was interrupted by introducing two DNA fragments (5'NoxR::5'HYG and 3' NoxR::3'HYG) into protoplasts of the EV-MIL31 strain (Fig. 2-6A). In total, 39 transformants were recovered from hygromycin-containing medium, tested for H_2O_2 sensitivity and examined by PCR using different primer pairs (Fig. 2-6B). Two strains (DR2 and DR5) displaying wild-type growth on PDA, but an elevated sensitivity to H_2O_2 had an expected *HYG* integration at the *AaNoxR* site. An expected 1.8-kb fragment was amplified from wild-type with the primers NoxR-ATG and NoxR-tail. In contrast, an expected 3.8-kb fragment was amplified from DNA purified from both DR2 and DR5. Northern blot hybridization of total RNA prepared from the wild-type strain and two putative *AaNoxR* disruptants with an *AaNoxR*-specific probe revealed that the 1.7-kb transcript of *AaNoxR* was detected in wild-type strain (Fig. 2-6C). In contrast, a truncated transcript (about 1-kb) was detected in DR2 and DR5 strains, confirming the successful disruption of *AaNoxR* in *A. alternata*.

The genetically complemented fungal strains CpA16, CpB24 and CpR40 displaying wild-type growth on PDA plates or displaying wild-type resistance to H_2O_2 were identified by transforming a wild-type copy of *AaNoxA*, *AaNoxB* and *AaNoxR* into protoplasts prepared from the DN2, DB5 and DR2 mutants, respectively.

The NADPH Oxidases Contribute to the ROS Production

Accumulation of H_2O_2 within fungal hyphae was examined by staining with 3,3'-diaminobenzidine (DAB). H_2O_2 induced polymerization of DAB, resulting in a dark-brown pigmentation. The H_2O_2 levels, based on the intensity of pigmentation, were much higher within incipient hyphae of the wild-type strain than those seen for *AaNoxA*

mutants (DN2, DN6), *AaNoxB* mutants (DB5 and DB6) or *AaNoxR* mutants (DR2 and DR5) (Fig. 2-7). Hyphae of the genetically complemented strains CpA16, CpB24 and CpR40 were stained dark brown, indicating restoration of H_2O_2 accumulation within hyphae. Quantitative analysis revealed a reduced accumulation of H_2O_2 within fungal hyphae of *AaNoxA* mutants (DN2 and DN6) relative to the wild-type (Fig. 2-8A). The genetically rescued strain CpA16 accumulated intracellular H_2O_2 at level similar to that of the wild-type. The amount of H_2O_2 secreted into the medium by the *AaNoxA* mutants was lower than that produced by the wild-type (Fig. 2-8B). The complementation strain CpA16 accumulated extracellular H_2O_2 at level that resembled the wild type.

Cellular superoxide anions were detected by nitroblue tetrazolium (NBT). NBT, when reduced by superoxide, precipitated as a dark blue, water-insoluble formazan. The staining of fungal hyphae with NBT indicated that the *AaNoxA* mutants (DN2 and DN6) seemingly produced less superoxide than the wild-type (Fig. 2-9). Microscopic examination revealed that the dark precipitate observed within the hyphae of wild-type (WT) has higher intensity than those of the DN2 and DN6 mutants. Hyphae of the *AaNoxA* complementation strain (CpA16) were stained dark blue by NBT.

The accumulation of ROS within fungal hyphae was examined further with the H_2DCFDA fluorescent probe. Based on fluorescent intensity, ROS accumulated within incipient hyphae of the wild-type and the rescued (CpA16) strains were apparently higher than those of *AaNoxA* mutants (DN2 and DN6) (Fig. 2-10). The *AaNoxA* mutants emitted fainter fluorescence, which diffused along the hyphal cytoplasm. These results confirm that NADPH oxidases play an important role in ROS generation in *A. alternata*.

Discussion

The phagocytic Nox system of mammalian is a multi-enzyme complex that is responsible for ROS generation. I report here the cloning and characterization of the *A. alternata NoxA* and *NoxB* genes, homologous to the mammalian gp91^{phox} and the *A. alternata NoxR* gene homologous to the mammalian p67^{phox}. Genetic analyses of fungal mutants defective for each of the *Nox* genes revealed that three Nox components are required for the accumulation of ROS, consistent with the findings with the Nox complex in animals and plants.

AaNoxA and AaNoxB contain six transmembrane-spanning domains and cytosolic FAD/NADPH binding domains, which are commonly found in fungal NADPH oxidases. Compared with NoxA, all fungal NoxB homologs have an extra 40-50 amino acids in the N termini with no known function (Takemoto et al., 2007). However, as with other fungal Noxs, no putative calcium binding EF-hand motif has been found in these AaNox and AaNoxB proteins. The *A. alternata NoxA* and *NoxB* were independently inactivated with the split marker approach using two DNA fragments overlapping within the selection marker. Fungal strains carrying a disrupted copy of *AaNoxA* or *AaNoxB* displayed slightly reduced growth compared with the wild-type progenitor. Further analyses using PCR with different primer sets and Northern blotting confirm the successful integration of the *HYG* marker gene within the *AaNox* genes.

Compared to yeasts, genetic transformation and targeted disruption or replacement in filamentous fungi can be problematic due to high frequency of ectopic integrations and low efficiency of homologous recombination (Pratt and Aramayo, 2002). Integration of foreign genes in filamentous fungi is a rather rare process presumably influenced by a nonhomologous end joining (NHEJ) mechanism (Ninomiya et al., 2004).

The split-marker recombination strategy, using truncated DNA fragments overlapping within the selectable marker gene, was originally developed for rapid gene deletions and gap-repair cloning in yeast (Fairhead et al., 1996), and later was successfully applied to diverse filamentous fungi (Kuck and Hoff, 2010). Several studies from different fungal species have revealed that the split-marker approach increases the frequency of homologous recombination while decreases ectopic integration (Catlett et al., 2003; Jeong et al., 2007; You et al., 2009). This approach has been shown to increase the frequency of targeted gene disruption and homologous integration to as high as 100% in the tangerine pathotype of *A. alternata* (Lin et al., 2009; Lin and Chung, 2010; Lin et al., 2010; Wang et al., 2010; Yago et al., 2011).

NADPH oxidases have been studied in various fungal species (Tudzynski et al., 2012). Fungal Noxs have been characterized to be required for ROS production in fungi, including *As. nidulans*, *E. festucae*, and *S. sclerotiorum* (Lara-Ortiz et al., 2003; Tanaka et al., 2006; Kim et al., 2011). In this chapter, I assess the potential role for Nox in the formation of ROS based on *in situ* detection of H_2O_2 and superoxide using different staining methods and comparison of wild type and Nox-disrupted mutants of *A. alternata.* The results clearly show that Nox is a key generator of ROS in the tangerine pathotype of *A. alternata* because mutational inactivation of each of the Nox components resulted in fungi that accumulate lower ROS compared to wild type. Surprisingly, deletion of a *NoxA* homolog in *P. anserina* and *M. grisea* increases ROS production (Malagnac et al., 2004; Egan et al., 2007), suggesting the presence of an alternative source for ROS generation that is up-regulated as a consequence of the loss

of Nox genes (Egan et al., 2007). In *B. cinerea,* Nox homologs appear to play no role in ROS production (Segmuller et al., 2008).

Although two p67^{phox} family members, p67^{phox} and NOXA1, are found in mammalian genomes (Lambeth, 2004), only a single NoxR is identified in filamentous fungi (Takemoto et al., 2006). As with all p67^{phox} homologs, *A. alternata* NoxR contains four tetratricopeptide repeats (TPR) and a putative Nox activation domain (Fig. 2-3). In contrast, AaNoxR has no Scr homolog 3 (SH3) and PB1 domains commonly found in the human p67^{phox}. SH3 and PB1 domains interact, respectively, with p47^{phox} and p40^{phox} (Lambeth, 2004). The absence of these domains implicated in the protein-protein interaction in fungal NoxR suggests that filamentous fungi likely have no *p47^{phox}* and *p40^{phox}* homologs (Takemoto et al., 2007). However, fungal NoxR contains a different type of PB1 domain in the C terminus (Noda et al., 2003). This finding indicates that the regulatory partners involved in the activation of fungal NoxR are different from those of p67^{phox} (Takemoto et al., 2006). NoxR has been shown to be required for the activation of *NoxA* and *NoxB* in *As. nidulans*, *E. festucae*, *N. crassa*, *P. anserina* and *B. cinerea* (Cano-Dominguez et al., 2008; Segmuller et al., 2008; Semighini and Harris, 2008; Tanaka et al., 2008; Brun et al., 2009). However, I found that the *A. alternata* NoxR does not impact the expression of *NoxB*, but negatively regulates *NoxA* (see Chapter 3 for details). Although Nox components are well conserved in fungi that possess them, their true roles in the regulation of physiological and developmental processes may vary considerably in different species. In the present studies, mutational inactivation of *NoxA*, *NoxB* or *NoxR* in *A. alternata* resulted in fungal strains that are impaired for ROS accumulation. The phenotypes could be fully restored in each of the

mutants by re-introducing a functional copy of the gene into the respective mutant, strongly indicating that Nox enzymes are one of the key ROS generators in *A. alternata*.

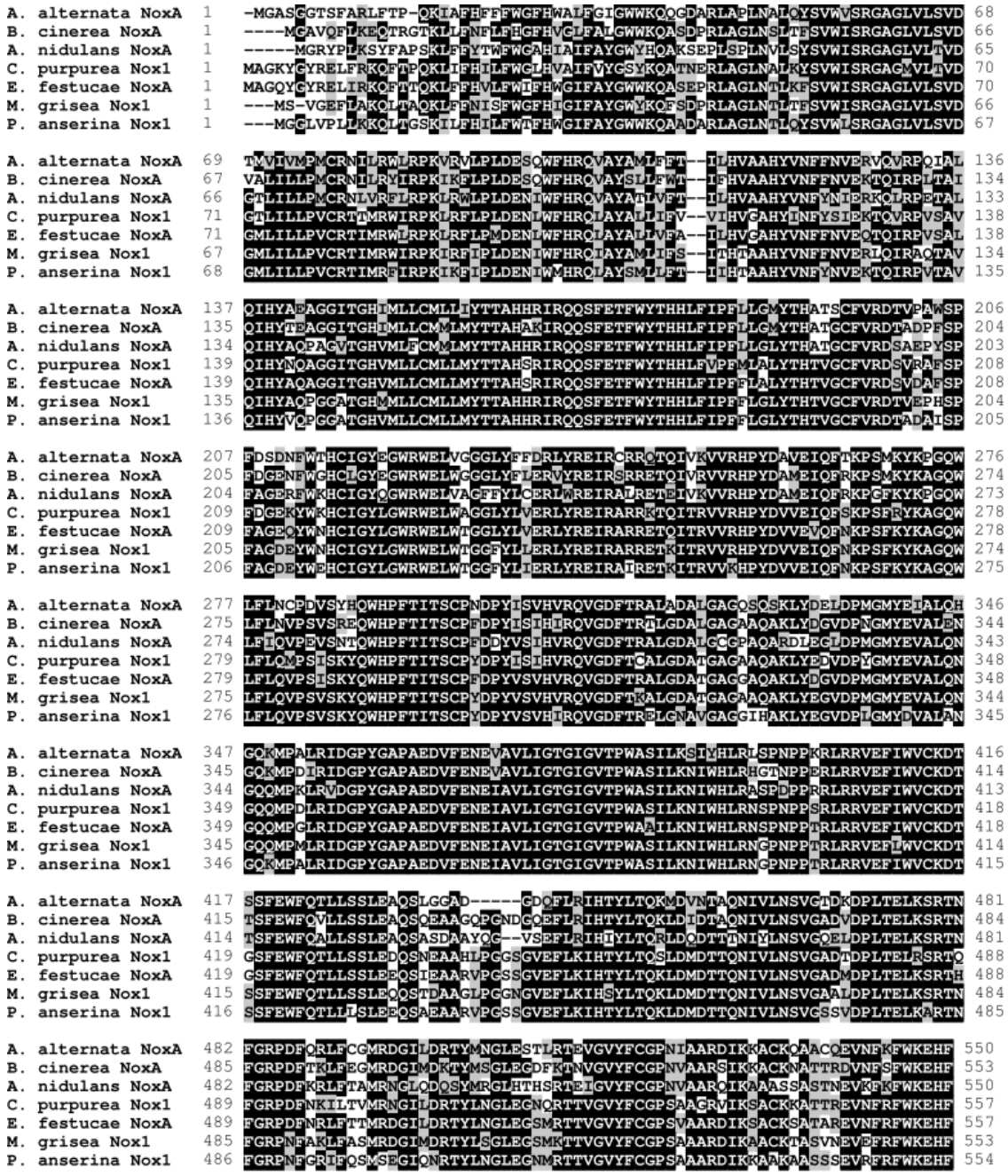


Figure 2-1. Alignment of fungal NADPH oxidases NoxA and Nox1. Conserved amino acids are shaded. Alignment was carried out by BioEdit using CLUSTALW.

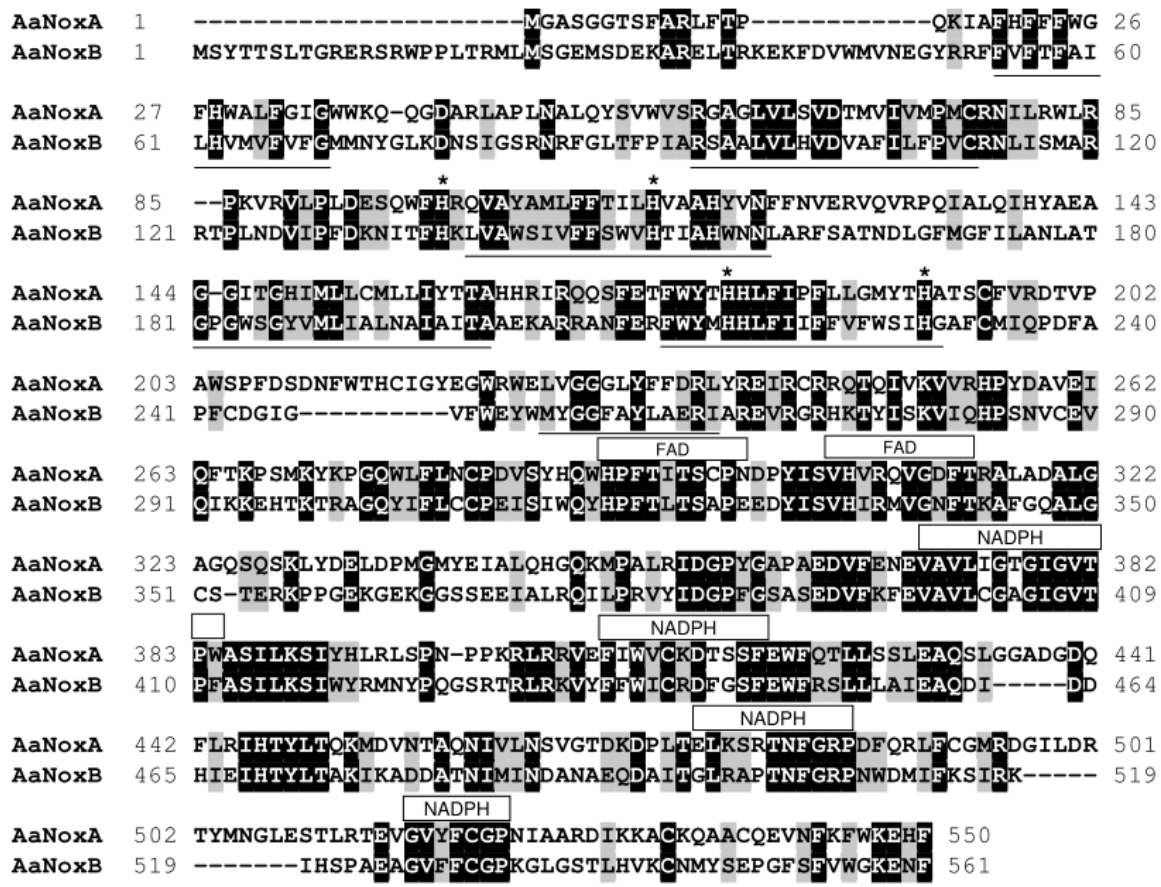


Figure 2-2. Alignment and comparison of the deduced amino acid sequences of NoxA and NoxB of *Alternaria alternata*. Conserved amino acids are shaded. Four conserved histidine residues potentially for haem binding are indicated by asterisks. Six putative transmembrane domains are underlined. Putative FAD- and NADPH-binding domains are also indicated.

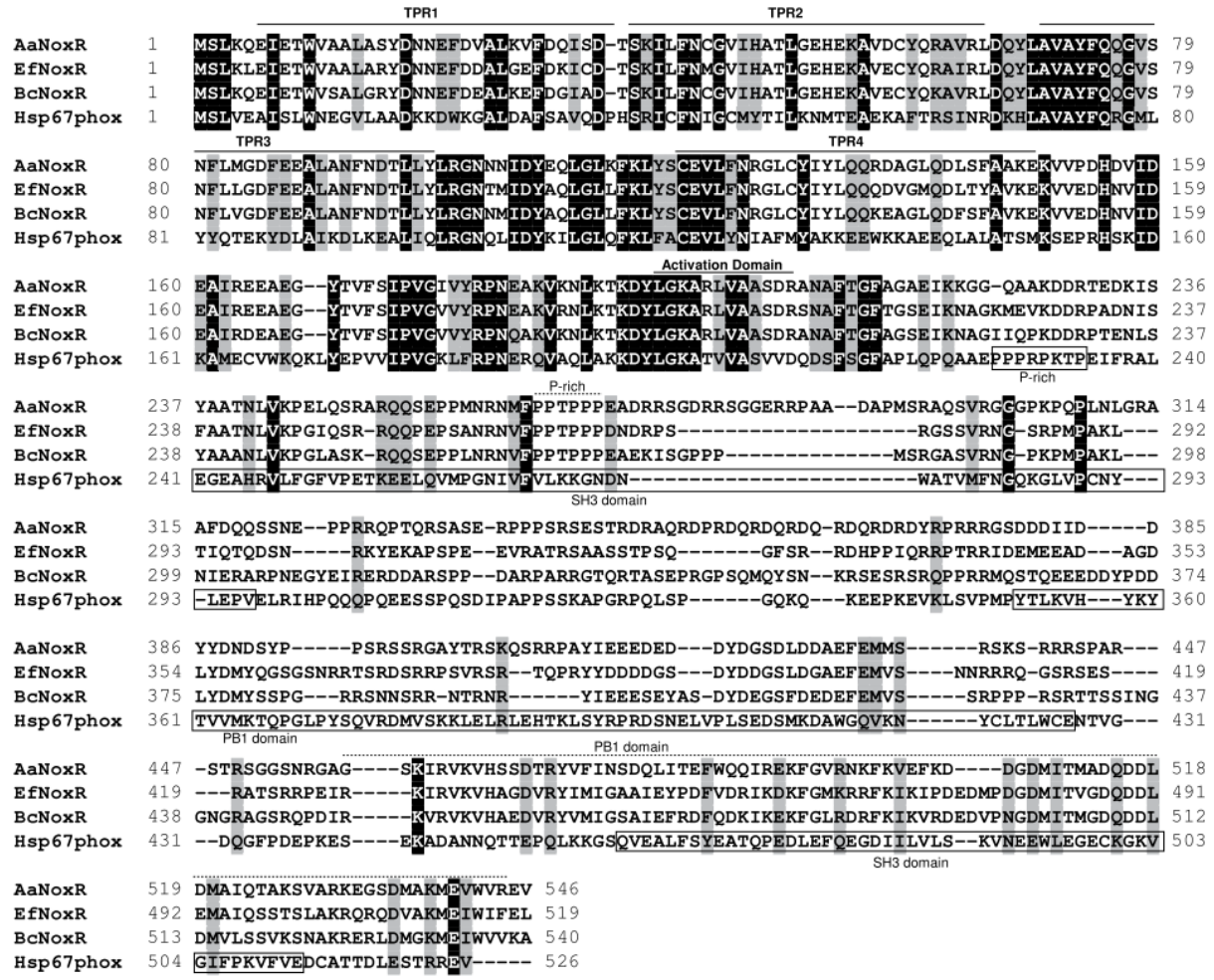


Figure 2-3. Alignment of the deduced amino acid sequence of the *Alternaria alternata* NoxR with the *Epichloë festucae* EfNoxR, *Botrytis cinerea* BcNoxR and human Hsp67^{phox}. Conserved amino acids are shaded. Proline-rich (P-rich), Src homology 3 (SH3) and Phox1 and Bem1 (PB1) domains of human p67^{phox} are boxed. Four tetratricopeptide repeats (TPR) and a putative Nox activation domain are also indicated.

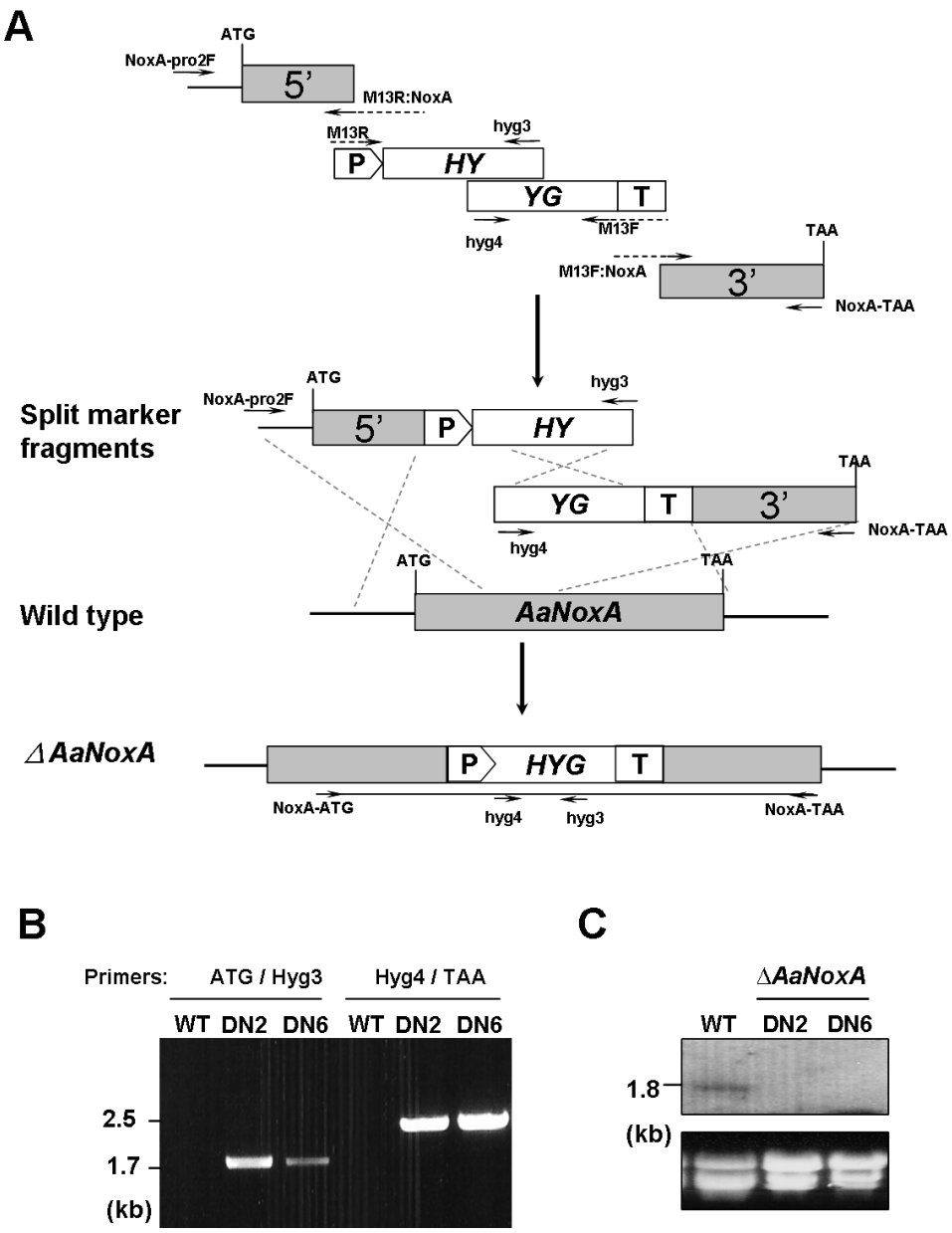


Figure 2-4. Targeted disruption of *AaNoxA* in *Alternaria alternata*. (A) Schematic illustration of a split marker strategy for disruption of *AaNoxA* by inserting a hygromycin phosphotransferase gene (*HYG*) under the control of the *Aspergillus nidulans* trpC promoter (P) and terminator (T). Oligonucleotide primers used to amplify each fragment are also indicated. (B) Image of DNA fragments amplified from genomic DNA of different fungal strains with the primers indicated. (C) Image of an RNA gel blot, probed with a DIG-labeled *AaNoxA*, showing a 1.8-kb transcript from the wild-type strain (WT) but not from two *AaNoxA* disruption mutants (DN2 and DN6). Ribosomal RNA stained with ethidium bromide shows relative loading of the RNA samples.

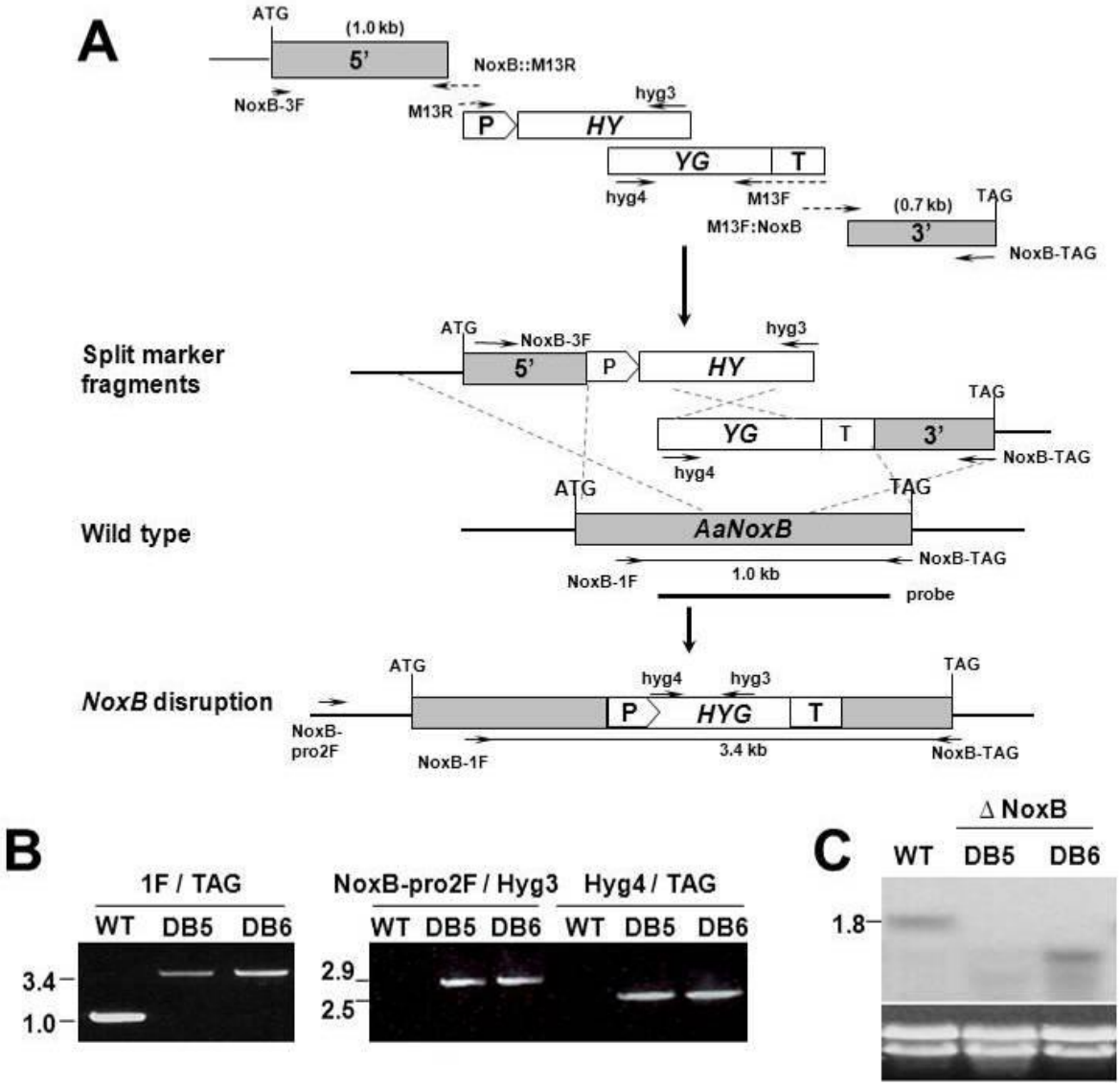


Figure 2-5. Targeted disruption of *NoxB* gene in *Alternaria alternata* using a split marker approach. (A) Schematic depiction of the generation of truncated, but overlapping, hygromycin phosphotransferase gene (*HYG*) under the control of the *Aspergillus nidulans* trpC promoter (P) and terminator (T), and gene disruption within *AaNoxB*. Oligonucleotide primers used to amplify each fragment are also indicated. (B) Image of DNA fragments amplified from genomic DNA of different fungal strains with the primers indicated. The primer NoxB-pro2F sequence is not present in the split marker fragment. (C) RNA gel blotting, probed with a DIG-labeled *AaNoxB*, showing a 1.8-kb transcript from the wild-type strain (WT) but not from two AaNoxB disruption mutants (DB5 and DB6). The lower panel shows ribosomal RNA as loading controls.

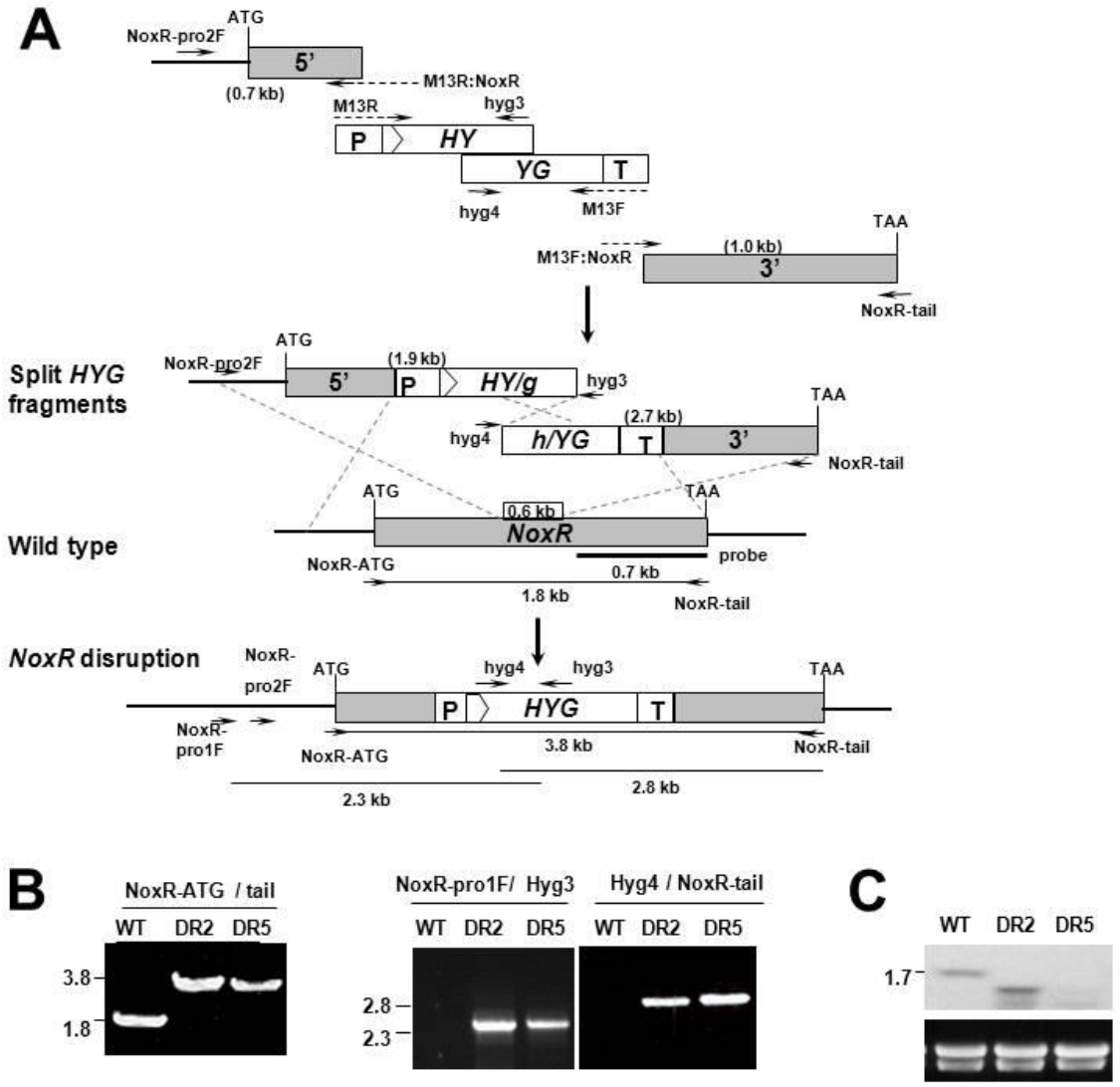


Figure 2-6. Targeted disruption of *NoxR* in *Alternaria alternata* using a split marker approach. (A) Schematic illustration of a split marker strategy for disruption of *AaNoxR* by inserting a hygromycin phosphotransferase gene (*HYG*) under the control of the *Aspergillus nidulans* trpC promoter (P) and terminator (T). Oligonucleotide primers used to amplify each fragment are also indicated. (B) Image of DNA fragments amplified from genomic DNA of fungi with the primers indicated. (C) Image of an RNA gel blotting, probed with a DIG-labeled AaNoxR, showing a 1.7-kb transcript from the wild-type strain (WT) but not from two *AaNoxR* disruption mutants (DR2 and DR5). Ribosomal RNA stained with ethidium bromide shows relative loading of the RNA samples.

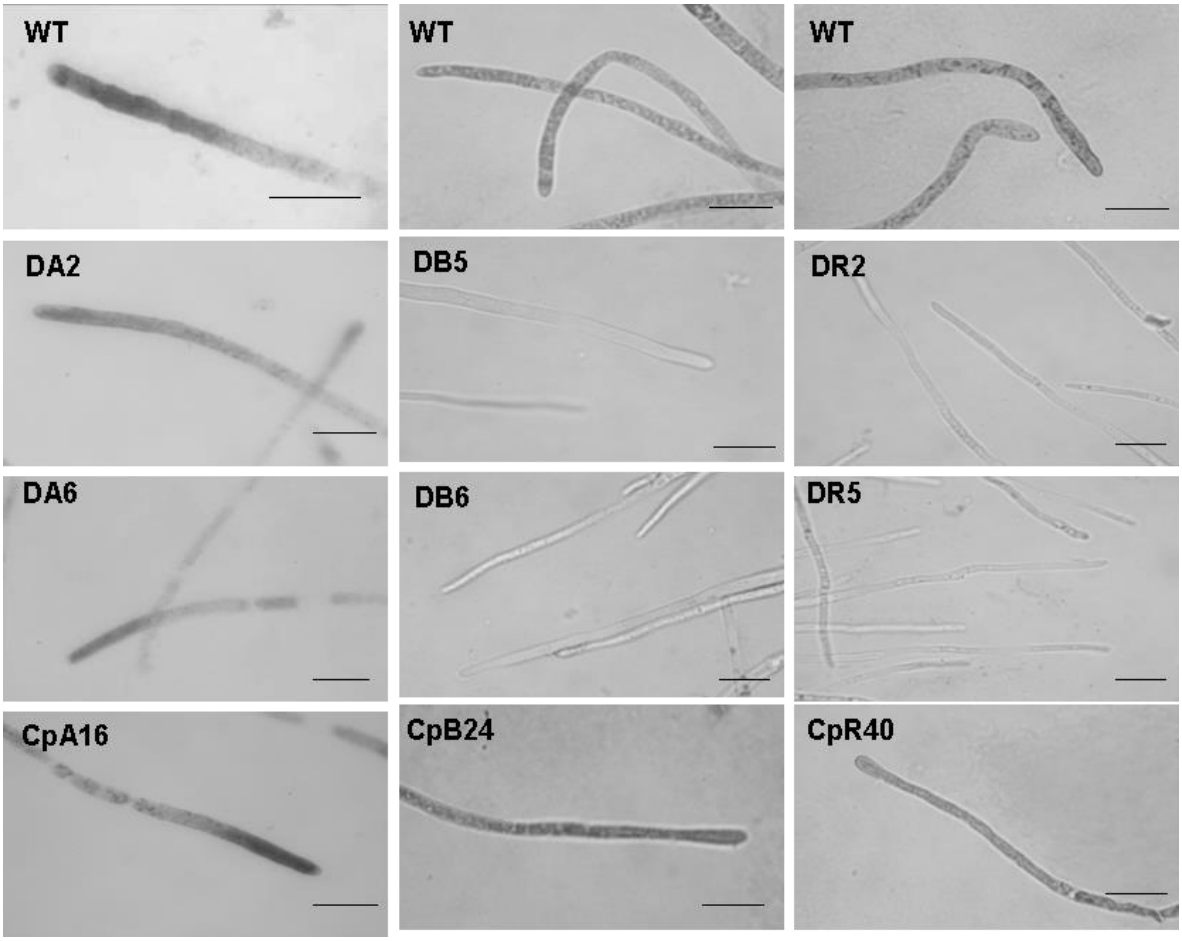


Figure 2-7. NoxA, NoxB and NoxR are required for the accumulation of cellular H_2O_2. The wild-type EV-MIL31 strain (WT), the strains lacking *NoxA* (DN2, DN6), the strains lacking *NoxB* (DB5, DB6), the strains lacking *NoxR* (DR2, DR5) and the corresponding rescued strains (CpA16, CpB24 and CpR40) of *A. alternata* were grown on PDA for 5 days, stained with 5mM 3,3'-diaminobenzidine (DAB) solution for 12 to 18 hours and examined microscopically. Bar = 10 μm.

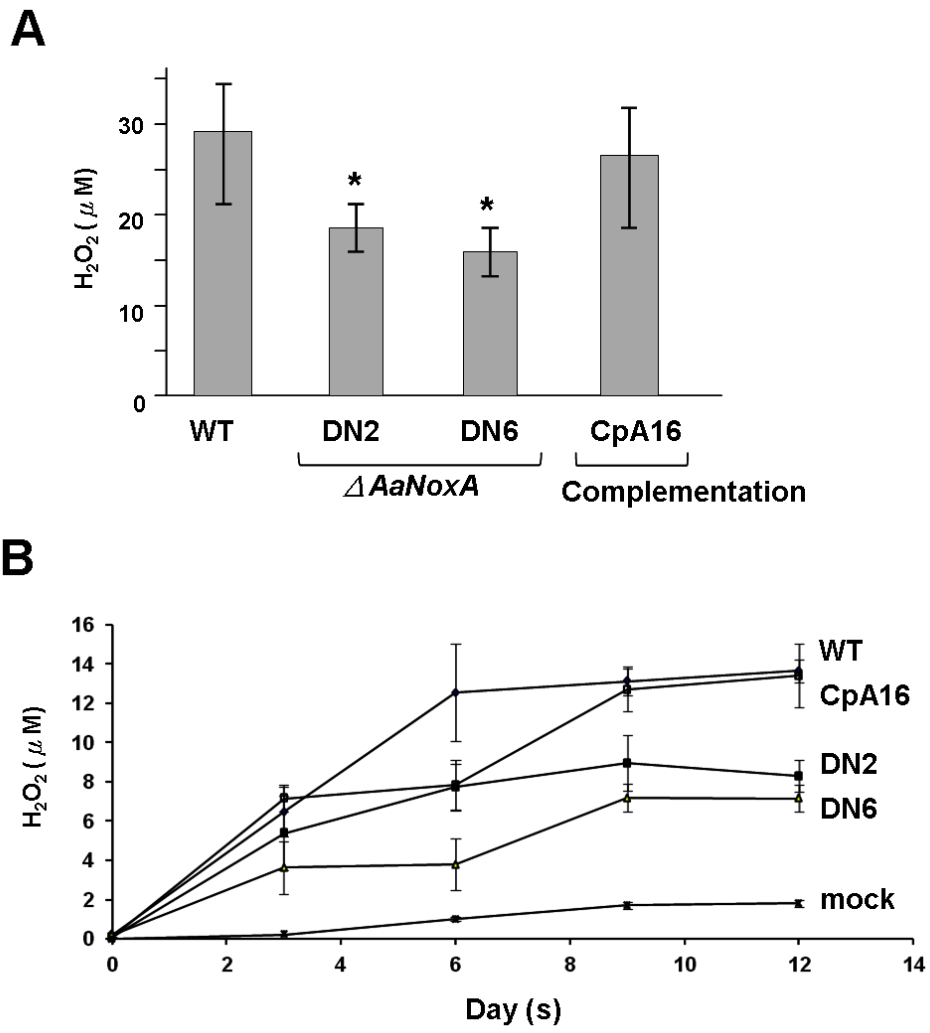


Figure 2-8. Quantitative analysis of H_2O_2. (A) Quantitative analysis of intracellular H_2O_2 among the wild-type (WT), two *AaNoxA* mutants (DN2 and DN6) and genetically complementation strain (CpA16) using a xylenol orange assay. Asterisks indicate the values, separated by Student's t-test ($P \leq 0.05$), that are significantly different from those of the wild-type. (B) Quantitative analysis 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. Each column or points indicate the mean number of H_2O_2 ± the standard deviation from two independent experiments with at least three replicates.

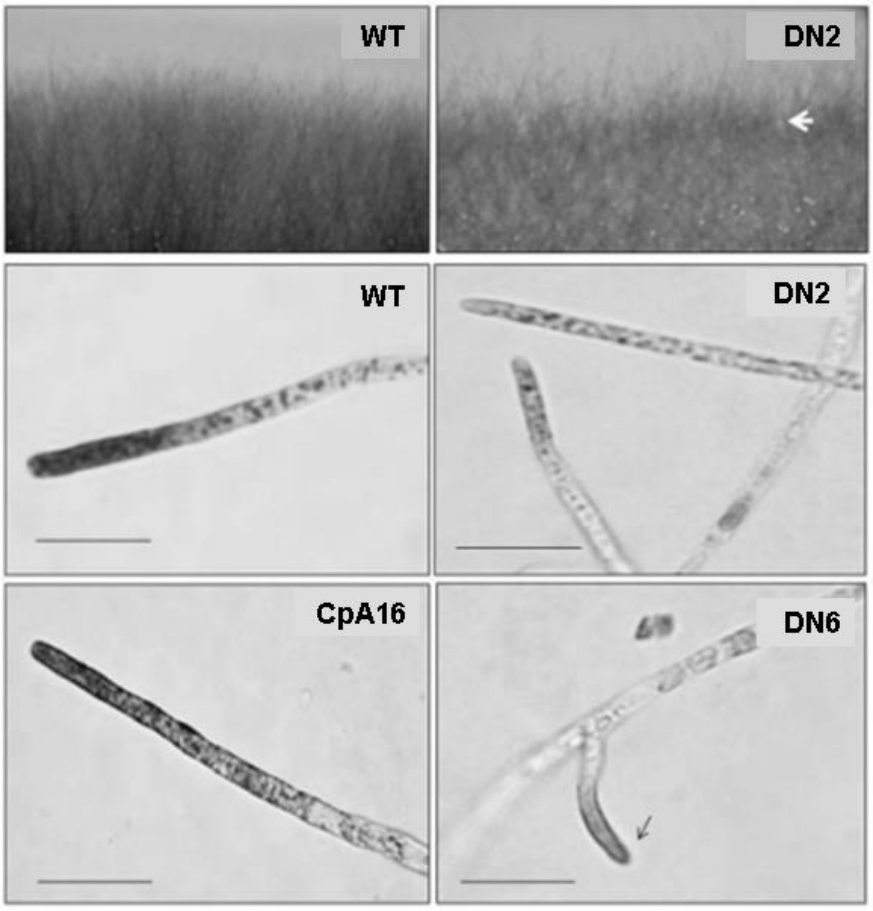


Figure 2-9. Accumulation and detection of superoxide within the hyphae of the wild-type strain (WT), the *AaNoxA* mutants (DN2 and DN6) and the rescued strain (CpA16). Fungal strains were grown on PDA for 6 days and stained with nitroblue tetrazolium (NBT). Top panels show the edge of fungal colonies after staining with NBT. Microscopic images of fungal hyphae tips showing the deposition of water-insoluble formazan after staining with NBT. Bar = 20 µm.

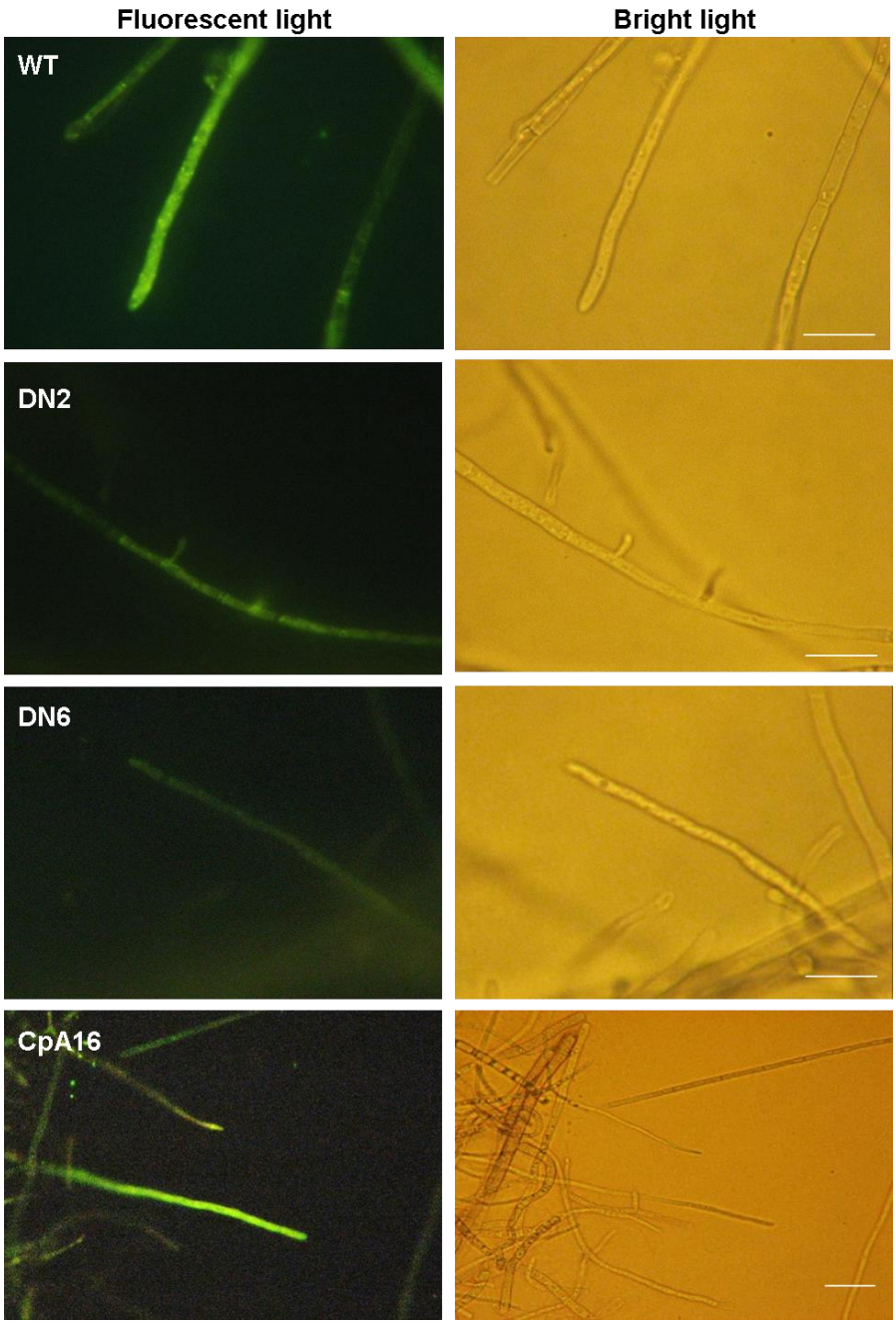


Figure 2-10. Detection of ROS within *Alternaria alternata* hyphae staining with the fluorescent probe H_2DCFDA. Fungal mycelium was collected from wild-type (WT), the *AaNoxA* mutants (DN2 and DN6) and the genetically rescued strain (CpA16) grown on PDA for 6 days, stained with 2.5 µg/mL H_2DCFDA for 15 min, and examined with a microscope equipped with a 450-490 nm excitation filter and a 520 nm barrier filter. Bar = 20 µm.

CHAPTER 3
NADPH OXIDASES ARE REQUIRED FOR OXIDATIVE STRESS RESISTANCE AND FULL VIRULENCE IN THE CITRUS FUNGUS *Alternaria alternata*

Introduction

The production of reactive oxygen species (ROS), including superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$), are inevitable in all organisms with an aerobic lifestyle (Apel and Hirt, 2004). The ROS scavenging or detoxification systems are essential to maintain the reduced redox states within subcellular environments and repair of ROS-triggered damage. These processes are absolutely required to ensure the fitness of organisms under aerobic conditions (Mittler, 2002). Even though highly toxic to cells, ROS can serve diverse roles in cellular defense and in the signaling transduction pathways controlling cell differentiation or developmental modifications (Aguirre et al., 2005).

Fungal pathogens of plants often encounter a wide range of potentially harmful environmental obstacles during the course of host colonization. Such environmental changes are often associated with an oxidative burst that results in the production and accumulation of highly toxic ROS, which can lead to programmed cell death and is a characteristic feature of the hypersensitive reaction (HR) (Shetty et al., 2008). HR plays a key role in plant defense responses against saprophytic microorganisms and noncompatible (avirulent) or biotrophic pathogens, yet it has a modest effect against necrotrophic phytopathogens (Mayer et al., 2001; Glazebrook, 2005; Rivas and Thomas, 2005; Unger et al., 2005; Walz et al., 2008). HR may actually enhance colonization by necrotrophic phytopathogens, as they acquire nutrients and energy primarily from dead cells (Divon and Fluhr, 2007). Because many necrotrophic pathogens are able to

produce and secrete HSTs that kill host cells prior to colonization, leading to the accumulation of ROS, the pathogens must have evolved an effective means to suppress the toxicity of ROS, which would allow them to proliferate inside the host plant (Mayer et al., 2001; Glazebrook, 2005).

The membrane-bound NADPH oxidase (Nox) is one of the key enzymes for ROS production (Babior et al., 2002; Lambeth, 2004). It has been well accepted that ROS play critical roles in defense mechanism of animals and plants. ROS are required for establishing successful invasion and colonization of microbes in their hosts. In the rice blast pathogen *M. grisea*, both Nox1 and Nox2 are crucial for appressorium-assisted penetration to rice (Egan et al., 2007). In the necrotrophic fungus *B. cinerea*, Nox2 regulates formation of an appressorium-like structure and Nox1 is required for *in-planta* growth (Segmuller et al., 2008). In *Sclerotinia sclerotiorum*, *Nox1* is associated with the production of oxalic acid, one of the primary virulence contributors in this necrotroph (Kim et al., 2011). Likewise, *NoxA* homolog of the biotrophic pathogen *Claviceps purpurea* is required for full virulence in cereals (Giesbert et al., 2008). Recent studies in the fungal endophyte *Epichloë festucae* and its grass host revealed that ROS restricts fungal proliferation and thus, the fungus maintains a mutualistic association with the host plant (Takemoto et al., 2006; Tanaka et al., 2006). Interestingly, Nox is necessary for normal tolerance to oxidative stress in *B. cinerea* and *C. purpurea*. Increased sensitivity of Nox mutants of *B. cinerea* and *C. purpurea* is in agreement with the gene expression patterns. This is a rather unexpected result since Nox is involved in ROS generation (Giesbert et al., 2008; Segmuller et al., 2008). Gene inactivation of fungal Nox homologs often leads to the reduced production of H_2O_2 (Lara-Ortiz et al., 2003;

Takemoto et al., 2006; Cano-Dominguez et al., 2008; Semighini and Harris, 2008; Kim et al., 2011). Similarly, I have demonstrated that the *A. alternata* Nox system is involved in ROS production in Chapter 2.

Previous studies have also demonstrated the essential role of the *A. alternata YAP1* gene, encoding a redox-responsive *YAP1*-like transcription factor, for ROS detoxification and fungal pathogenicity (Lin et al., 2009; Yang et al., 2009). The *A. alternata* YAP1 regulates a number of enzymatic activities, including catalase, superoxide dismutase (SOD), glutathione-S-transferase, glutathione peroxidase, glutathione reductase and ligninolytic peroxidase (Lin et al., 2011). Thus the *A. alternata YAP1* is required for the detoxification of H_2O_2, and perhaps, other ROS. Further studies have revealed that a *HOG1* mitogen-activated protein (MAP) kinase and a SKN7 response regulator are also required for cellular resistance to oxidative stress and pathogenicity in *A. alternata* (Lin and Chung, 2010; Chen et al., 2013). These results confirm further that *A. alternata* is able to detoxify or obviate the ROS-mediated plant defense barriers to successfully establish disease. In order to understand further the mechanisms underlying oxidative stress resistance and to determine the role of Nox system in fungal virulence, I characterized the Nox components (NoxA, NoxB and NoxR) focusing on their roles for stress response and pathogenesis in the tangerine pathotype of *A. alternata*. Genetic analyses revealed that Nox is absolutely required for resistance to ROS and full virulence. I also showed that the expression of the *AaAP1* and *AaHOG1* genes is regulated by Nox in *A. alternata*, suggesting that Nox system is involved in sensing and responding to ROS.

Materials and Methods

Fungal Strains and Culture Conditions

The wild-type EV-MIL31 strain of *Alternaria alternata* (Fr.) Keissler and the genetically altered strains (Table A-1), *AaNoxA* mutants (DN2, DN6), *AaNoxB* mutants (DB5, DB6), *AaNoxR* mutants (DR2, DR5) and the corresponding complementation strains (CpA16, CpB24 and CpR40) were generated from previous studies (Yang and Chung, 2012, 2013). Fungal mutants disrupted at the *AaAP1* gene, encoding an oxidative stress-responsive transcription activator and the *AaHOG1* gene, encoding a HOG1 MAP kinase, were created in separate studies (Lin et al., 2009; Lin and Chung, 2010). Conidia were harvested by flooding with sterile water followed by low speed centrifugation (3,000 × *g*) from fungal cultures grown on PDA under constant fluorescent light for 3 to 4 days. For expression experiment in response to oxidative stress, fungal strains were grown on cellophane overlaid onto PDA for 2 days, shifted to PDA amended with different oxidants at appropriate concentration, and incubated for an additional 24 hours. RNA was isolated with Trizol reagent (Molecular Research Center, Cincinnati, OH, USA) and used for a Northern hybridization with *NoxA*, *NoxB*, *NoxR*, *YAP1* or *HOG1* probes.

Creation and Identification of Double Mutants

Fungal strains mutated at *NoxA NoxB* or *NoxA NoxR* were created by transforming split *SUR* marker fragments fused with truncated *NoxA* into protoplasts prepared from a *AaNoxB* mutant (DB5) or a *AaNoxR* mutant (DR2). Two truncated fragments, SUr and sUR, overlapping within the *SUR* gene, encoding an acetolactate synthase that confers sulfonylurea resistance (Sweigard et al., 1997), were amplified by PCR with the primers sur1 pairing with surR and sur2 pairing with surF (Fig. 3-1). The 5’

NoxA fragment was amplified with the primers NoxA-pro2F and NoxA:sur1 and fused with the SUr fragment. The 3' *NoxA* fragment was amplified with the primers sur2:NoxA and NoxA-TAA and fused with the sUR fragment. Fungal transformation using $CaCl_2$ and PEG was performed as described in Chapter 2. Transformants were recovered from medium supplemented with 5 µg/mL sulfonylurea (Chem Service, West Chester, PA, USA) and tested for elevated sensitivity to H_2O_2 (Fisher Scientific, Pittsburgh, PA) compared to its progenitor. Successful integration of *SUR* gene within the targeted gene was examined by PCR with two sets of primers.

Manipulation of Nucleic Acids

Isolation of fungal genomic DNA and RNA, purification of plasmids and Northern blot analyses were performed as described in Chapter 2.

Sensitivity Test

Chemical sensitivity was conducted by transferring fungal hyphae/conidia as a toothpick point inoculation onto PDA medium amended with test compounds at appropriate concentration. Fungal radial growth was measured at 4 to 7 days. The growth difference of the disrupted mutants relative to that of the wild-type grown on the same plate was calculated. The percentage change in growth, which could be positive or negative, was determined by dividing the relative difference of the growth by that of the wild-type, followed by multiplication of 100. The significance of the treatments was determined by analysis of variance, and treatment means were separated by Tukey's test ($P \leq 0.05$).

Virulence Test

Evaluation of fungal virulence was performed on detached calamondin (*Citrus mitis* Blanco) or Minneola (*Citrus paradisi* Macfad. x *Citrus reticulata* Blanco) leaves

inoculated with conidial suspension ($1x10^4$ conidia/mL) or mycelial mass. Conidial suspension (5 µL) or mycelial mass (with agar removed) was applied to detached citrus leaves and incubated in a moist chamber for 3 to 4 days for lesion development.

Results

Generation of Nox Double Mutants

Transforming split *Sur* marker fragments fused with truncated *NoxA* into protoplasts prepared from DB5 and DR2 identified fungal strains *NoxA NoxB* (ΔnoxAB1 and AB6) and *NoxA NoxR* (ΔnoxAR2 and AR3), respectively. PCR diagnosis confirmed successful integration of SUR within the NoxA locus (Fig. 3-1B). An expected 2.4-kb fragment was amplified from DNA of double mutants AB1, AB6, AR2 and AR3 with the primers surF and NoxA-TAA. Likewise, an expected 3.4-kb fragment was amplified from DNA of AB1, AB6, AR2 and AR3 with the primers NoxA-pro1F and surR. The primer NoxA-pro1F sequence is not present within the split marker fragment. No fragment was amplified from the wild-type strain using these two primer sets.

Nox Contributes to Oxidative and Nitrosative Stress Resistance

Nox mutants grew slightly slower than the wild-type strain. *AaNoxA* and *AaNoxB* mutants showed reduced growth on PDA by 12% and 11%, respectively. *AaNoxR* mutant displayed no significant growth reduction compared to wild-type strain. The double mutant strains (noxAB and noxAR) showed reduced growth by approximately 20%. Growth reduction of the mutant greater than the respective baseline threshold was considered hypersensitive to the compounds tested. Mutational inactivation of *Noxs* in *A. alternata* resulted in an increase sensitivity to various ROS-inducing compounds to varying degrees (Fig. 3-2). *AaNoxA*, *AaNoxB* or *AaNoxR* mutants were hypersensitive to H_2O_2, Cumyl-H_2O_2, rose Bengal (RB), as well as superoxide-generating agents

diamide, menadione (MND) and potassium superoxide (KO_2) to varying degrees. *AaNoxA* and *AaNoxB* single mutant strains were more sensitive than the *AaNoxR* mutant to H_2O_2. *AaNoxB* and *AaNoxR* mutant strains displayed greater sensitivity than the *AaNoxA* mutant to Cumyl-H_2O_2. *AaNoxA* and *AaNoxR* single mutant strains were more sensitive than *AaNoxB* to diamide. *AaNoxB* and *AaNoxR* mutant strains were less sensitive than *AaNoxA* to KO_2. All phenotypes seen in the *AaNoxA*, *AaNoxB* and *AaNoxR* mutant strains were fully or near fully restored, as seen in the genetically complementation CpA16, CpB24 and CpR40 strains, expressing a wild-type copy of the corresponding genes. The *NoxA NoxB* and *NoxA NoxR* double mutant strains displayed severe hypersensitivity to H_2O_2 and RB compared with the sensitivity observed in the strains mutated at *AaNoxA*, *AaNoxB* or *AaNoxR* alone. The *NoxA NoxR*, but not *NoxA NoxB* double mutant strain displayed greater sensitivity than *AaNoxA* or *AaNoxR* to Cumyl-H_2O_2 and diamide. The *NoxA NoxB* and *NoxA NoxR* double mutants displayed MND and KO_2 sensitivity similar to the strains carrying a single gene mutation.

Nox mutants displayed high sensitivity to diamide, a compound that is well known to stimulate the formation of superoxide anion ($O_2^{\cdot-}$) and nitric oxide ($NO^{\cdot}$) (de Lamirande et al., 2009). *Nox* mutants were further tested for sensitivity to different nitrosative stress generating compounds. Sodium nitroprusside (SNP) and hydroxylamine hydrochloride (HDA) are capable of producing $NO^{\cdot}$. L-arginine (Arg) is an $NO^{\cdot}$ synthase substrate and nitro-arginine methyl ester (N-Arg) is a $NO^{\cdot}$ synthase inhibitor (Rees et al., 1990; Cayatte et al., 1994). The *A. alternata NoxA* and *NoxR* mutants were moderately sensitive to SNP, HDA, Arg and N-Arg. *AaNoxB* mutant also displayed moderate sensitivity to all of the tested compounds but Arg (Fig. 3-3).

Expression of Nox Genes Is Responsive to Oxidative and Nitrosative Stress

Northern blot analyses were performed to assess the expression of the *AaNoxA*, *AaNoxB* and *AaNoxR* genes in response to H_2O_2 and other compounds related to oxidative stress in axenic cultures. The 1.8-kb *AaNoxA* gene transcript was barely detectable when the wild-type strain was grown on PDA, but was elevated in the presence of H_2O_2, KO_2, MND, *tert*-butyl H_2O_2 (t-BHP) or hematoporphyrin (HP) (Fig. 3-4). Accumulation of the 1.8-kb *AaNoxB* transcript was detected abundantly when the fungus was grown on PDA, and was elevated to varying degrees when the fungus was shifted to medium supplemented with KO_2, t-BHP or HP. *AaNoxR* was weakly expressed when the fungus was grown on PDA. The accumulation of the 1.7-kb *AaNoxR* gene transcript was apparently elevated in the wild-type strain responding to KO_2, HP or t-BHP. In contrast, H_2O_2 and MND had a moderate effect on the expression of *AaNoxB* and *AaNoxR*.

Nox Regulates the YAP1 Transcription Factor and the HOG1 MAP Kinase

Northern blot analyses showed that the expression of both redox responsive transcription factor *YAP1* and MAP kinase *HOG1* genes was down-regulated in the *A. alternata* strains lacking *NoxA*, *NoxB* or *NoxR* (Fig. 3-4). Re-introduction of a functional copy of the *Nox* gene into the respective mutant restored the expression of the *AaAP1* and *AaHOG1*. In contrast, there was no difference in the expression of the *AaNoxA* gene between the wild-type, *AaAP1* mutants and genetically rescued strain CpY, or between *AaHOG1* mutants (Fig. 3-4C). Deletion of *AaAP1* or *AaHOG1* apparently promoted the expression of both *AaNoxB* and *AaNoxR* genes.

Expression of AaAP1

Expression of the *AaAP1* gene was investigated further in the wild type (WT) and *AaNoxA* mutant (DN2) strains in response to ROS-generating compounds (Fig. 3-5). Northern blot analyses showed that the expression of *AaAP1* was induced in the *AaNoxA* mutant in response to H_2O_2 and other oxidative stress generating compounds (Fig. 3-5A). The increased level of the *AaAP1* gene transcript in the wild-type strain was much greater than that seen in the *AaNoxA* mutant in response to H_2O_2 (Fig. 3-5B, C), further indicating that the activation of *AaAP1* gene expression was partially regulated by *Nox*.

Nox Mutants Appear to be Unable to Penetrate Citrus Leaves

Since the *AaNoxB* single and *NoxA NoxB* double mutant strains did not produce any conidia, fungal pathogenicity was assayed by placing a mycelial mass (with agar removed) on calamondin leaves. Necrotic lesions induced by the wild-type strain appeared at 2 to 4 days post-inoculation (dpi). In contrast, the *AaNoxB* single and *NoxA NoxB* double mutant strains did not produce visible lesions at 4 dpi (Fig. 3-6B and Table 3-1). The *AaNoxB* rescued strain (CpB24) produced necrotic lesions comparable with those induced by the wild-type. *AaNoxB* and *NoxA NoxB* mutant strains induced necrotic lesions similar to those produced by the wild-type and the CpB24 strains on pre-wounded calamondin leaves.

Pathogenicity assays using a point inoculation method with conidial suspension revealed that *AaNoxA* mutant strains produced necrotic lesions on detached calamondin leaves at frequencies ranging from 76% to 81%. The lesions induced by the *AaNoxA* mutant were significantly smaller than those induced by the wild-type and the CpA16 strain re-carrying a functional copy of *AaNoxA* (Fig. 3-6A). In contrast, the

AaNoxR single and *NoxA NoxR* double mutant strains induced pinpoint or no visible lesions on detached leaves inoculated with conidial suspension. The wild-type and the genetically reverted CpR40 strains induced visible lesions at 3 dpi (Fig. 3-6C). When tested on pre-wounded leaves, the strains lacking *AaNoxA*, *AaNoxR* or both induced necrotic lesions at frequencies and magnitudes similar to those of the wild-type and the complementation strains at 2 dpi.

Discussion

In this chapter, the roles of three Nox components (*NoxA*, *NoxB* and *NoxR*) in relation to oxidative and nitrosative stress resistance as well as pathogenesis of *A. alternata* were investigated. Using a loss-of-function mutation in each of these *Nox* genes, the requirement of NOX for resistance to oxidative/nitrosative stress and full virulence in the tangerine pathotype of *A. alternata* were demonstrated. Disruption of the *AaNoxA*, *AaNoxB* or *AaNoxR* gene with a hygromycin-resistance cassette yielded mutants that displayed pleiotropic phenotypes. The phenotypes associated with mutational inactivation of the *Nox* gene were fully restored in the respective complementation strains (CpA16, CpB24 and CpR40), further confirming that the *AaNoxA*, *AaNoxB* or *AaNoxR* disruption was indeed responsible for the observed phenotypes.

The *A. alternata* Nox system is required for vegetative growth and cellular resistance to ROS in axenic cultures. These deficiencies strongly resemble the phenotypes previously seen for the *A. alternata ap1* null mutant defective at a YAP1-like transcription regulator and for the *A. alternata hog1* mutant defective at a HOG1-like MAP kinase (Lin et al., 2009; Lin and Chung, 2010). Although AaNoxA, AaNoxB and AaNoxR are core components of the Nox system, each may have shared or unique

functions in response to different environmental stimuli, because the degree of impairment varied considerably among individual *Nox* mutants. For example, *AaNoxA* and *AaNoxB* mutants display greater growth reduction and cellular sensitivity than the *AaNoxR* mutant to H_2O_2. In contrast, *AaNoxA* and *AaNoxR* mutants are more sensitive to diamide than *AaNoxB* mutant. However, *NoxA NoxB* or *NoxA NoxR* double mutation strains display greater sensitivity to H_2O_2, menadione (MND), diamide and rose Bengal (RB) than the single gene-mutated strains, indicating an additive effect between Nox components. Likewise, *BcNoxA* and *BcNoxB* act additively for H_2O_2 resistance in the necrotrophic fungal pathogen *B. cinerea* (Segmuller et al., 2008). *A. alternata Nox* mutants are hypersensitive to menadione. Similar results have been observed in *Cpnox1* mutant in *C. purpurea* (Giesbert et al., 2008). However, *BcNoxA* and *BcNoxB* mutants of *B. cinerea* are sensitive to H_2O_2 but not menadione (Segmuller et al., 2008). Expression of the *AaNoxA*, *AaNoxB* and *AaNoxR* genes in *A. alternata* was up-regulated in response to a number of ROS compounds, confirming further the involvement of Nox enzymes in cellular resistance to ROS. Consistently, transcript levels of *BcNoxA* and *Cpnox1* were also increased during oxidative stress mediated by H_2O_2 (Giesbert et al., 2008; Segmuller et al., 2008). Moreover, disruption of *AaNoxA*, *AaNoxB* or *AaNoxR* resulted in fungi showing an increased sensitivity to the $NO^{\cdot}$ donors (SNP and HDA), and the $NO^{\cdot}$ synthase substrate (L-arginine) and inhibitor (nitro-L-arginine), implying a possible involvement of Nox system in the $NO^{\cdot}$ signaling pathways. The important role of Nox in response to $NO^{\cdot}$ is further supported by the observations that the expression of *AaNoxA* was elevated by SNP, HAD and Arg. Expression of

AaNoxA was almost undetectable in the wild-type treated with an NO˙ synthase inhibitor nitro-L-arginine.

Little is known about how the Nox complex is regulated in fungi. In mammalian systems, p38 MAP kinase (HOG1 homolog) has been shown to activate the Nox complex via phosphorylation of $p47^{phox}$ and $p67^{phox}$ (NoxR homolog) (Brown et al., 2004). Furthermore, a p21-activated kinase (Pak) was also known to regulate the mammalian Nox complex (Martyn et al., 2005). Deletion of a *Pak* homolog in the ergot fungus *C. purpurea* repressed accumulation of the *Nox1* gene transcript (Rolke and Tudzynski, 2008). In the filamentous fungus *Aspergillus nidulans*, expression of a *NoxA* homolog has been shown to be suppressed by SakA, a HOG1 MAP kinase homolog (Lara-Ortiz et al., 2003). In the endophytic fungus *E. festucae*, inactivation of a *SakA* homolog did not alter expression of *NoxA* or *NoxR*. It was speculated that the interaction might occur at the post-translational level (Eaton et al., 2008). Studies with *Podospora anserina* indicate that *NoxA* facilitates nuclear localization of the PaMpk1 MAP kinase (Malagnac et al., 2004; Kicka et al., 2006). Experimental evidence derived from the present study reveals a regulatory expression of the *AaAP1* and *AaHOG1* genes by *Nox* in response to ROS and chemical stimuli, implying a close interaction between ROS production and resistance. Although impairment of *AaAP1* or *AaHOG1* has no impact on the expression of *AaNoxA*, both *AaAP1* and *AaHOG1* repressed the expression of *AaNoxB* and *AaNoxR*. This transcriptional feedback loop could avoid the excessive production of ROS.

NoxR is homologous to the $p67^{phox}$ regulatory subunit and presumably required for activation of NoxA and NoxB. *NoxA* have been shown to be regulated by *NoxR* in

As. nidulans and *E. festucae* (Takemoto et al., 2006; Semighini and Harris, 2008) and both *NoxA* and *NoxB* are regulated by *NoxR* in *N. crassa*, *B. cinerea* and *P. anserina* (Cano-Dominguez et al., 2008; Segmuller et al., 2008; Brun et al., 2009). In this chapter, I found that AaNoxR transcriptionally suppresses *AaNoxA*, because disruption of *AaNoxR* resulted in an elevated expression of *AaNoxA*, but not *AaNoxB*. Unlike the findings with *N. crassa*, *B. cinerea* and *P. anserina* (Cano-Dominguez et al., 2008; Segmuller et al., 2008; Brun et al., 2009), the phenotypes seen for the *AaNoxR* single and the *NoxA NoxB* double mutation strains of *A. alternata* were also very different, indicating further that *AaNoxR* may not directly regulates *AaNoxA* and *AaNoxB*. It is surprising that the *A. alternata NoxR* has no impact on the expression of *AaNoxB*, but negatively regulates the expression of *AaNoxA*. In this context, regulation of the Nox system in response to environmental stimuli in *A. alternata* might be somewhat unique. It is noteworthy that some fungi, such as *Rhizopus oryzae*, *Yarrowia lipolytica* and *Phycomyces blakesleeanus*, have NoxR-like proteins but lack *NoxA and NoxB* homologs (Takemoto et al., 2007). Analysis of the deduced amino acids sequences of these NoxR-like proteins revealed no Nox activation domain, implying that NoxR may have functions in addition to regulating the Nox system.

A. alternata secretes a host-selective toxin to rapidly kill host cells prior to invasion and obtains nutrients exclusively from dead tissues. As a result of cell death, a considerable amount of H_2O_2 was detected in the vicinity of the infection area (Lin et al., 2011). Hence, *A. alternata* must have evolved an effective ROS detoxification system to successfully colonize within the oxidative environment of necrotic tissues. The fungal YAP1 redox-responsive transcriptional regulator and the HOG1 MAP kinase-mediated

signaling pathways are absolutely required for *A. alternata* pathogenicity in citrus. *AaAP1* and *AaHOG1* mutants are impaired in both penetration and colonization stages (Lin et al., 2009; Yang et al., 2009; Lin and Chung, 2010). However, Nox-mutated strains apparently are arrested in the penetration stage. Although *AaNoxA*, *AaNoxB* or *AaNoxR* mutants cause significantly smaller or no lesions on unwounded citrus leaves, all mutants induced lesions, which are comparable to wild-type on pre-wounded leaves. Similarly, the *B. cinerea* strains lacking *BcNoxB* or *BcNoxR* display deficiency in virulence due to their impairment of penetration on bean leaves (Segmuller et al., 2008). In summary, it was demonstrated that a close interaction among the Nox system, the YAP1 redox responsive transcription factor and the HOG1 MAP kinase-mediated signaling pathway is critical for fungal resistance to ROS and pathogenesis in *A. alternata*. This study underlines an important regulatory role of Nox enzymes in fungi.

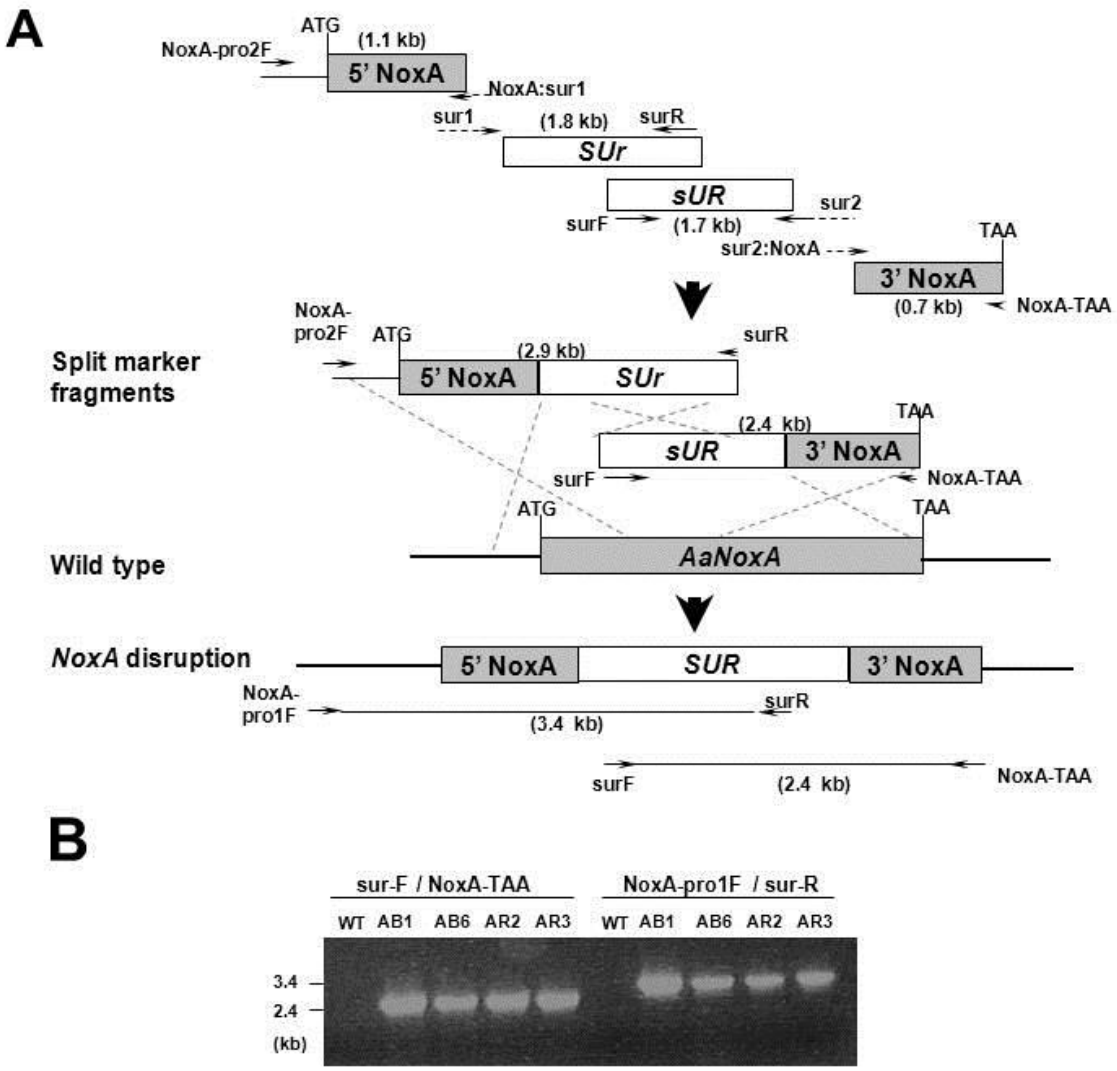


Figure 3-1. Targeted disruption of *AaNoxA* in a fungal strain lacking *NoxB* (DB5) or *NoxR* (DR2) of *Alternaria alternata* using a split marker approach. (A) Schematic depiction of the generation of truncated, but overlapping, sulphonylurea-resistance gene (*SUR*), and gene disruption within *AaNoxA*. Primers used to amplify each fragment are indicated. (B) Image of DNA fragments amplified from genomic DNA of fungi with the primers indicated. The primer NoxA-pro1F sequence is not located within the split marker fragment. Fragment patterns indicate successful disruption at the *AaNoxA* locus. Fungal strains AB1 and AB6 carry impaired *NoxA* and *NoxB* genes; strains AR2 and AR3 carry impaired *NoxA* and *NoxR* genes.

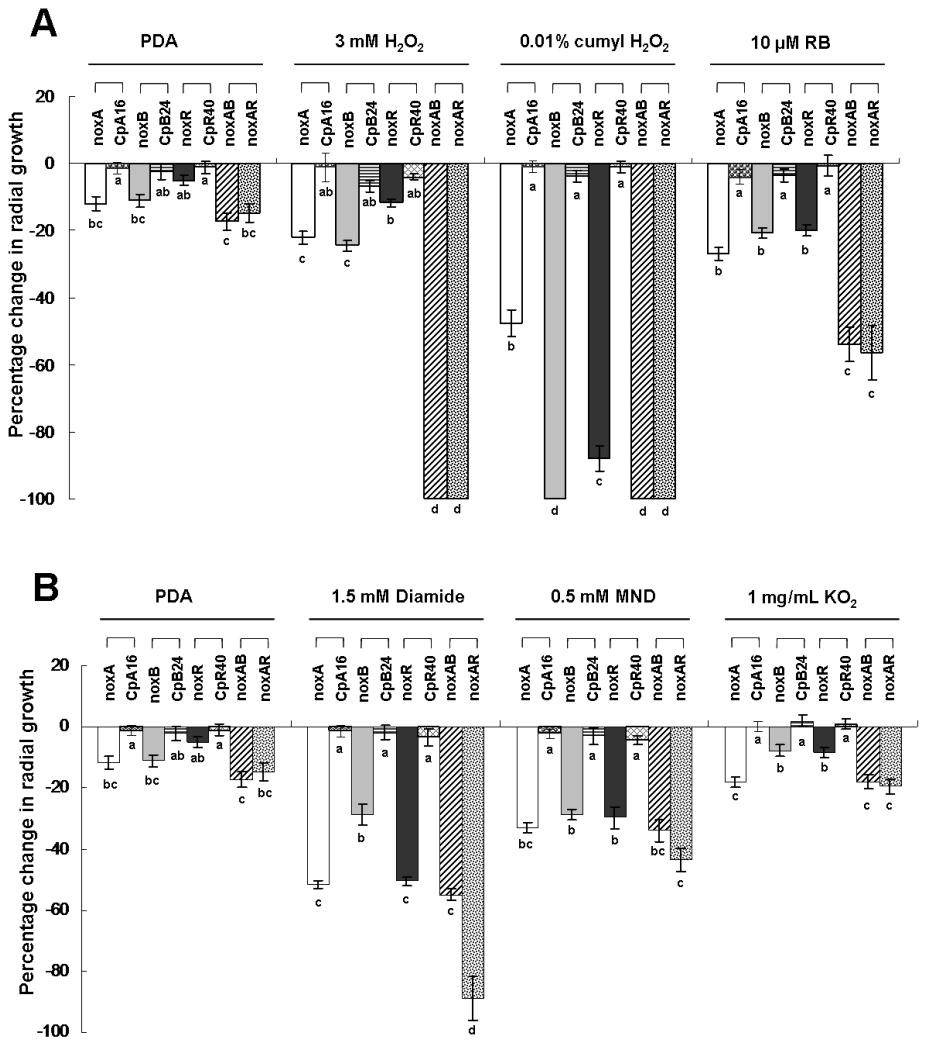


Figure 3-2. Sensitivity assays of the wild-type (WT), the strains carrying a single deletion of *NoxA*, *NoxB* or *NoxR*, the genetically complementation (CpA16, CpB24 and CpR40), and the double mutation *NoxA NoxB* (noxAB) and *NoxA NoxR* (noxAR) strains of *Alternaria alternata*. Two independent mutant strains of each type were used for the assays. Fungi were grown on PDA amended with the test compounds. Radial growth was measured at 3-6 days. The percentage change in radial growth was calculated as the percentage of growth of the deletion mutants in relation to the wild-type grown on the same plate. A negative percentage change indicates growth reduction in relation to the wild-type. The mutant was considered to be hypersensitive to the test compounds when the percentage change in growth reduction was greater than that measured in untreated PDA. The data presented are the mean and standard error of two independent mutants with at least two replicates of two experiments (n = 8). For each test compound, means indicated by the same letter within a test compound are not significantly different from one another, $P \leq 0.05$. RB, rose Bengal; MND, menadione.

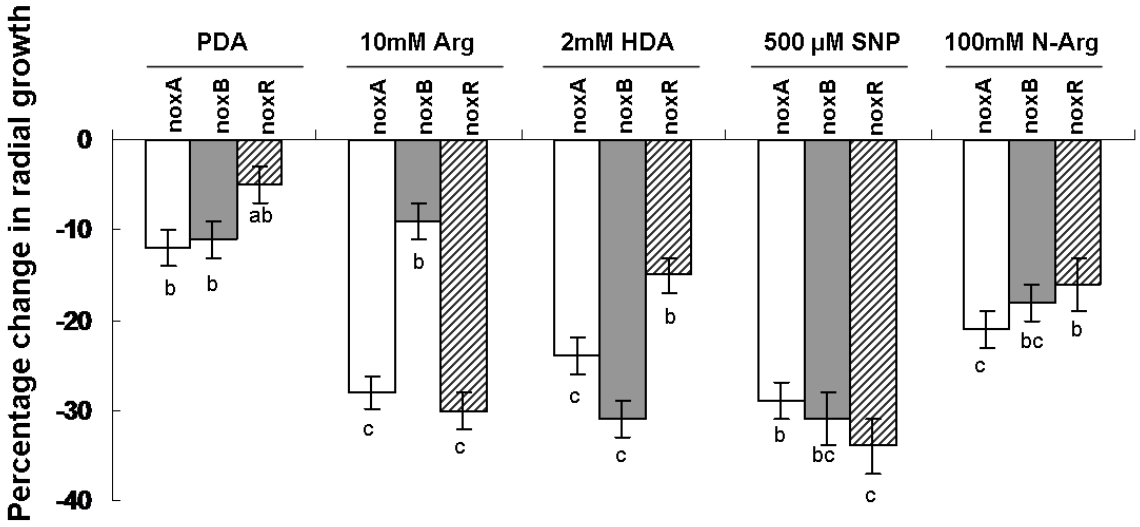


Figure 3-3. Sensitivity assays of the wild-type (WT), the strains carrying a single deletion of *NoxA*, *NoxB* or *NoxR* of *A. alternata*. Two independent mutant strains of each type were used for the assays. Fungi were grown on PDA amended with the test compounds. Radial growth was measured at 3-6 days. The percentage change in radial growth was calculated as the percentage of growth of the deletion mutants in relation to the wild-type grown on the same plate. A negative percentage change indicates growth reduction in relation to the wild-type. The mutant was considered to be hypersensitive to the test compounds when the percentage change in growth reduction was greater than that measured in untreated PDA. The data presented are the mean and standard error of two independent mutants with at least two replicates of two experiments (n = 8). For each test compound, means indicated by the same letter within a test compound are not significantly different from one another, $P \leq 0.05$. Arg, L-arginine; HDA, hydroxylamine hydrochloride; SNP, Sodium nitroprusside; N-Arg, nitro-arginine methyl ester.

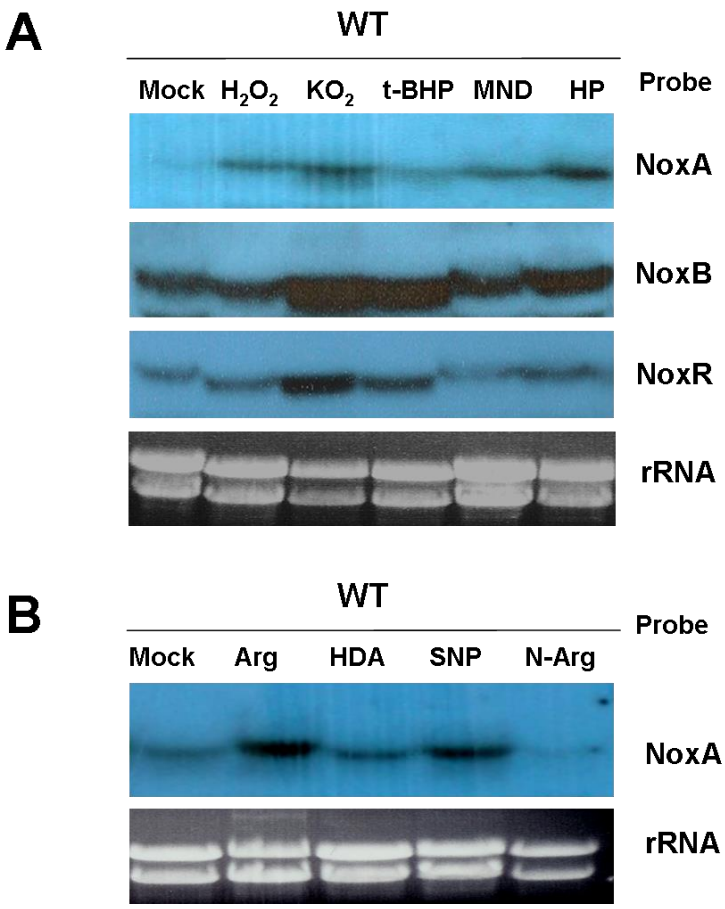


Figure 3-4. Images of RNA gel blotting. (A) Northern blot analyses showing the expression of the *NoxA*, *NoxB* and *NoxR* genes in response to ROS. (B) Northern blot analyses for the expression of *NoxA* gene in response to nitrosative stress-related compounds. The mock controls were grown on PDA only.

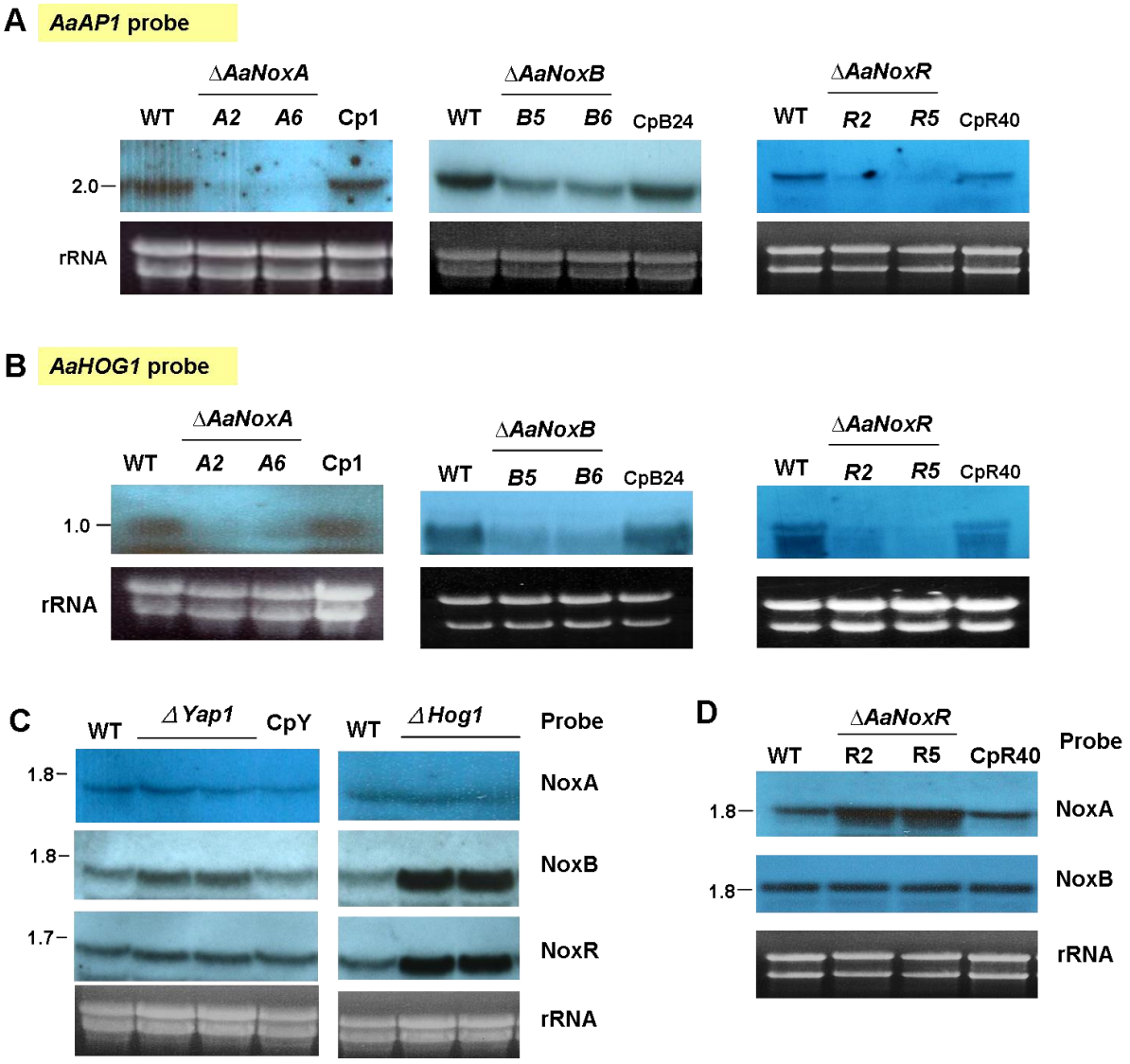


Figure 3-5. Images of RNA blotting. Fungal RNA purified from the wild-type (WT), *NoxA* mutants (A2, A6), *NoxB* mutants (B5, B6) and *NoxR* mutants (R2, R5), as well as the respective genetically complementation strains (CpA16, CpB24 and CpR40), was electrophoresed in a formaldehyde-containing gel, blotted and hybridized with an *AaAP1* (A) or *AaHOG1* (B) gene probe. (C) Fungal RNA purified from WT, two *AaAP1* mutants and its complementation strain (CpY), as well as two *AaHOG1* mutants was hybridized to a *NoxA*, *NoxB* or *NoxR* gene probe. (D) Total RNA purified from WT, *NoxR* mutants and its rescued strain (CpR40) was hybridized to a *NoxA* or *NoxB* 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).

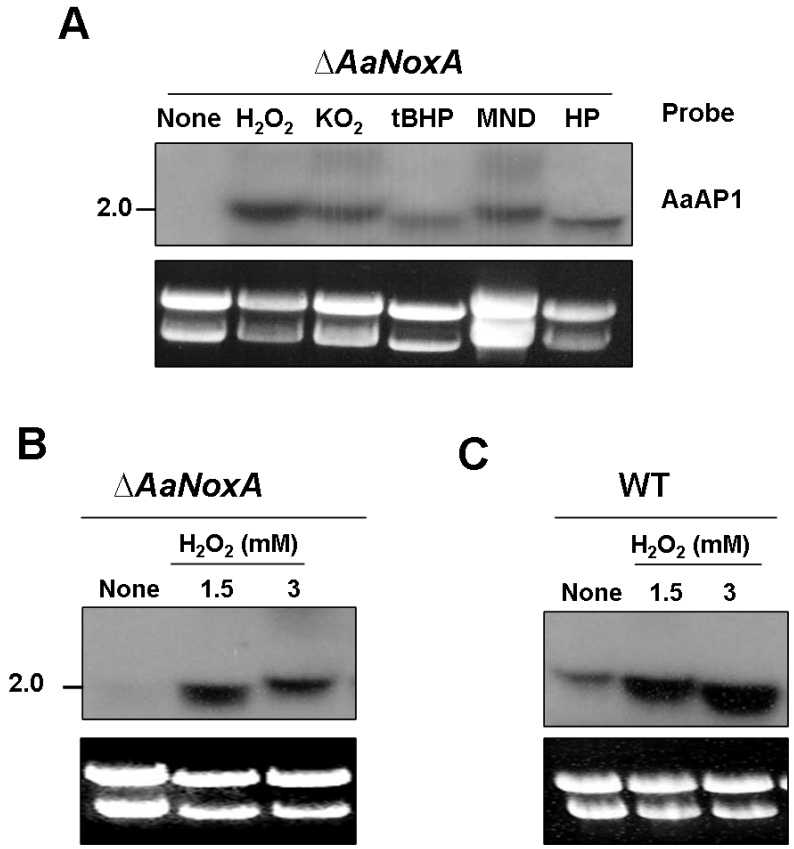


Figure 3-6. RNA gel blotting indicates the expression of *AaAP1*. (A, B) The *Alternaria alternata NoxA* mutant (DN2) was grown on cellophane overlaid onto PDA for 2 days, shifted to PDA containing H_2O_2 (1 or 3 mM), KO_2 (0.5 mg/mL), *tert*-butyl-hydroperoxide (t-BHP, 0.05%), menadione (MND, 1 mM) or hematoporphyrin (HP, 50 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 (WT) grown on PDA or H_2O_2, 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).

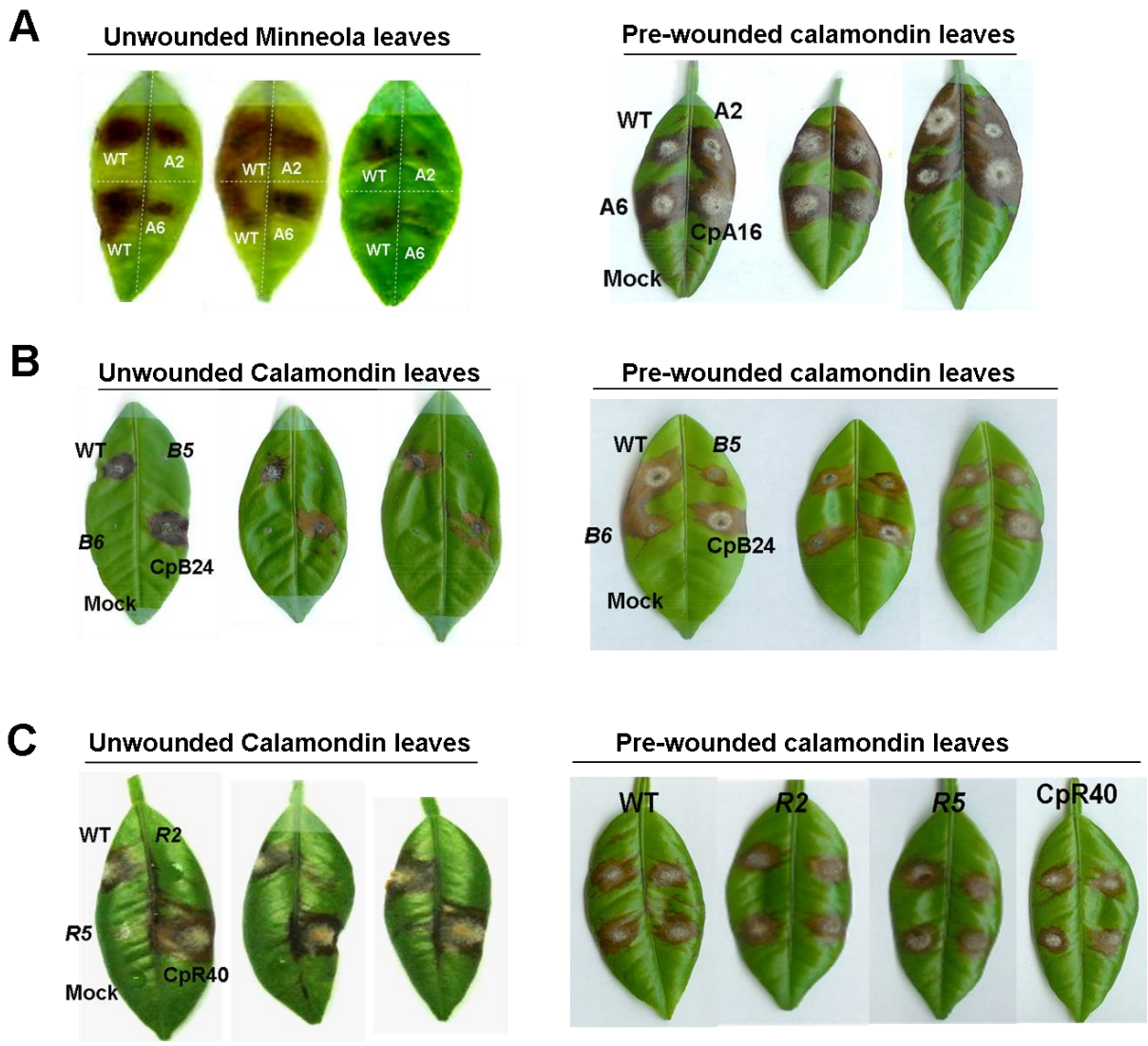


Figure 3-7. Pathogenicity of the *NoxA*, *NoxB* and *NoxR* mutants of *A. alternata* assayed on unwounded or pre-wounded calamondin or Minneola leaves. Conidia suspension or mycelial mass of *NoxA* mutants (A2, A6), *NoxB* mutants (B5, B6) and *NoxR* mutants (R2, R5), as well as the respective genetically complementation strains (CpA16, CpB24 and CpR40) was inoculated on detached citrus leaves. Inoculation of wild-type (WT) strain served as control. The mock controls were treated with water only. Photographs were taken at 2 to 4 days after inoculation.

Table 3-1. Pathogenicity assayed on detached Minneola or calamondin leaves inoculated with the wild-type and the genetically modified strains of *Alternaria alternata*

Fungal Strain	Genotype	Inoculum	Disease incidence (%)*	
			Unwounded	Wounded
Mock		H_2O	0/26 (0)	0/15
EV-MIL31	Wild-type	Mycelial mass	32/32 (100)	17/17
DB5	*NoxB* disruption		0/22 (0)	11/11
DB6			0/22 (0)	11/11
AB1	*NoxA NoxB* double disruption		0/11 (0)	10/10
AB6			0/11 (0)	10/10
CpB24	*NoxB* rescued		21/22 (95.5)	11/11
Mock		H_2O	0/24 (0)	0/12
EV-MIL31		Conidia	63/63 (100)	20/20
DN2	*NoxA* disruption#		32/42 (76.2)※	20/20
DN6			39/48 (81.3)※	20/20
CpA16	*NoxA* rescued#		40/45 (88.9)	16/16
DR2	*NoxR* disruption		5/27 (18.5)	24/24
DR5			6/27 (22.2)	24/24
AR2	*NoxA NoxR* double disruption		3/44 (6.8)	10/10
AR3			3/44 (6.8)	10/10
CpR40	*NoxR* rescued		27/27	20/20

* Pathogenicity was assayed on detached calamondin leaves by placing mycelial mass or 5 µL of conidial suspension (10^4 conidia/mL) in each of the spots. The inoculated leaves were incubated in a mist chamber for lesion development. The number of necrotic lesions and the total number of inoculated spots on leaves are to the right and left of the slash (/), respectively. Except for double-mutant strains, all strains were tested at least twice. The mock control was treated with water only.

※ Lesions induced by NoxA mutant were significantly smaller (by ~40%) than those induced by the wild-type.

NoxA disruption and *NoxA* rescued strains were tested on Minneola leaves. Other strains were tested on calamondin leaves.

CHAPTER 4
THE INVOLVEMENT OF NADPH OXIDASES IN DEVELOPMENTAL AND PHYSIOLOGICAL PROCESSES OF *Alternaria alternata*

Introduction

Reactive oxygen species (ROS) play important roles in cellular signaling in different aerobic organisms ranging from bacteria to mammalian cells (Mittler et al., 2011). The non-toxic, steady-state level of ROS in cells is maintained by a complex interplay between the ROS producing pathways and the ROS scavenging mechanisms. Through highly regulated networks, cells are able to generate and accumulate ROS in specific compartments (Lambeth, 2004; Mittler et al., 2004; Mittler et al., 2011). ROS are produced by NADPH oxidase (Nox) in animals, plants and some fungi. Nox plays a critical role in numerous biological processes (Brown and Griendling, 2009; Aguirre and Lambeth, 2010). Plant NADPH oxidases, so-called respiratory burst oxidase homologs (RBOH), are involved in regulating a myriad of developmental processes. These include polarized cell expansion of root hair, pollen tube growth, cell wall lignification as well as seed ripening and germination (Foreman et al., 2003; Potocky et al., 2007; Hamann et al., 2009; Muller et al., 2009).

In filamentous fungi, Nox was first shown to be required for cleistothecia development in *Aspergillus nidulans* (Lara-Ortiz et al., 2003). NoxA (Nox1) plays an essential role in perithecia development and NoxB (Nox2) is required for ascospore germination in both *Podospora anserina* and *Neurospora crassa* (Malagnac et al., 2004; Cano-Dominguez et al., 2008). Moreover, NoxA-mediated ROS production is involved in the regulation of apical dominance in hyphal tips in *As. nidulans* (Semighini and Harris, 2008). *Nox* genes of *P. anserina* are involved in the degradation of cellulose by regulating the formation of needle-like hyphae (Brun et al., 2009). In *Botrytis cinerea*, *S.*

sclerotiorum and *Claviceps purpurea*, NoxA and NoxB are required for sclerotia formation (Giesbert et al., 2008; Segmuller et al., 2008; Kim et al., 2011). In the rice blast pathogen *Magnaporthe oryzae*, Nox enzymes are required for appressoria formation (Egan et al., 2007).

The maintenance of cell wall integrity is essential in plants and fungi during growth, morphogenesis and exposure to diverse environmental stresses (Levin, 2005; Ringli, 2010; Seifert and Blaukopf, 2010). Cells change the physical structure and chemical composition of cell wall, and orchestrate cellular metabolism to maintain the integrity of the cell wall (Hamann et al., 2009; Levin, 2011). In Arabidopsis, RBOH-generated ROS- along with jasmonic acid- (JA) and Ca^{2+}-signaling mechanisms are involved in maintaining plant cell wall integrity (Hamann et al., 2009; Denness et al., 2011; Hamann and Denness, 2011). In *M. oryzae*, Nox1, but not Nox2, is required for cell wall biosynthesis and remodeling during appressorium formation (Egan et al., 2007).

The tangerine pathotype of *Alternaria alternata* has no known sexual cycle. Environmental stimuli such as light, oxygen and nutrition increase sporulation (Rotem, 1994). The formation and dispersal of conidia are essential for the completion of the life cycle, as well as the onset of Alternaria brown spot in citrus. Previous studies have demonstrated that conidia formation is positively regulated by the G protein-controlled cAMP level, as well as by the FUS3 and SLT2 mitogen-activated protein kinases (MAPK)-mediated signaling pathways in *A. alternata* (Lin et al., 2010; Wang et al., 2010; Yago et al., 2011). The G protein/cAMP-dependent protein kinase A (PKA) is important for conidiation in filamentous fungi (Bolker, 1998). The PKA complex, containing two regulatory and two catalytic subunits, forms an inactive tetramer when the cellular cAMP

levels are low. Elevated cAMP levels activate PKA by separating the catalytic subunits from the regulatory subunits, resulting in the activation of downstream enzymes or transcriptional regulator by phosphorylation (Gerits et al., 2008). Recent studies conducted in our lab revealed that PKA negatively regulates conidiation in *A. alternata*. Inactivation of the PKA catalytic subunit gene (*PKA*^{cat}) resulted in no detectable PKA activity and hyperconidiation (Tsai et al., 2013). Fungal mutants lacking a PKA regulatory subunit gene (*PKA*^{reg}) produced detectable PKA activity but no mature conidia.

The objective of this study is to determine the role of Nox enzymes in morphogenesis and conidiation in *A. alternata*. I provide genetic evidence to define the central role of *NoxA, NoxB* and *NoxR* in the development of conidia. I also show a novel role of *NoxB* in cell wall integrity and fungicide sensitivity. The results highlight a dramatic flexibility and uniqueness of NOX components in developmental and physiological processes in the tangerine pathotype of *A. alternata*.

Materials and Methods

Fungal Strains and Chemical Sensitivity Tests

Alternaria alternata (Fr.) Keissler strains used in this study and their genotypes are given in Table A-1. The genetically altered strains — *AaNoxA* mutants (DN2, DN6), *AaNoxB* mutants (DB5, DB6), *AaNoxR* mutants (DR2, DR5) and the corresponding complementation strains (CpA16, CpB24 and CpR40), as well as the double mutant *NoxA NoxB* (noxAB1 and AB6) and *NoxA NoxR* (noxAR2 and AR3) strains — were generated from previous studies (Chapter 2 and 3). Fungal mutants disrupted at the gene encoding a MAPK (*AaFUS3*), a cAMP-dependent protein kinase A (PKA) catalytic subunit (*AaPKA*^{cat}) or a PKA regulatory subunit (*AaPKA*^{reg}) were created in separate

studies (Lin et al., 2010; Tsai et al., 2013). Assays for chemical sensitivity to cell wall-targeting compounds, sodium dodecyl sulfate (SDS), calcofluor white (CFW) and Congo red (CR), as well as fungicides vinclozolin and fludioxonil, were conducted by transferring fungal hyphae/conidia as a tooth pick point inoculation onto PDA containing the test chemicals at appropriate concentrations. Fungal radial growth was measured from 3 to 6 days. The percentage change in growth of the disrupted mutants relative to that of the wild-type grown on the same plate was calculated. The significance of the treatment was determined by analysis of variance and the treatment means were separated by Tukey's test ($P \leq 0.05$).

Isolation and Quantification of Conidia

Conidia were harvested by flooding with sterile water and low speed centrifugation (3,000 × *g*) from fungal cultures grown on PDA under constant fluorescent light for 3 to 4 days. The concentration of conidia was determined with the aid of a haemocytometer. Conidial morphology was examined using a Leitz Laborlux phase-contrast microscope (Leica Microsystems, Exton, PA, U.S.A.).

Extraction and Quantification of Fungal Chitin

Fungal mycelium was ground in liquid nitrogen, mixed with 500 µL cell-wall extraction buffer (50 mM Tris-HCl pH8.0, 2% SDS, 0.3 M β-mercaptoethanol, 1 mM EDTA), and boiled at 100 °C for 15 min (Selvaggini et al., 2004). Cell wall pellets were collected by centrifugation at 8,000 × *g* for 10 min, washed three times with distilled water, and dried completely in 60 °C oven. Cell wall material (5-10 mg) was resuspended in 200 µL 6 M HCl and hydrolyzed at 100 °C for at least 4 hours. Sample was then dried completely in 60 °C oven and resuspended in 300 µL distilled water. Chitin was determined and quantified by measuring the glucosamine released from

chitin after hydrolysis using *p*-dimethylaminobenzaldehyde as a chromogen. Crude extracts of fungal cell wall (100 µL) were mixed with equal volume of 1.5 M Na_2CO_3 dissolved in 4% (v/v) acetylacetone and heated at 100 °C for 20 min. Samples were mixed with 700 µL of ethanol and 200 µL of Ehrlich's reagent (26 mg/mL *p*-dimethylaminobenzaldehyde dissolved in 5.8 M HCl, and 50% ethanol) and measured spectrophotometrically for absorbance at 520 nm. The quantity of glucosamine was calculated by reference to a regression line established using pure glucosamine as a standard. The significance of the treatment was determined by analysis of variance and the treatment means were separated by Tukey's test ($P \leq 0.05$).

Manipulation of Nucleic Acids

Isolation of fungal RNA and Northern blot analyses were performed as described in Chapter 2.

Protein Purification and Western Blot Analysis

Western-blot analysis was performed to determine if mutational inactivation of *AaNoxB* will affect the phosphorylation of AaFUS3. Fungal isolates were grown on liquid complete medium (CM) for 3 days (Chung, 2003). Total proteins were extracted from fungal mycelium in a buffer containing 10 mM Tris-HCl (pH7.5), 150 mM NaCl, 5mM EDTA, 10 mM NaN_3, and 1% Triton X-100, followed by centrifugation at 10,000 × *g* for 15 min at 4 °C. Protein concentrations were determined by a protein assay kit (Bio-Rad, Hercules, CA). The protein samples were denatured in 2x SDS sample buffer heated at 100 °C for 10 min. Proteins were electrophoresed in a 12% SDS-polyacrylamide gel set at 25 mA for 60-90 min. Proteins were electroblotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) and incubated with 1:1000 dilutions of rabbit anti-phosphate-p44/42 MAP kinase antibody or FUS3 (y-40) rabbit polyclonal antibody. The anti-rabbit-

IgG antibody conjugated horseradish peroxidase (HRP) at a 1:2000 dilution was used as a secondary antibody. All antibodies used in this study were purchased from Cell Signaling Technology (Boston, MA). Detection of the HRP was performed using LumiGLO (Signaling Technology, Boston, MA) as a chemofluorescent substrate.

Results

Nox Is Involved in Conidia Formation

Compared with the wild-type strain grown on PDA in the light, *AaNoxA* mutant (DN2 and DN6) and *AaNoxR* mutant (DR2 and DR5) formed light brown colonies and *AaNoxB* mutant (DB5 and DB6) accumulated little or no pigmentation (Fig. 4-1A, 4-2A and 4-3A). Quantitative analysis of conidia formation revealed that *AaNoxA* mutants were reduced in conidiation by 49% (Fig. 4-1B). Light microscopy analysis revealed that the wild-type strain grown on PDA often formed multicellular, elliptical and melanized conidia with both vertical and transverse septae. In contrast, *AaNoxA* mutant produced light-colored conidia with less distinct septae (Fig. 4-1C). The complementation strain CpA16 produced wild-type levels of conidia with both vertical and transverse septae and dark pigmentation. *AaNoxB* mutants failed to produce any conidia. Pigmentation and conidiation were restored to wild-type levels in the complementation strain CpB24 (Fig. 4-2B and C). *AaNoxR* mutants reduced conidiation by 90%. Conidia produced by *AaNoxR* mutants were similar to those produced by wild-type. The complementation strain CpR40 produced wild-type levels of conidia (Fig. 4-3B and C). Mutational inactivation of *AaNoxA* or *AaNoxR* did not affect conidial germination. The *NoxA NoxB* double mutant strain did not produce any conidia. The *NoxA NoxR* double mutant strain produced conidia at levels similar to the *AaNoxR* mutant.

NoxB Plays a Negative Role in Cell Wall Integrity and Fungicide Sensitivity

Fungi were tested for sensitivity to cell wall-targeting compounds, including SDS, calcofluor white (CFW) and Congo red (CR). The wild-type strains were reduced in radial growth by 35%-40% when grown on medium amended with 0.01% SDS. Fungal strains impaired for each of the *Nox* genes displayed an increased sensitivity to SDS (Fig. 4-4A). The complementation strains (CpA16, CpB24 and CpR40) displayed wild-type level sensitivity to SDS. The *NoxA NoxB* (noxAB) and the *NoxA NoxR* (noxAR) double mutant strains were more sensitive to SDS than the single gene mutant strains.

The wild-type strain of *A. alternata* displayed reduced radial growth by 34%-40% when grown on medium amended with 100 µM CFW or 60 µM CR. Surprisingly, *AaNoxB* and *noxAB* mutant strains showed increased resistance to CFW and CR compared with the wild-type (Fig. 4-4A). In contrast, mutant strains lacking *NoxA*, *NoxR* or both displayed slightly increased growth in the presence of CFW, but had elevated sensitivity to CR, when compared with the wild type. The complementation strains (CpA16, CpB24 and CpR40) displayed sensitivity to CFW and CR at levels not significantly different from the wild type.

The wild-type strain of *A. alternata* displayed reduced radial growth by 26%-32% and 38%-44% when grown on medium amended with 10 µg/mL vinclozolin and 0.1 µg/mL fludioxonil fungicides, respectively. The complementation strains (CpA16, CpB24 and CpR40) displayed wild-type level sensitivity to these fungicides, whereas *AaNoxB* and *noxAB* mutant strains were insensitive to them (Fig. 4-4B). Disruption of *AaNoxA* or *AaNoxR* had a lesser effect on fungicide resistance. Fungal strains lacking *NoxA*, *NoxR* or both displayed slightly increased resistance to vinclozolin compared with the same strains grown on untreated PDA. On PDA amended with fludioxonil, *AaNoxR, AaNoxB*

and *noxAB* mutant strains displayed significantly increased resistance (a positive percentage change) compared with the wild type.

Quantitative assays revealed that fungal strains carrying defective *NoxB* or *NoxA* *NoxB* had significantly higher chitin content compared to the levels measured in the wild-type and other *Nox* disrupted strains (Fig. 4-5). The chitin level detected in the complementation strain CpB24 was not significantly different from that of wild-type. The *NoxA* or *NoxR* disruptants had lower chitin content than the wild type and the CpR40 complementation strains.

Gene Expression

Northern blot analyses were performed to determine possible interplays at transcriptional levels among *NoxB*, *FUS3*, PKA^{cat} and PKA^{reg} (Fig.4-6A). The results indicated that accumulation of the *FUS3* and PKA^{reg} gene transcripts was elevated in the *NoxB* mutant. Inactivation of *NoxB* had little or no effect on the expression of PKA^{cat}. The complementation strain CpB24 displayed wild-type expression of both *FUS3* and PKA^{reg}. Inactivation of *FUS3* reduced the level of *NoxB* and PKA^{cat} gene transcripts and had no effect on the expression of the PKA^{reg} (Fig.4-6B). Deletion of PKA^{cat} or PKA^{reg} apparently reduced the expression of *NoxB* gene (Fig.4-6C, D). The accumulation of the *FUS3* and PKA^{reg} gene transcripts was not affected in the PKA^{cat} mutants. However, inactivation of PKA^{reg} promoted the expression of both *FUS3* and PKA^{cat} genes.

AaNoxB Negatively Regulates Phosphorylation of AaFUS3

The phosphate-p44/42 MAP kinase monoclonal antibody detected faint bands in the protein samples prepared from the wild-type strain and the *NoxB* complementation strain CpB24 of *A. alternata* (Fig.4-7). Phosphorylation of AaFUS3 was elevated in fungal strains lacking *AaNoxB*. The intensity of band detected by the phosphate-p44/42

MAPK antibody was apparently stronger in the protein sample prepared from the *NoxB* mutants. The anti-FUS3 antibody detected protein bands representing total AaFUS in each sample.

Discussion

In this chapter, I investigated the roles of Nox components (NoxA, NoxB and NoxR) in developmental and physiological functions of *A. alternata*. Fungal strains impaired for *AaNoxA*, *AaNoxB* or *AaNoxR* reduced growth slightly compared with the wild-type progenitor and formed light brown colonies on PDA. Nox plays no role in hyphal branching. Reduction in growth was observed in *Nox* mutants of *B. cinerea* and *N. crassa* (Cano-Dominguez et al., 2008; Segmuller et al., 2008), but not in the rice blast pathogen *M. oryzae*. The *M. oryzae Nox1* mutants grow slightly faster than wild type and *Nox2* mutants exhibit wild-type growth rate (Egan et al., 2007). *Nox1* mutants of *P. anserina* and *M. oryzae* produce lightly pigmented hyphae (Malagnac et al., 2004; Egan et al., 2007). In addition to radial growth and pigmentation, *A. alternata* Nox is required for conidiation. Similarly, disruption of either *Nox1* or *NoxR* in *N. crassa* impacts conidia production. *M. oryzae* mutant impaired for both *NoxA* and *NoxB* displays a dramatic reduction in conidiogenesis. However, mutational inactivation of *Nox* genes in *B. cinerea*, *Nox2* in *N. crassa* and *Nox1* or *Nox2* in *M. oryzae* has little or no effect on conidia formation (Egan et al., 2007; Cano-Dominguez et al., 2008; Segmuller et al., 2008). Moreover, *N. crassa Nox1*, *M. oryzae Nox2* and *Epichloë festucae NoxA* mutants are all defective for the formation of aerial hyphae (Egan et al., 2007; Cano-Dominguez et al., 2008). In contrast, the *Nox* gene product plays no role in vegetative growth in *As. nidulans* (Lara-Ortiz et al., 2003). These divergent phenotypes

suggest that the well-conserved Nox enzymes may have varying impacts on vegetative growth and development in different fungi.

AaNoxA, AaNoxB and AaNoxR may independently and cooperatively interact with other yet unidentified components during different developmental stages or under different environmental conditions. As shown in this study, *A. alternata NoxB*, but not *NoxA*, has a unique role in cell wall integrity and fungicide sensitivity. Similarly, *M. oryzae Nox1* is involved in cell wall biochemistry (Egan et al., 2007). Calcofluor white (CFW) preferentially binds to polysaccharides containing 1,4-linked D-glucopyranosyl units and alters the assembly of chitin microfibrils in fungi (Elorza et al., 1983). Both *AaNoxB* and *NoxA NoxB* mutants have higher chitin content than the wild-type and the complementation strain CpB24. The results suggest a negative regulatory role of *AaNoxB* in cell wall integrity. This novel phenotype has not been identified to my knowledge in other fungi. Previous studies have revealed that mutational inactivation of the *A. alternata SLT2* MAPK causes reduced accumulation of chitin (Yago et al., 2011). *AaNoxB* and *NoxA NoxB* mutants, as well as fungal strains lacking the gene encoding a class III histidine kinase (*HSK1*), a response regulator (*SKN7*) or a *HOG1* MAP kinase all exhibit elevated resistance to fungicides vinclozolin and fludioxonil in varying degrees (Lin and Chung, 2010; Chen et al., 2012). These results imply a possible interplay between these different signaling elements in the context of cell wall integrity and fungicide sensitivity. The association of *Nox* with fungicide sensitivity, to my knowledge, has not been demonstrated in fungi. In the cereal pathogen *Fusarium graminearum*, elevated accumulation of H_2O_2 was observed after the application of a triazole fungicide prothioconazole that is acting as an inhibitor of ergosterol biosynthesis in fungi

(Audenaert et al., 2010). It will be of interest to determine whether or not AaNoxB is a target of vinclozolin and fludioxonil fungicides in the future.

The Nox-derived ROS contributes to diverse developmental processes in many organisms. In mammalian cells, Nox is required for reconstructing the cytoskeleton organization (Brown and Griendling, 2009). In *Caenorhabditis elegans*, Nox-generated ROS are required for tyrosine cross-linking within the cuticular extracellular matrix (Edens et al., 2001). H_2O_2 causes the rapid cross-linking of cell wall structural proteins, leading to a strengthened cell wall in soybean (Brisson et al., 1994; Lamb and Dixon, 1997). NoxA-derived ROS have also been suggested to be involved in cell wall cross-linking during cleistothecia formation (Lara-Ortiz et al., 2003). Recent studies with *M. oryzae* and *S. cerevisiae* provide novel insights into the molecular mechanisms of how Nox mediates fungal differentiation. In *M. oryzae*, Nox is required for the differentiation of penetration peg at the base of appressoria by remodeling the F-actin cytoskeleton and initiating the polarized growth from the appressorium (Dagdas et al., 2012; Ryder et al., 2013). Nox2/NoxR complex is involved in septin-mediated F-actin assembly at the appressorium pore. The Nox1-mediated production of ROS is required for oxidative cross-linking of cell wall proteins and polarized growth. In *S*. *cerevisiae*, a newly identified Nox homolog, *YNO1*, is also involved in the formation of actin cable (Rinnerthaler et al., 2012). Whether or not *A. alternata Nox* plays a role in cytoskeleton reorientation or polarized growth remains to be determined.

Formation of conidia in fungi is tightly regulated process, which is often modulated by different signaling pathways in a given species. Studies with *A. alternata* have revealed that G protein, cAMP-dependent PKA, FUS3 MAPK and SLT2 MAPK

signaling pathways are all required for conidia formation. Fungal strains impaired for *FUS3* gene fail to produce mature conidia (Lin and Chung, 2010). *SLT2* mutants produce fewer but slightly larger conidia with fewer transverse septae (Yago et al., 2011). *AaGα1* (a Gα subunit-coding gene) mutants reduce conidiation (Wang et al., 2010). PKA^{cat} mutants produce abundant conidia and PKA^{reg} mutants produce no mature conidia (Tsai et al., 2013). The present study indicates that the *A. alternata* strains lacking *Nox* are also defective in conidia production. Nox and its ability to produce ROS apparently play a crucial role in conidia formation. NoxB is likely the primary determinant for the initiation of conidiation because inactivation of *NoxB* completely blocked the formation. Impairment of *NoxA* or *NoxR* also resulted in a severe reduction in conidia formation. These findings suggest that conidia formation is governed by a complex and intertwined regulatory network, where NOX appears to play a central role in *A. alternata*.

Based on the observed phenotypes derived from previous and present studies, a regulatory network involved in conidia formation in *A. alternata* is proposed. Upon exposure to light or upon receiving signal cues from environmental stimuli, the activated G-protein elevates the cellular level of cAMP, which in turns binds to PKA and triggers disassociation of the PKA tetramer by separating the catalytic subunits from the regulatory subunits. The released catalytic subunits are capable of activating downstream enzymes or transcriptional regulators. The expression of *AaNoxB* is apparently down-regulated in fungal strains lacking PKA catalytic subunits. However, *NoxB* has little effect on the expression of PKA-coding genes, indicating that *PKA* is epistatic to *NoxB*. Moreover, the expression of *AaNoxB* was activated by *FUS3*.

Expression of *Nox* genes has been shown to be regulated by SLT2 and FUS3/KSS1 in other fungi (Cano-Dominguez et al., 2008; Segmuller et al., 2008). Intriguingly, expression of *FUS3* gene and phosphorylation of FUS3 MAPK were elevated in the *AaNoxB* mutant background. Fungal cells might be compensating for the absence of *NoxB*, by elevating expression of its partner genes. Both FUS3 and PKA^{reg} may play a role in conidia maturation. The *AaFUS3*- or PKA^{reg}-deficient mutants never produce fully developed conidia, instead produce highly melanized segments, with distinct septae that often expanded into globular swellings and occur in chains (Lin et al., 2010; Tsai et al., 2013). Interconnected regulation between the MAPK and cAMP/PKA signaling pathways have also been implicated in governing conidia germination in *B. cinerea* (Doehlemann et al., 2006), appressorium formation in *M. oryzae* (Xu and Hamer, 1996) and sclerotium formation in *S. sclerotiorum* (Chen and Dickman, 2005).

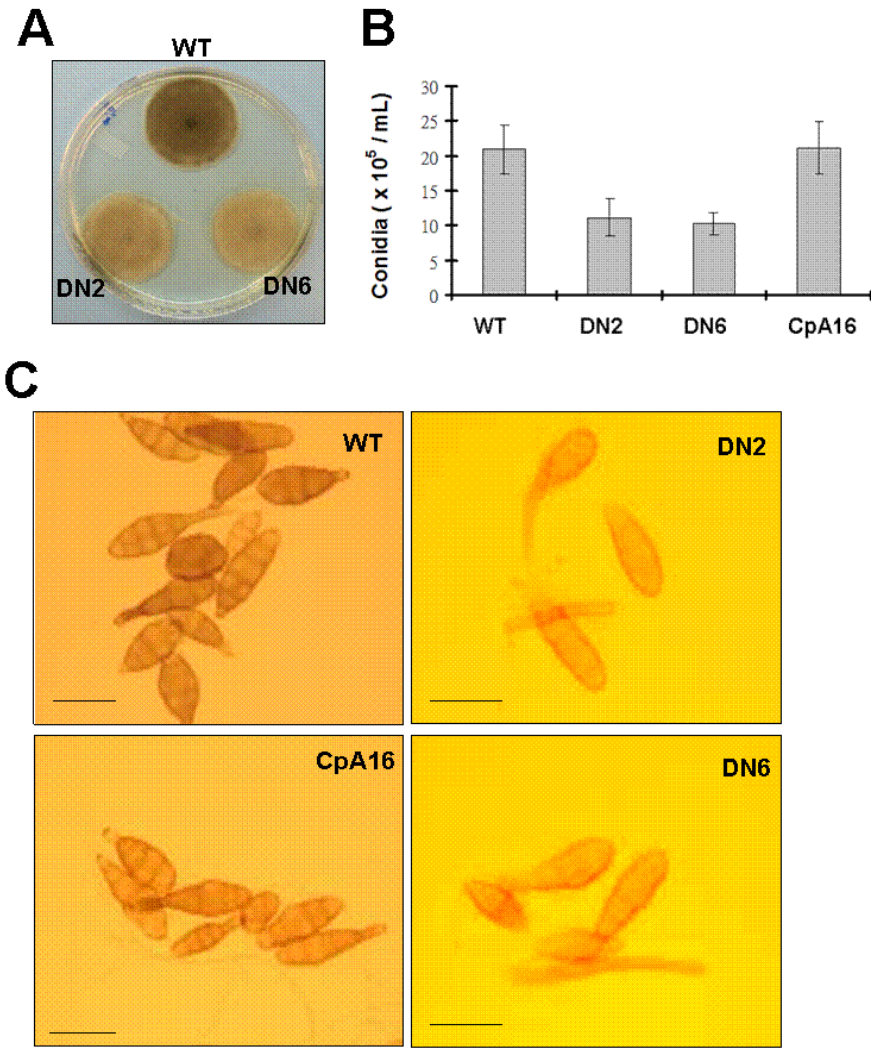


Figure 4-1. Reduced pigmentation and conidiation in *NoxA* mutant of *A. alternata*. (A) Morphological appearance of the wild-type (WT) and two *NoxA* mutants (DN2 and DN6) grown on PDA. (B) Quantitative analysis of conidia produced by the WT, *NoxA* mutants and the genetically reverted strain CpA16. (C) Images of conidia under light microscope. *NoxA* mutants produce light-colored conidia with less distinct septae. Only representative replicates are shown. Bar = 20 µM.

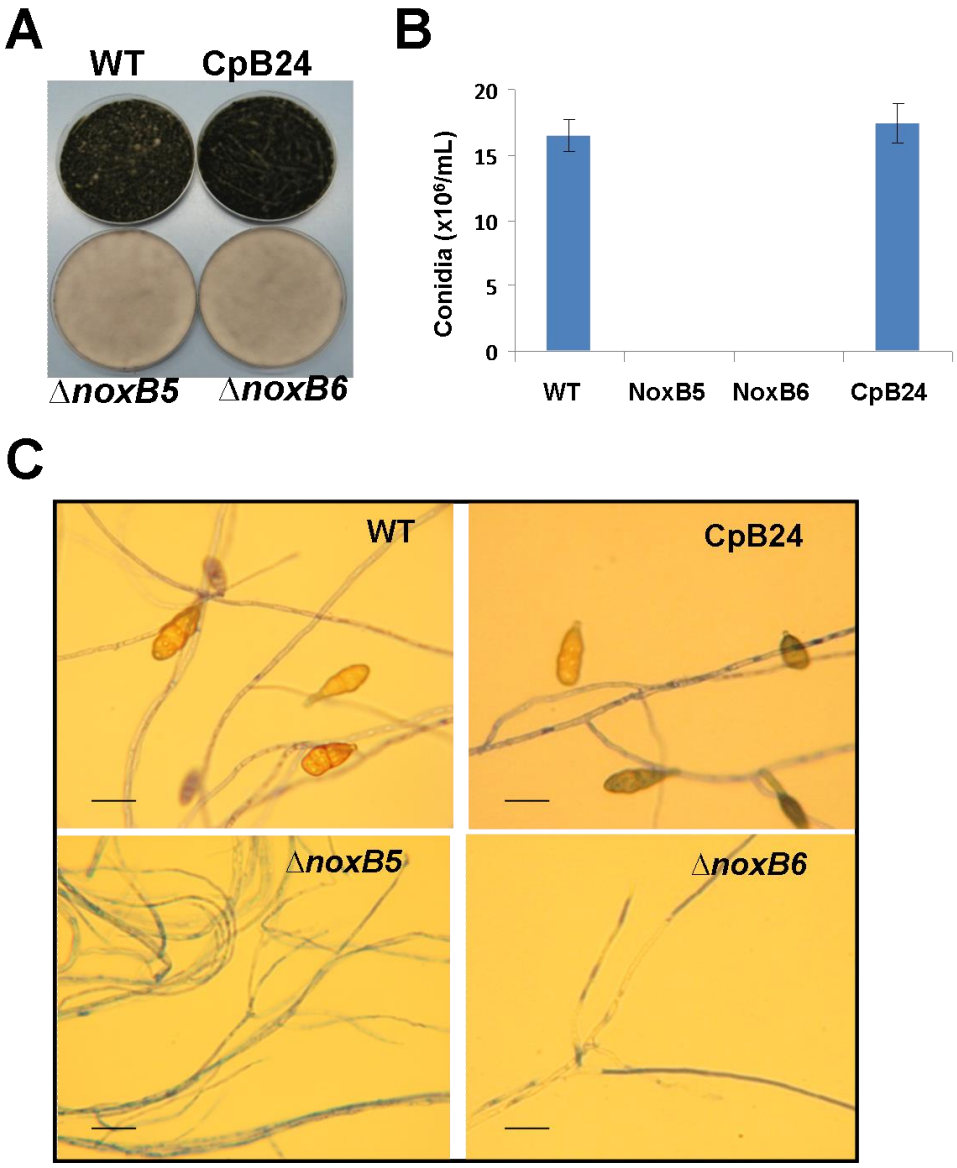


Figure 4-2. Conidia produced by the wild-type strain (WT), the strains lacking *AaNoxB* and the complementation strain CpB24 of *A. alternata*. (A) Conidiation was evaluated by growing the wild-type (WT), two *NoxB* mutants (*noxB5* and *noxB6*) and a complementation strain CpB24 on PDA plates in the light for 3 days. (B) Quantification of conidia. Each column represents the mean number of conidia ± the standard deviation from two independent experiments, with at least three replicates. (C) Images of conidia. *NoxB* mutants fail to produce conidia. Only representative replicates are shown. Bar = 20 µM.

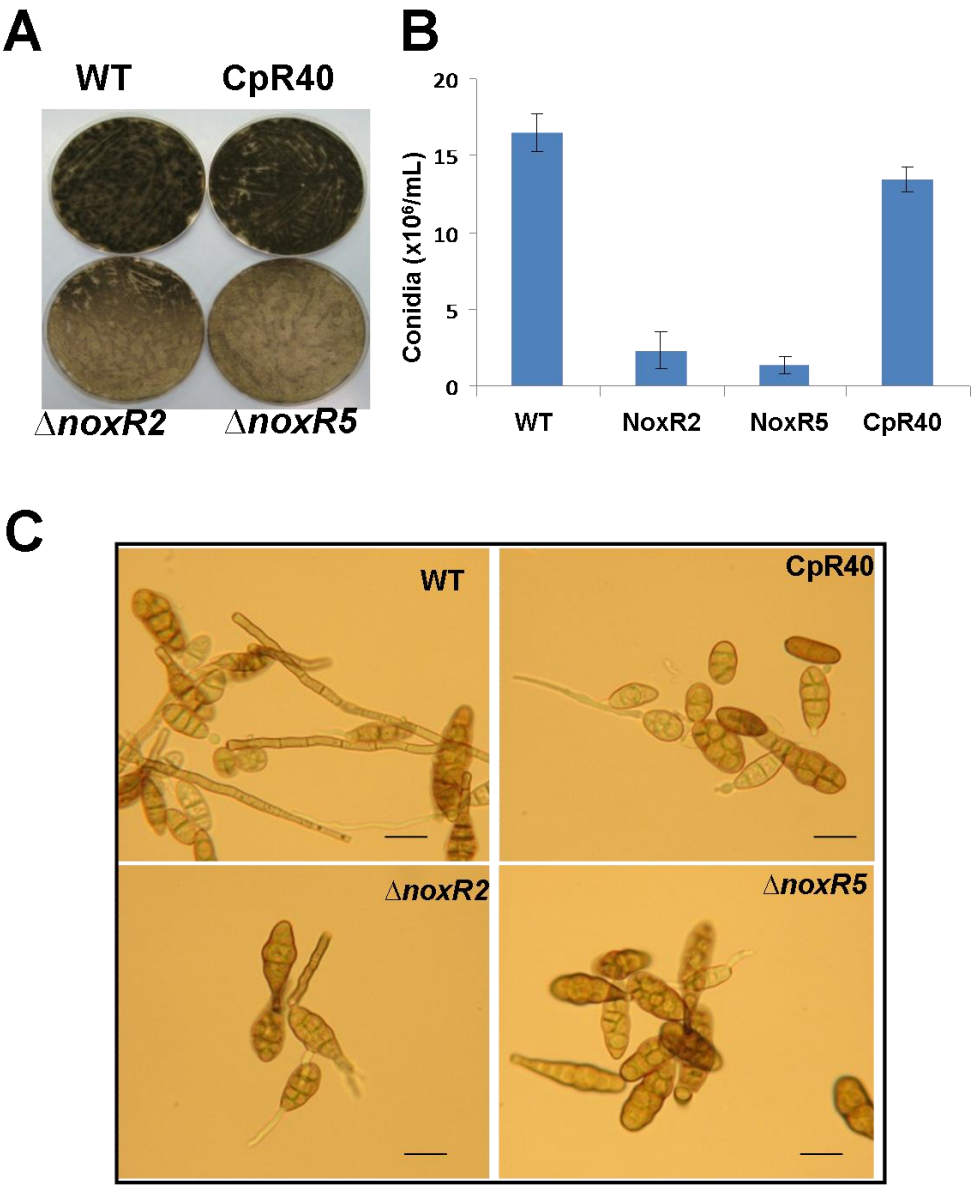


Figure 4-3. Conidia produced by the wild-type strain (WT), the strains lacking *AaNoxR* and the complementation strain CpR40 of *A. alternata.* (A) Conidiation was evaluated by growing the wild-type (WT), two *NoxR* mutants (*noxR2* and *noxR5*) and a complementation strain CpR40 on PDA plates in the light for 3 days. (B) Quantification of conidia. Each column represents the mean number of conidia ± the standard deviation from two independent experiments, with at least three replicates. (C) Images of conidia. Only representative replicates are shown. Bar = 20 µM.

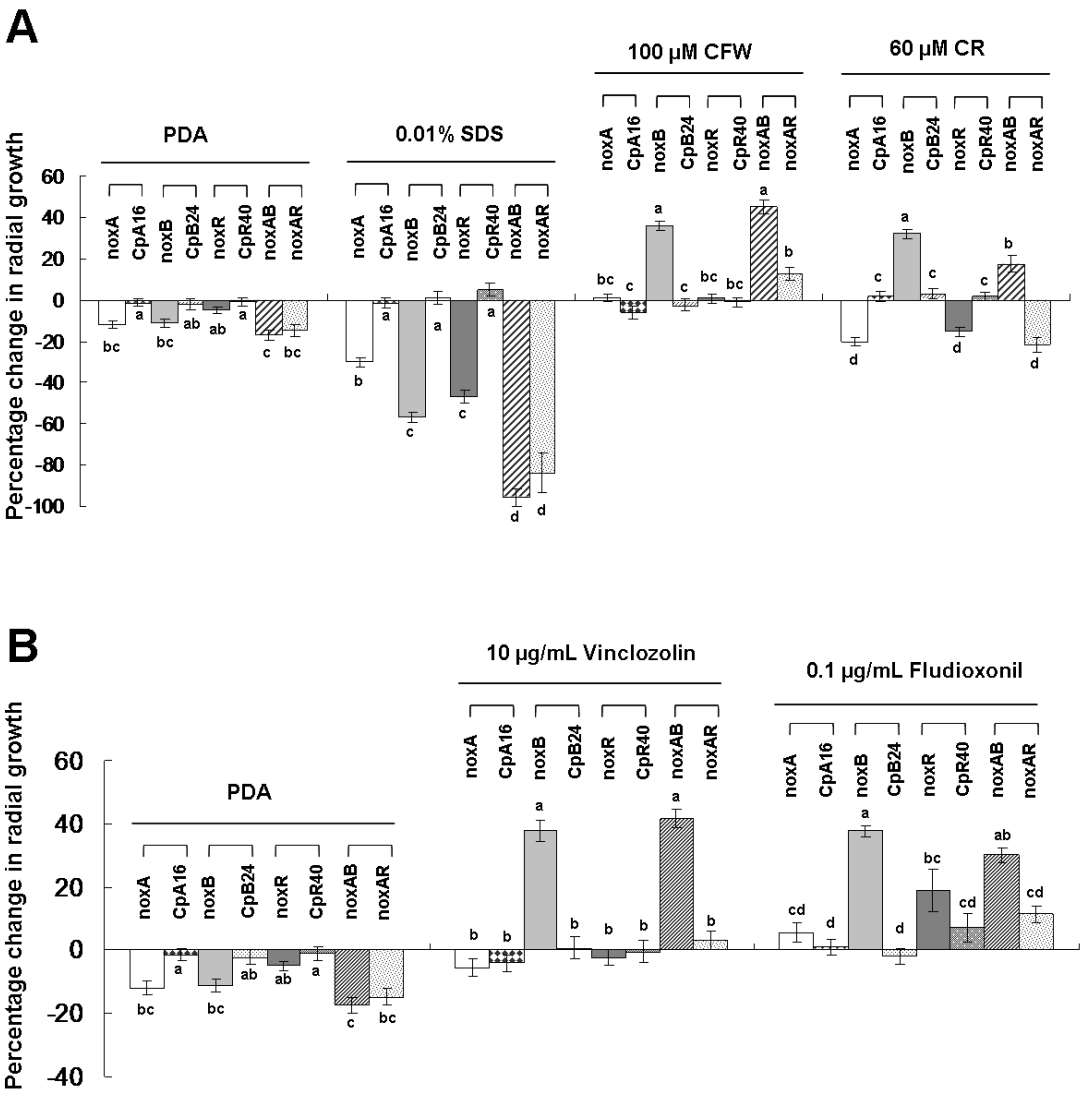


Figure 4-4. Sensitivity assays of the *A. alternata* strains carrying a single *Nox* gene mutation (noxA, noxB and noxR), the corresponding rescued strains (CpA16, CpB24 and CpR40) and the *NoxA NoxB* (noxAB) and *NoxA NoxR* (noxAR) double mutant strains to the (A) cell wall-targeting compounds sodium dodecyl sulphate (SDS), calcofluor white (CFW) and Congo red (CR), as well as (B) vinclozolin and fludioxonil fungicides. Fungal strains were grown on PDA amended with or without chemicals for 3 to 6 days. The percentage change in radial growth was calculated as the percentage of growth of the genetically modified fungal strains in relation to the wild-type grown on the same plate. The data presented are the means and standard errors of two independent mutants with at least two replicates of two independent experiments (n = 8). For each test compound, means indicated by the same letter within a test compound are not significantly different from one another, Tukey's test ($P \leq 0.05$).

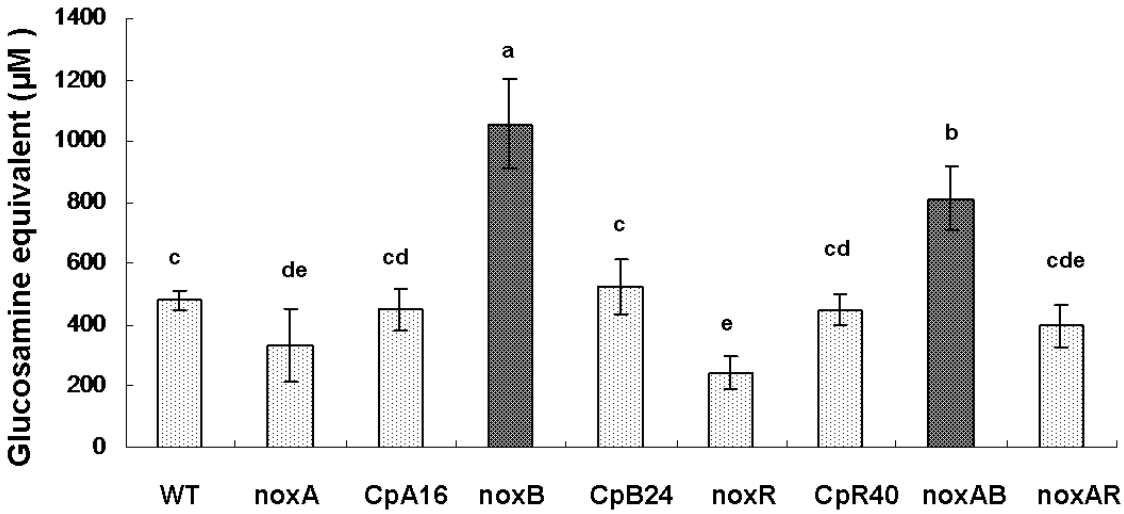


Figure 4-5. Quantification of chitin in the cell wall obtained from the wild-type (WT), the strains carrying single *Nox* gene mutation (noxA, noxB and noxR), the rescued strains (CpA16, CpB24 and CpR40) and the *NoxA NoxB* (noxAB) and *NoxA NoxR* (noxAR) double mutant strains of *A. alternata*. Means indicated by the same letter are not significantly different from one another, Tukey's test ($P \leq 0.05$).

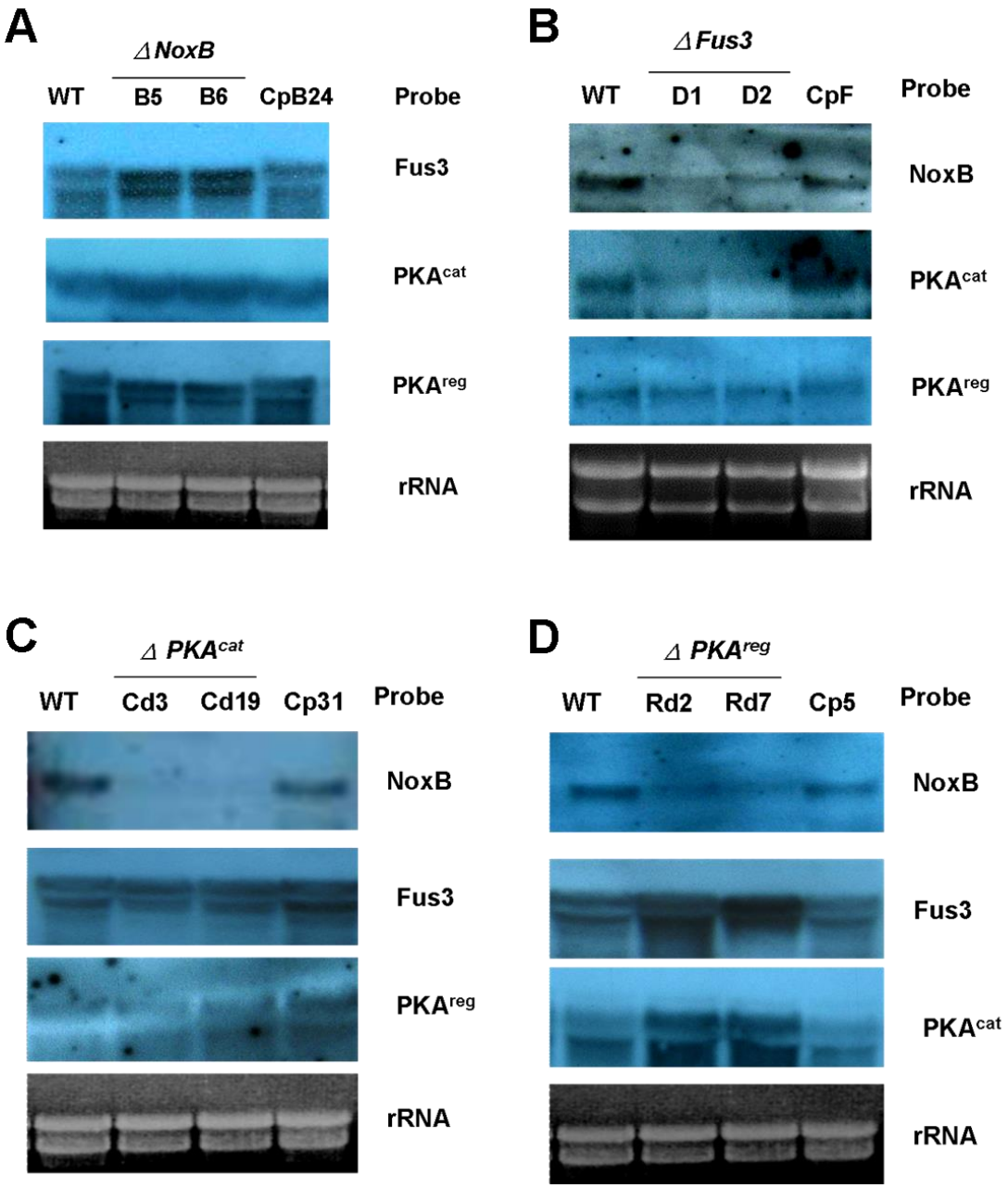


Figure 4-6. Images of RNA blotting. Fungal RNA was purified from the wild-type of *A. alternata* (WT), (A) from the *NoxB* mutants (B5, B6), the *NoxB* rescued strain (CpB24), (B) from the *FUS3* mutants (D1, D2) and the *FUS3* rescued strain (CpF), (C) from the *PKA*^{cat} mutants (Cd3, Cd19) and the *PKA*^{cat} rescued strain (Cp31), and (D) from the *PKA*^{reg} mutants (Rd2, Rd7) and the *PKA*^{reg} rescued strain (Cp5) of *A. alternata*. RNA was electrophoresed, blotted and hybridized to gene specific probes as indicated.

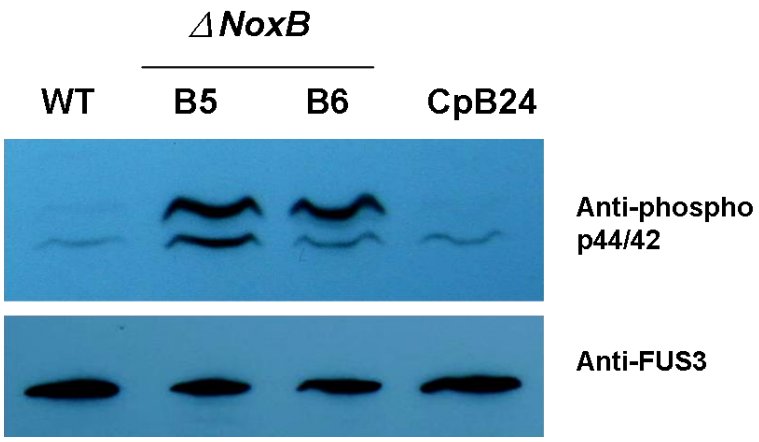


Figure 4-7. Immunological detection of the AaFUS3 protein in *A. alternata*. Fungal proteins were purified from wild-type (WT), two *AaNoxB* mutants (B5 and B6) and the genetically rescued strain (CpB24) grown on complete medium (CM) for 3 days. Proteins were fractionated in a denaturing 12% SDS-polyacrylamide gel, electroblotted onto a nitrocellulose membrane, and probed with anti-phospho-p44/42 monoclonal or anti-FUS3 polyclonal antibody.

CHAPTER 5
GLUTATHIONE PEROXIDASE–MEDIATED OXIDATIVE STRESS RESISTANCE IS REGULATED BY NADPH OXIDASE IN *Alternaria alternata*

Introduction

All cells have effective mechanisms to protect themselves against toxic reactive oxygen species (ROS). Studies with *A. alternata* have demonstrated that both YAP1 and HOG1 are essential for cellular resistance to oxidative stress induced by H_2O_2 and several superoxide-generating compounds (KO_2, menadione and diamide) and for pathogenicity in citrus (Lin et al., 2009; Lin and Chung, 2010). In earlier chapters it was demonstrated that Nox plays a dual role in ROS production and resistance and that Nox regulates the expression of *YAP1* and *HOG1* in *A. alternata*. Thus, *A. alternata* has evolved sophisticated mechanisms for cellular protection against the toxicity of ROS. The *A. alternata* YAP1 is involved in detoxification of H_2O_2 and perhaps other oxidants through the regulation of catalase, superoxide dismutase (SOD), glutathione-S-transferase, glutathione peroxidase (GPx), glutathione reductase and ligninolytic peroxidase (Lin et al., 2011). The results suggest that the glutathione system might play an important role in the regulation of redox homeostasis and oxidative resistance in *A. alternata*.

In eukaryotic cells, glutathione (GSH)-dependent GPx is one of the primary defenses against the toxicity of peroxides. The tripeptide glutathione (*γ*-L-glutamyl-L-cysteinyl-glycine) containing a free sulfhydryl group is an electron donor for GPx, which catalyzes reduction of H_2O_2 to water. GSH is concomitantly oxidized to glutathione disulfide (GSSG), which is then recycled back to GSH by glutathione reductase using NADPH as an electron donor (Meister and Anderson, 1983). Cells need to maintain a highly reduced environment; cells are under oxidative stress as the ratio of GSH to

GSSG decreases (Schafer and Buettner, 2001; Blokhina et al., 2003). The budding yeast *S. cerevisiae* has three *GPx* genes (*GPx1*, *GPx2* and *GPx3*) homologous to mammalian *GPx* (Inoue et al., 1999). Unlike its counterpart in mammalian, the yeast GPx lacks selenium in the active sites (Mullenbach et al., 1987). The *S*. *cerevisiae GPx3* also termed Sc*HYR1* (hydrogen peroxide resistance), is constitutively expressed regardless of the presence of oxidants. In contrast, *GPx1* is induced by glucose depletion and *GPx2* is induced by H_2O_2 and *tert*-butyl-hydroperoxide in a YAP1-dependent manner. The *GPx3* mutant is hypersensitive to peroxides, whereas *GPx1* and *GPx2* single mutants and *GPx1 GPx2* double mutants do not show obvious phenotypes. *GPx3* functions as redox-transmitter for YAP1. In the presence of peroxides, ScHYR1/GPx3 is oxidized at Cys-36 to form a cysteine sulfenic acid (-SOH), which then forms an intermolecular disulfide bond with Cys-598 of YAP1. The activated YAP1 subsequently triggers expression of the genes required for resistance to oxidative stress. YAP1 has also been known to regulate cellular ROS homeostasis and the synthesis of gluthathione (Inoue et al., 1999; Delaunay et al., 2002). In the human pathogenic fungus C*ryptococcus neoformans*, *GPx1* and *GPx2* are required for resistance to peroxides but play no role in virulence (Missall et al., 2005). In the rice blast pathogen *M. oryzae*, a yeast GPx3/HYR1 homolog, is required for H_2O_2 resistance *in vitro* and *in planta* and plays a role in fungal virulence (Huang et al., 2011).

In this chapter, a *HYR1/GPx3* gene homolog, designated *AaGPx* (*A. alternata* glutathione peroxidase) was characterized in order to understand further the molecular mechanisms involved in cellular response to oxidative stress in the tangerine pathotype of *A. alternata*. Genetic evidence is provided to define the central role of *AaGPx* in the

development of conidia, resistance to peroxides and virulence. These studies also reveal that *AaGPx* plays a negative role in chitin accumulation. This phenotype has not been previously identified in any fungus to my knowledge. Expression of *AaGPx* is coordinately regulated by NADPH oxidase, YAP1 and HOG1. The results derived from this dissertation research further support the important role of ROS detoxification during *A. alternata* pathogenesis in citrus.

Materials and Methods

Fungal Strains and Chemical Sensitivity Tests

Fungal strains and their genotypes used in this study are given in Table A-1. The wild-type EV-MIL31 strain of *A. alternata* and the genetically altered strains altered in Nox components have been described in Chapters 2 and 3. Fungal mutants, impaired for the *AaAP1* or the *AaHOG1* gene, were created in separate studies (Lin et al., 2009; Lin and Chung, 2010). Fungal strains were grown on PDA at 28 ºC. Assays for chemical sensitivity were performed as described in Chapter 3.

Molecular Cloning and Sequence Analysis

All oligonucleotide primers used in this study are compiled in Table B-1. A 2.2-kb DNA fragment containing the entire *AaGPx* ORF and its 5' and 3' untranslated regions was amplified from genomic DNA of *A. alternata* by PCR with primers GPx-1F and GPx-1R. Bioinformatics using on-line software were carried out as described in Chapter 2. AaGPx sequence can be retrieved from the GenBank/EMBL Data Libraries under Accession No. ACY73852.

AaGPx Gene Inactivation

AaGPx gene was disrupted in the wild-type EV-MIL31 strain as described in Chapter 2. Split marker fragments were jointed and amplified by two-round PCR. A 2.1-

kb 5'GPx::PHY fusion fragment (0.9 + 1.2-kb) was amplified with the primers GPx-1F, M13R:GPx, M13R and hyg3. A 2.7-kb 3'GPx::YGT fusion fragment (0.9 + 1.8-kb) was amplified with the primers hyg4, M13F, M13F:GPx and GPx-1R (Fig. 5-2A). Preparation and transformation of fungal protoplasts were conducted as previously described (Chung et al., 2002). Transformants recovered from hygromycin-containing medium were tested for H_2O_2 sensitivity and analyzed by PCR and Northern blotting. A *GPx* gene-specific probe used for Northern blotting was labeled with DIG and amplified by PCR with the primers GPx-2F and GPx-2R. A functional *GPx* gene under control of its endogenous promoter were amplified with the primers GPx-1F and GPx-1R and co-transformed into protoplasts prepared from an AaGPx mutant with the pCB1532 plasmid for genetic complementation.

Miscellaneous

Procedures used for isolation of fungal genomic DNA and RNA, purification of plasmids, Northern blot analyses, quantification of conidia and chitin as well as assays for fungal virulence were performed as described in the previous chapters. The significant differences compared with wild-type were determined by student t-test ($P \leq 0.05$).

Results

Cloning and Characterization of *AaGPx*

Sequence analysis revealed that the *A. alternata GPx* gene (*AaGPx*) contains a 669-bp ORF interrupted by two introns (58 and 104 bp) and encodes 168 amino acids. AaGPx contains a glutathione peroxidase active site (GKvVLVvNTaSkCGfT; a.a. 27-42) and a glutathione peroxidase signature (IGFPCNQF; a.a. 68-73) (Fig. 5-1A). BlastP search and alignment using CLUSTALW revealed these domains are highly conserved

among filamentous fungi (Fig. 5-1B). AaGPx is most similar to the GPx1 of *Pyrenophora tritici-repentis* (96% similarity and 92% identity). AaGPx displays strong similarity to GPx or HYR1 homologs of filamentous fungi and yeasts (74 to 92%) (Table 5-1). AaGPx contains three conserved cysteines, in which two of them Cys39 and Cys88 correspond to Cys36 and Cys82 in the active sites of yeast HYR1.

Targeted Disruption of *AaGPx*

Transformation of two truncated but overlapping HYG fragments (Fig. 5-2A) into protoplasts of *A. alternata* recovered 36 transformants from medium containing hygromycin. Two strains (GPx10 and GPx22) displayed reduced growth by 12%-16% on PDA and increased sensitivity to H_2O_2. PCR diagnosis (Fig. 5-2B) with multiple sets of primers confirmed targeted disruption of *AaGPx* in the genome of *A. alternata*. Hybridization of fungal RNA to an *AaGPx*-specific probe identified a 0.5-kb *AaGPx* gene transcript from the wild type, but not from GPx10 and GPx22 strains (Fig. 5-2C), indicating that the two transformants are *AaGPx* null mutants.

***AaGPx* Is Required for Conidia Formation and Fungal Virulence**

The wild type formed heavily melanized colonies and *AaGPx* mutants formed light brown colonies when grown on PDA in the light (Fig. 5-3A). *AaGPx* mutants were reduced in conidiation by 98% (Fig. 5-3B). *AaGPx* mutants produced less melanized conidia compared to the wild type (Fig. 5-3C). Introduction and expression of a functional copy of *AaGPx* in a mutant fully restored conidiation.

The wild-type strain of *A. alternata* induced necrotic lesions on all inoculated spots (100%) at 3 days after inoculation (Fig. 5-4A). *AaGPx* mutants produced lesions on detached citrus leaves at frequencies ranging from 64% to 82%. The lesions induced by the *AaGPx* mutants were significantly smaller than those induced by the wild type

(Fig. 5-4B). The genetically complemented strain CpGPx16 induced necrotic lesions on calamondin leaves at frequencies and magnitudes similar to those induced by the wild type.

AaGPx Contributes to Resistance to Oxidative Stress

A. alternata GPx mutants exhibited reduced growth on PDA by 14 ± 5% relative to the wild type (Fig. 5-5). They were highly sensitive to the oxidants H_2O_2, Cumyl- H_2O_2, *tert*-butyl-hydroperoxide (tBH), and the superoxide-generating compounds menadione (MND) and potassium superoxide (KO_2). *AaGPx* mutants also displayed increased sensitivity to diamide, as well as the singlet oxygen-generating compounds hematoporphyrin (HP) and rose Bengal (RB). The phenotypes were fully restored in the CpGPx16 strain.

AaGPx Is Involved in Chitin Biosynthesis

The wild-type strain exhibited reduced growth by 36 to 50% on medium amended with calcofluor white (CFW) or Congo red (CR) (Fig. 5-5). Both GPx10 and GPx22 mutants were moderately sensitive to SDS, but displayed increased resistance to CFW anf CR. The genetically rescued strain CpGPx16 displayed sensitivity to SDS, CFW or CR at levels similar to that of wild type. Fungal strains defective for *GPx* had significantly higher chitin content compared with the levels measured in the wild type (Fig. 5-6). The chitin content detected in the genetically rescued strain CpGPx16 was not significantly different from that of wild type.

Expression of *AaGPx* Is Regulated by NOX, YAP1 and HOG1

The expression of *AaGPx* was nearly abolished in the *NoxA* mutants (Fig. 5-7A). Expression of the *AaGPx* gene transcript was down-regulated in fungal strains lacking *NoxB* or *NoxR*, as well as in double mutant strains *NoxA NoxB* or *NoxA NoxR*. Re-

introduction of a functional copy of the *Nox* gene into the respective single mutation strain restored the expression of the *AaGPx*. Similarly, *Yap1* or *Hog1* mutants had reduced levels of the *AaGPx* gene transcript, compared with the levels detected in the wild type and the complementation CpY strain (Fig. 5-7B). There was no difference in the expression of the *Yap1* gene among the wild type, the *GPx* mutant and the complementation strain (Fig. 5-7C).

Discussion

In this chapter, a glutathione peroxidase homolog, *AaGPx*, was functionally characterized in the tangerine pathotype of *A. alternata*. Through analyses of a loss-of-function mutation in *AaGPx* gene, it was demonstrated that the *AaGPx* is required for resistance to oxidative stress and full virulence.

AaGPx contains a glutathione peroxidase active site and a glutathione peroxidase signature, conserved domains commonly found in glutathione-dependent peroxidase homologs of fungi. Two cysteines, Cys39 and Cys88, found in AaGPx are likely essential for H_2O_2 detoxification as demonstrated in the yeast ScHYR1 (Delaunay et al., 2002; Zhang et al., 2008). The Cys36 residue in ScHYR1 has been shown to be required for YAP1 oxidation and Cys82 essential for peroxidase activity (Inoue et al., 1999). The yeast ScHYR1 is primarily responsible for H_2O_2 and tBH resistance. *AaGPx* appears to be required for cellular resistance to a broad range of oxidants, including H_2O_2, organic peroxides (Cumyl- H_2O_2 and tBH), superoxide-generating compounds (MND, KO_2 and diamide), as well as singlet oxygen-generating compounds (HP and RB). The *C. neoformans GPx1* mutant is sensitive to tBH and *GPx2* mutant displays sensitivity to Cumyl- H_2O_2. However, the *C. neoformans* strain impaired for *GPx1, GPx2*

or both displays wild-type sensitivity to H_2O_2 (Missall et al., 2005). These results suggest that the conserved *GPx* homologs have divergent roles in different fungi.

AaGPx mutant is hypersensitive to ROS and produces smaller lesions on citrus leaves, confirming that effective ROS scavenging function is required for pathogenesis of *A. alternata*. MoHYR1-mediated resistance to ROS is also required for full virulence in *M. oryzae* (Huang et al., 2011). In contrast, both *GPx1* and *GPx2* of *C. neoformans* are dispensable for virulence in mice (Missall et al., 2005). The expression of the *AaGPx* gene is co-regulated by AaNOX, AaAP1 and AaHOG1. In *S. cerevisiae*, only expression of the *GPx2* gene is regulated by YAP1 (Inoue et al., 1999). However, deletion of *MoHYR1* in *M. oryzae* down-regulates *YAP1* (Huang et al., 2011).

Inactivation of *GPx*, *YAP1* or *HOG1* in *A. alternata* results in fungal strains that are hypersensitive for ROS but defective in virulence at varying degrees. *AaAP1* and *AaHOG1* mutants are almost non-pathogenic to citrus. *AaGPx* mutant is reduced in virulence, producing fewer and smaller lesions than wild type when inoculated onto citrus leaves. Similar results were observed in *M. oryzae*, in which the *MoAP1* mutant is severely defective in pathogenicity whereas the *MoHYR1* mutant is reduced in virulence on both barley and rice (Guo et al., 2011; Huang et al., 2011).

A. alternata mutant strains impaired for *AaGPx* or *AaNoxB* show similar phenotypes in terms of conidiation and resistance to cell wall targeting compounds CFW and CR. In addition, both *GPx* and *AaNoxB* mutants accumulate higher chitin content than wild-type, suggesting that *AaGPx* and *AaNoxB* play a negative role in cell wall integrity. In contrast, the *M. oryzae MoHYR1* plays little or no role in fungal

development, as the *MoHYR1* mutants show wild-type growth, conidiation, and formation of germ tubes and appressoria (Huang et al., 2011).

In conclusion, NOX is one of key regulators in the production of ROS and for signal transductions leading to proper conidiation, fungal virulence, and cellular resistance to ROS and multidrug in *A. alternata*. These regulatory functions of AaNox conferring ROS resistance are likely governed partially by activating the *AaAP1, AaHOG1* and *AaGPx* genes. Maintaining ROS homeostasis in cells is critical for many physiological and developmental processes. The ability of ROS-detoxification mediated by the redox-responsive transcription regulator YAP1, the HOG1 MAP kinase, the NADPH oxidase and the GPx is absolutely required for *A. alternata* survival and pathogenesis. Lastly, a complex regulatory network involving NOX, G protein, cAMP-dependent protein kinase A, FUS3 MAP kinase and/or GPx is required for proper fungal development.

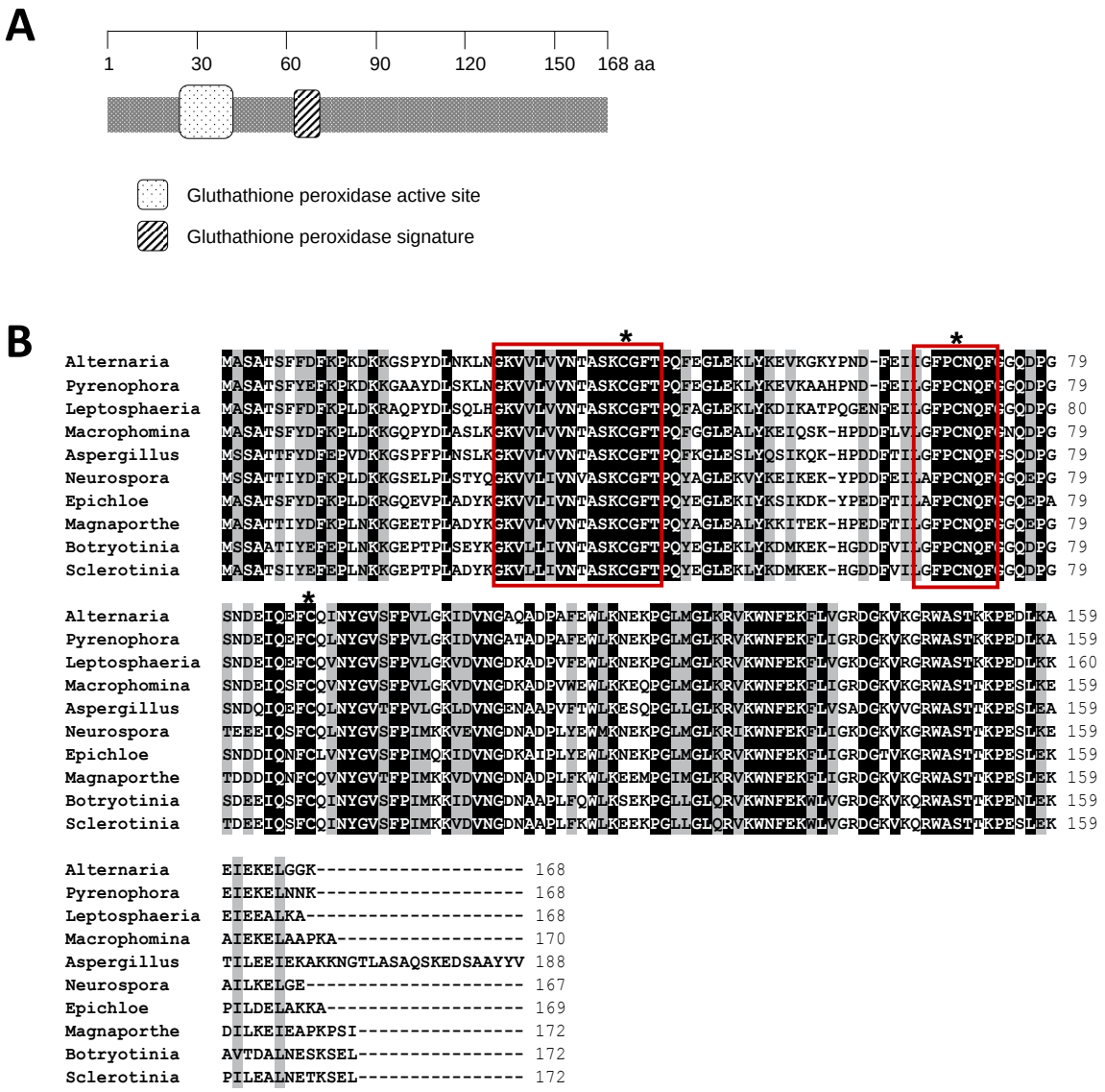


Figure 5-1. Functional domains of AaGPx identified in the tangerine pathotype of *A. alternata*. (A) AaGPx contains two glutathione peroxidase domains commonly found in the GPx/HYR1 of fungi and yeasts. (B) Alignment of the deduced amino acid sequence of AaGPx with other GPx/HYR1 homologs. Conserved amino acids are shaded. Alignment was obtained by BioEdit using CLUSTALW. Conserved glutathione peroxidase active site and signature domains are boxed. Asterisks indicate conserved cysteine residues.

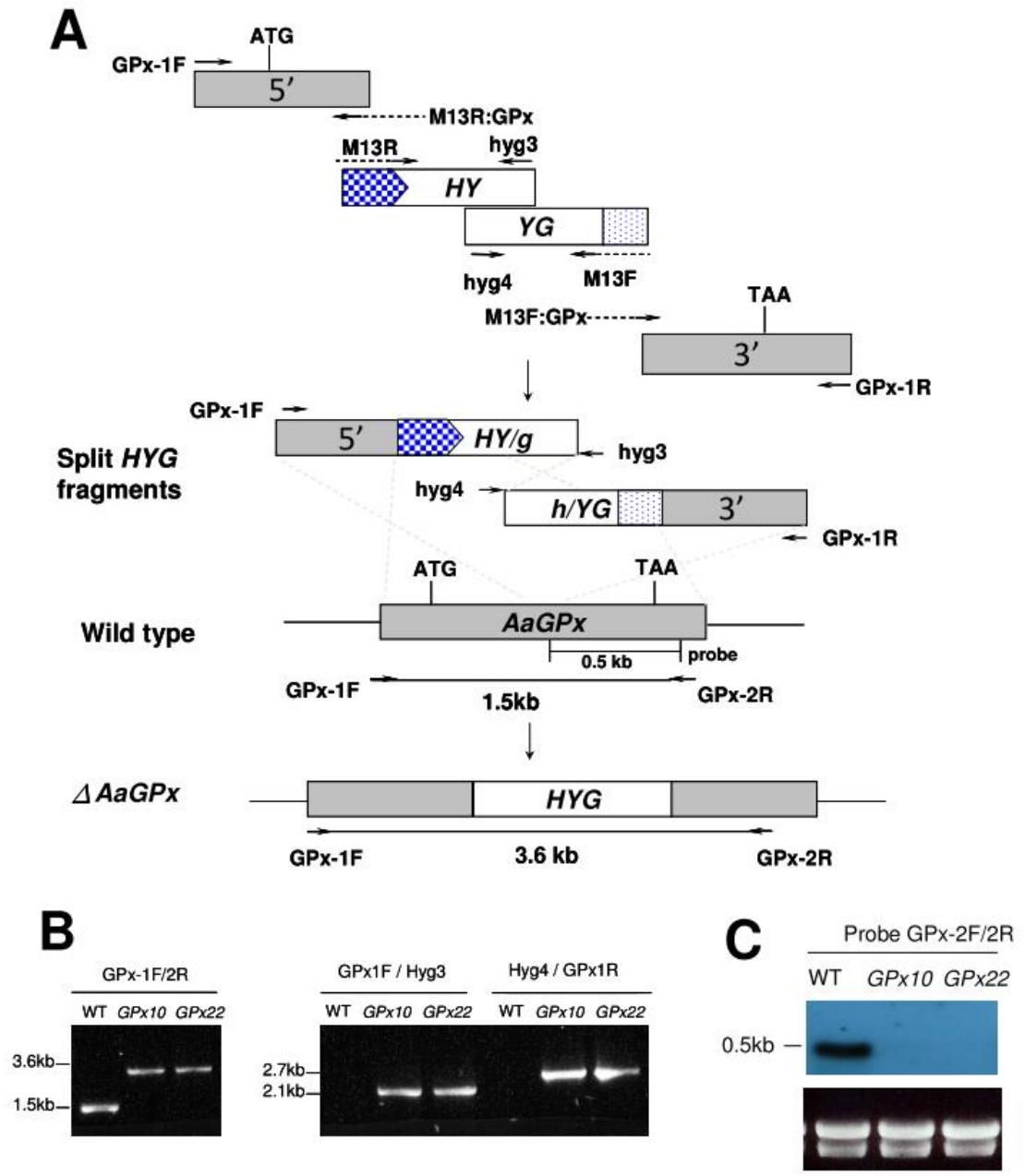


Figure 5-2. Targeted disruption of *AaGPx* in *Alternaria alternata*. (A) Schematic illustration of a split marker strategy for disruption of *AaGPx* by inserting a hygromycin phosphotransferase gene (*HYG*) under control of the *Aspergillus nidulans* trpC promoter and terminator. Oligonucleotide primers used to amplify each fragment are also indicated. (B) Image of DNA fragments amplified from genomic DNA of different fungal strains with the primers indicated. (C) Image of an RNA gel blotting, probed with a DIG-labeled *AaGPx*, showing a 0.5-kb transcript from the wild-type strain (WT) but not from two *AaGPx* disruption mutants (GPx10 and GPx22). Ribosomal RNA stained with ethidium bromide shows relative loading of the RNA samples.

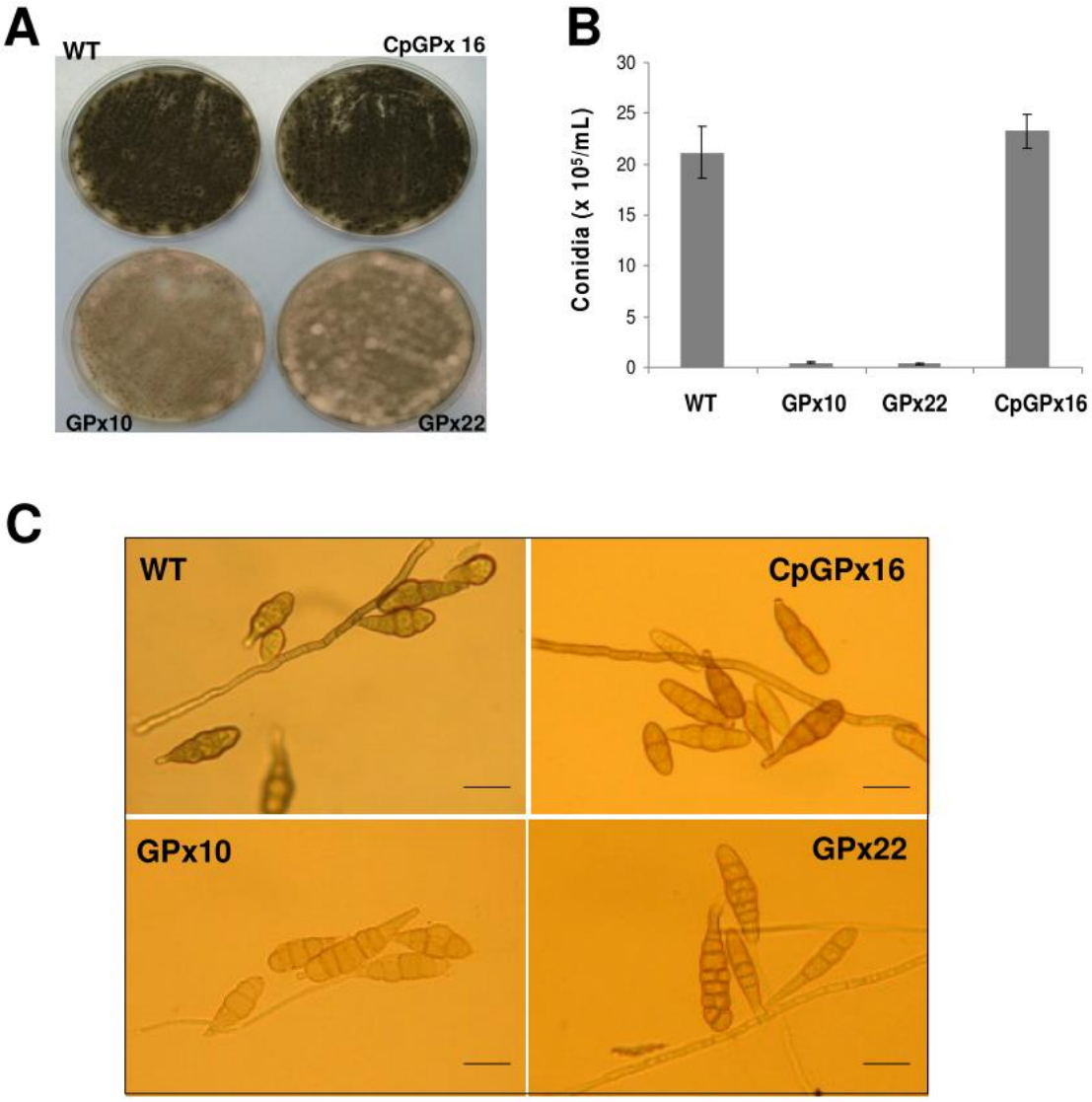


Figure 5-3. Reduced pigmentation and conidiation observed in the *GPx* mutants of *A. alternata*. (A) Conidiation was evaluated by growing the wild type (WT), two *GPx* mutants (GPx10, GPx22) and a complementation strain CpGPx16 on PDA plates in the light for 3 days. (B) Quantification of conidia. Each column represents the mean number of conidia ± the standard deviation from two independent experiments, with at least three replicates. (C) Images of conidia. *GPx* mutants produced less melanized conidia compared to the wild-type and CpGPx16. Only representative replicates are shown. Bar = 20 µM.

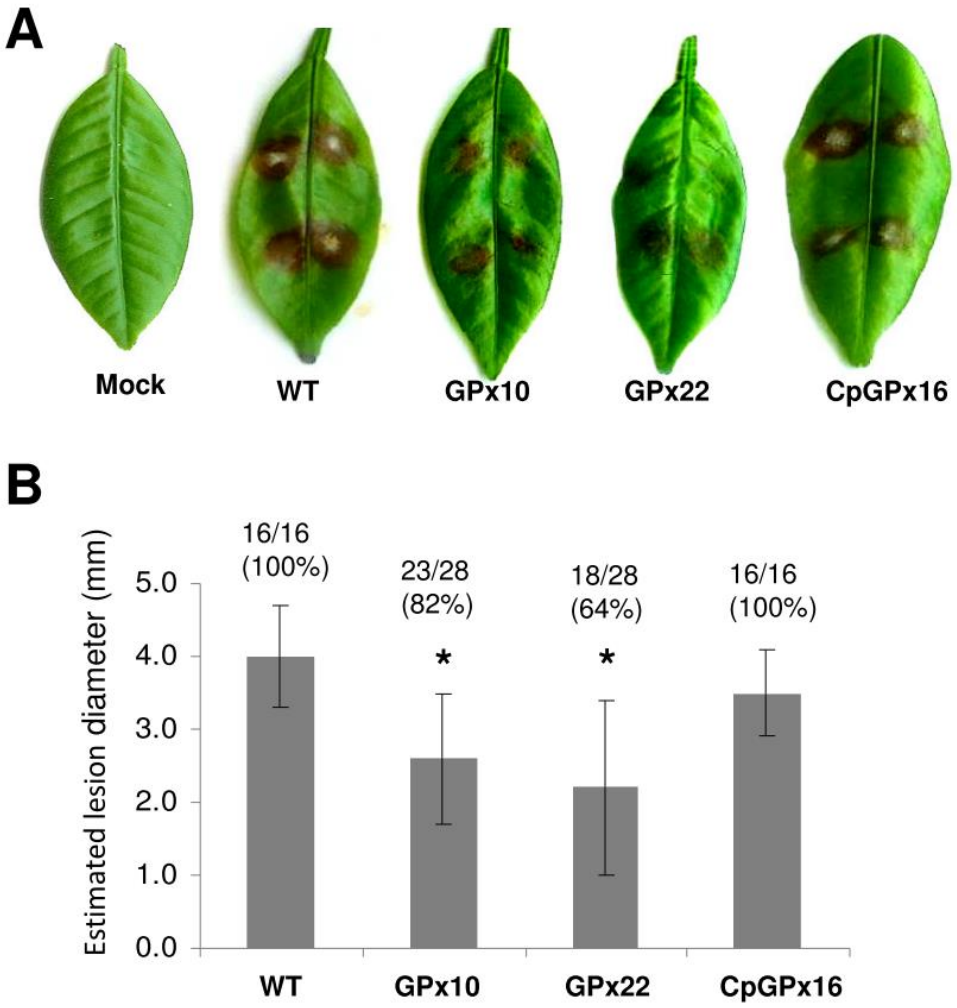


Figure 5-4. *AaGPx* mutants displayed reduced virulence. (A) Fungal pathogenicity was assayed on detached calamondin leaves inoculated with 5 µL of conidial suspension (10^4 conidia /mL) prepared from *A. alternata* wild type (WT), *GPx* disrupted mutants (GPx10 and GPx22) and complementation strain CpGPx16. The mock control was treated with water only. Photos were taken 3 days after inoculation. Only some representative replicates are shown. (B) Quantification of the mean size of necrotic lesions induced by wild-type, *GPx* mutants and CpGPx16 strain. Asterisks indicate a significant difference as determined by a student's t-test, $P \leq 0.05$. Disease incidence, defined by the number of inoculated leaf spots developing necrotic lesions relative to the total number of spots inoculated, is also indicated.

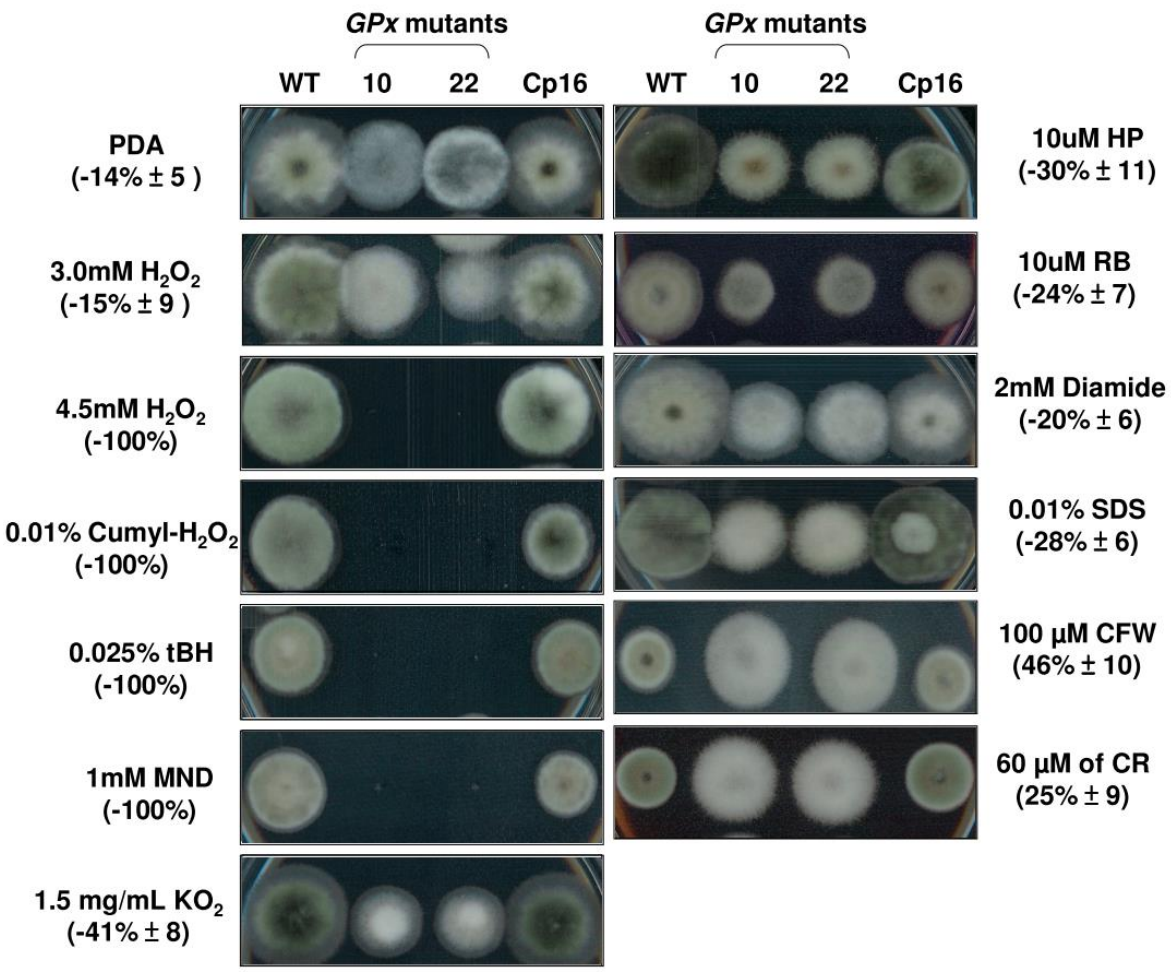


Figure 5-5. Assays for chemical sensitivity of the wild type (WT), two *AaGPx* mutants (10 and 22) and a complementation strain Cp16 of *A. alternata*. Mutational inactivation of *AaGPx* resulted in fungal strains that display hypersensitivity to oxidants and increased resistance to cell wall targeting compounds. Sensitivity (percentage growth reduction) or resistance (percentage growth increase) was calculated as cumulative percentage of growth of WT and *AaGPx* mutants grown on the same plate. Re-introduction and expression of a functional copy of *AaGPx* in the GPx10 mutant resulted in CpGPx16 strain with restored phenotypes. All tests were repeated at least twice with multiple replicates of each treatment. Only representative replicates are shown. tBH, tert-butyl-hydroperoxide; MND, menadione; HP, hematoporphyrin; RB, rose Bengal; SDS, sodium dodecyl sulfate; CFW, calcofluor white; CR, Congo red.

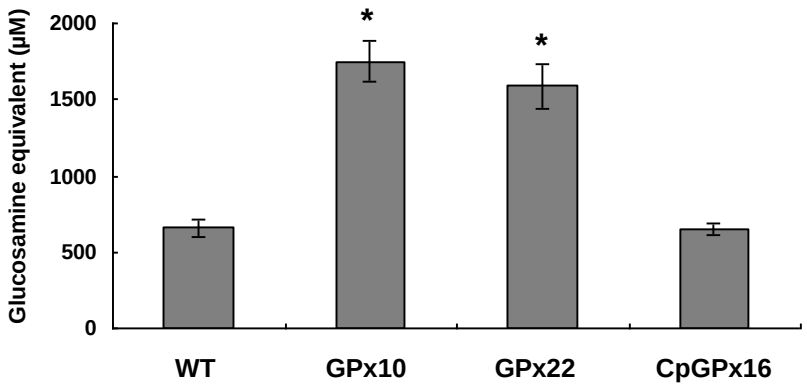


Figure 5-6. Quantification of chitin in the cell wall obtained from the wild type (WT), the strains carrying *GPx* gene mutation (GPx10 and GPx22) and the genetically rescued strain CpGPx16 of *A. alternata*. Asterisks indicate significant difference compared with wild-type by student t-test, $P \leq 0.05$.

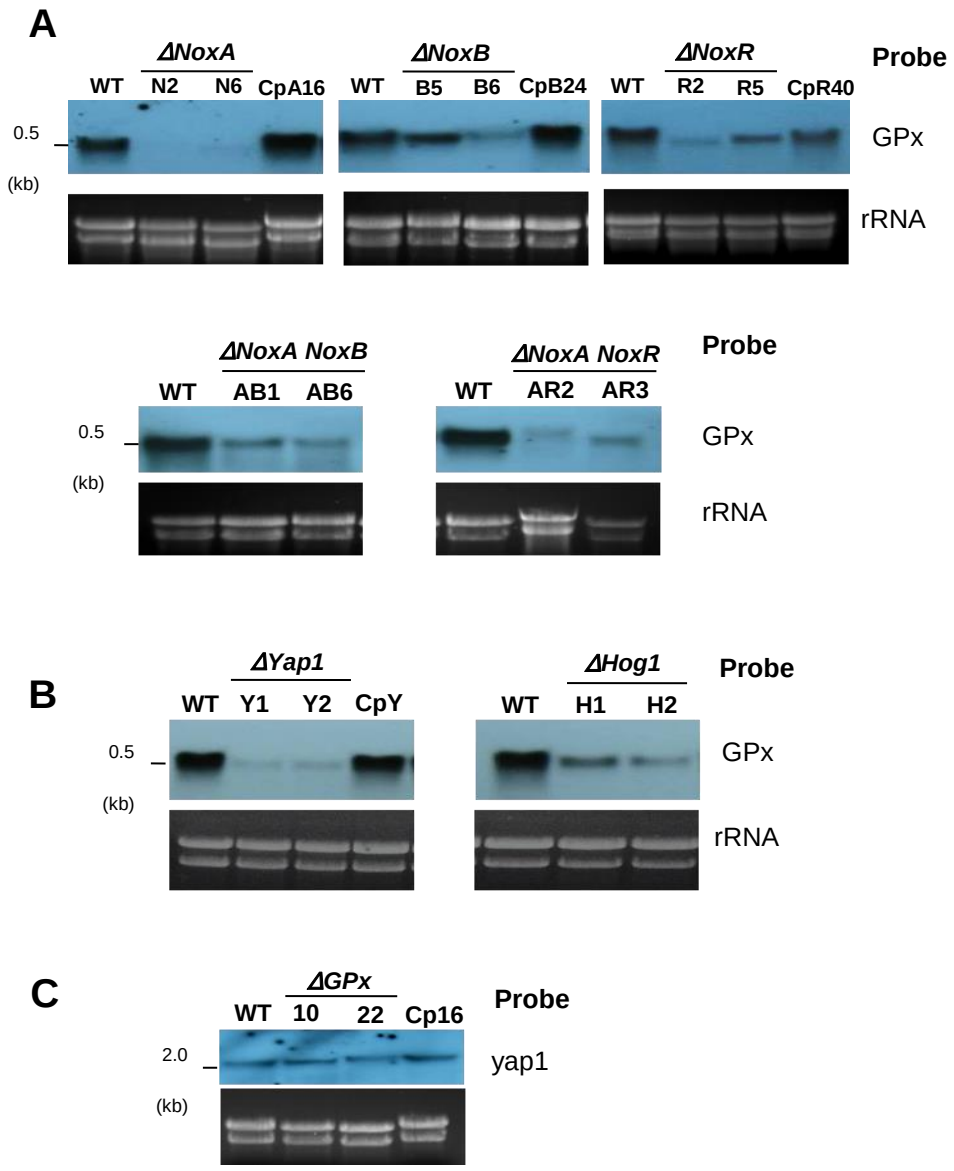


Figure 5-7. Images of RNA blotting. (A) Expression of *AaGPx* in wild-type (WT) and mutant strains impaired for *NoxA*, *NoxB*, or *NoxR*. (B) Expression of *AaGPx* in the *Yap1* mutants (Y1, Y2), the rescued strain CpY and in the *Hog1* mutants (H1, H2). (C) Expression of Yap1 in WT and two *AaGPx* mutants (GPx10, GPx22) and genetic reverted strain Cp16. Fungal RNA, was electrophoresed in formide-containing gels, blotted to nylon membrane and hybridized with DIG-labeled probe as indicated. Gels staining with ethidium bromide indicate relative loading of the RNA samples.

Table 5-1. *A. alternata* GPx is highly similar to other GPx/HYR1 homologs of fungi and yeast

ORGANISM	Protein	Accession	Similarity (%)	Identity (%)
Alternaria alternata	*GPx*	ACY73852	100	100
Pyrenophora tritici-repentis	GPx1	XP_001940175	96	92
Leptosphaeria maculans JN3	GPx	XP_003842197	92	83
Aspergillus terreus NIH2624	GPx	XP_001213339	86	74
Neurospora crassa OR74A	HYR1	XP_957919	86	72
Botryotinia fuckeliana B05.10	GPx	XP_001559367	85	68
Magnaporthe oryzae	HYR1	XP_003711364	84	70
Sclerotinia sclerotiorum 1980	GPx	XP_001598652	84	70
Epichloë festucae	GPx	AGC94642	82	73
Saccharomyces cerevisiae	GPX3/HYR1	NP_012303	74	56
Homo sapiens	GPx3	AAH13601	37	25

APPENDIX A
GENOTYPE OF *Alternaria alternata* STRAINS

Table A-1. Genotypes of *Alternaria alternata* strains used in this study

Strain	**Genotype**
WT	Wild-type of *Alternaria alternata*
AaNoxA (DN2 and DN6)	*AaNoxA* disruptant
CpA16	*AaNoxA* complementation
AaNoxB (DB5 and DB6)	*AaNoxB* disruptant
CpB24	*AaNoxB* complementation
AaNoxR (DR2 and DR5)	*AaNoxR* disruptant
CpR40	*AaNoxR* complementation
NoxA NoxB (NoxAB1 and NoxAB6)	*NoxA NoxB* double mutant
NoxA NoxR (NoxAR2 and NoxAR3)	*NoxA NoxR* double mutant
Yap1 (Y1 and Y2)	*AaYap1* disruptant
CpY	*AaYap1* complementation
Hog1 (H1 and H2)	*AaHog1* disruptant
AaFUS3 (D1 and D2)	*FUS3* MAP kinase mutant
CpF	*FUS3* complementation
$AaPKA^{cat}$ (Cd3 and Cd19)	cAMP-dependent protein kinase A (PKA) catalytic subunit mutant
Cp31	$AaPKA^{cat}$ complementation
$AaPKA^{reg}$ (Rd2 and Rd7)	cAMP-dependent protein kinase A (PKA) regulatory subunit mutant
Cp5	$AaPKA^{reg}$ complementation
AaGPx (GPx10 and GPx22)	*AaGPx* disruptant
CpGPx16	*AaGPx* complementation

APPENDIX B
PRIMER SEQUENCE

Table B-1. Sequence of oligonucleotide primers used in this study

Primer name	Sequence	Gene
M13R	5'-AGCGGATAACAATTTCACACAGGA-3'	HYG
M13F	5'-CGCCAGGGTTTTCCCAGTCACGAC-3'	HYG
hyg3	5'-GGATGCCTCCGCTCGAAGTA-3'	HYG
hyg4	5'-CGTTGCAAGACCTGCCTGAA-3'	HYG
NOXf1	5'-GAGACCTTYTGGTACACTCAYC-3'	NoxA
NOXr1	5'-GATYTCGTTCTCRAAGACGTC-3'	NoxA
NoxA-pro2F	5'-TGAGACGCTCTTCACACACTAGCTG-3'	NoxA
NoxA:M13R	5'-TCCTGTGTGAAATTGTTATCCGCTGGCGGACCTGCACACGTTCAACA -3'	HYG, NoxA
M13F:NoxA	5'-GTCGTGACTGGGAAAACCCTGGCGTCAAGTCGGTGACTTTACCCGC-3'	HYG, NoxA
NoxA-TAA	5'-TTAGAAATGCTCCTTCCAGAACTTGA-3'	NoxA
NoxA-ATG	5'-ATGGGAGCAAGCGGAGGCAC-3'	NoxA
NoxA:Sur1	5'-CGGTGAATCCAGCCGGGAGATGTGGGGCACGGCGGACCTGCACACGTTCAACA-3'	Sur, NoxA
Sur2:NoxA	5'-GATTATTGCACGGGAATTGCATGCTCTCACGTTCAAGTCGGTGACTTTACCCG-3'	Sur, NoxA
Sur1	5'-GTGCCCCACATCTCCCGGCTGGATTCACCG-3'	Sur
Sur2	5'-ACGTGAGAGCATGCAATTCCCGTGCAATAATC-3'	Sur
SurF	5'-ACTCCCATGGCCGACGCTCT-3'	Sur
SurR	5'-CGAAGCAACGTCGCCCTCAA-3'	Sur
NoxB-3F	5'-CGCGAGCGGTCAAGATGGC-3'	NoxB
M13R:NoxB	5'-TCCTGTGTGAAATTGTTATCCGCTCGACATACCTGTCCAGCAC-3'	HYG, NoxB
M13F:NoxB	5'-GTCGTGACTGGGAAAACCCTGGCGGCGAGAAGGGTGAGAAGG-3'	HYG, NoxB
NoxB-TAG	5'-CTAGAAGTTCTCCTTGCCCCATACG-3'	NoxB
NoxB-pro1F	5'-GGTGGGCTTGAATAGTCGCAAGC-3'	NoxB
NoxB-pro2F	5'-GCTCTGCCATTTCGCATGGC-3'	NoxB
NoxB-1F	5'-GTACTGGATGTACGGTGGATTTGC-3'	NoxB
NoxB-2F	5'-CATCCTCTTTCCAGTATGCAGGAAC-3'	NoxB
NoxB-1R	5'-ATACCAGCACCACAGAGAACGGC-3'	NoxB
p67f1	5'-AGGARATHGARACNTGGGT-3'	NoxR
p67r1	5'-TARCANAGACCNCGRTTRAA-3'	NoxR
NoxR-pro1F	5'-AGACCTCAAACGCCATCACTCG-3'	NoxR
NoxR-pro2F	5'-CCAACTGTGCCTTAATTCCCACAC-3'	NoxR

Table B-1. Continued

Primer name	Sequence	Gene
M13R:NoxR	5’-TCCTGTGTGAAATTGTTATCCGCTAGTTTGAGACGCCCTGTTGG-3’	HYG, NoxR
M13F:NoxR	5’-GTCGTGACTGGGAAAACCCTGGCGGCGAGAAGGGTGAGAAGG-3’	HYG, NoxR
NoxR-tail	5’-CGTTCGTAAATATGCGTGATGCTG-3’	NoxR
NoxR-ATG	5’-ATGTCTCTGAAGCAGGTGCGCTC-3’	NoxR
GPx1F	5’-TGTATTGTCACCATTACCCGCCG -3’	GPx
GPx2F	5’-CCCAGTTCGAGGGTCTGGAGA-3’	GPx
GPx:M13R	5’-TCCTGTGTGAAATTGTTATCCGCTTCGAGGCGGTGTTTACGACGA -3’	HYG, GPx
M13F:GPx	5’-GTCGTGACTGGGAAAACCCTGGCGGCGGCCTCAAGCGCGTAAAGTGGAAC-3’	HYG, GPx
GPx1R	5’-GCTTCGTCGGCTGTCCTGAAGA-3’	GPx
GPx2R	5’-CGCAATCATTCCAAACTCGCG-3’	GPx

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BIOGRAPHICAL SKETCH

Siwy Ling Yang, daughter of Hoong Yang and Yek Ying Goh, was born in Kuala Lumpur, Malaysia in 1982. Siwy Ling attended Chong Hwa Independent High School in Kuala Lumpur and graduated in 2000. She attended National Dong Hwa University at Hualien, Taiwan where she majored in Life Science and was awarded a Bachelor of Science degree in June 2005 and a Master of Science in Biotechnology in January 2007. Siwy Ling began working as Associate researcher at Stone and Resource Industry Research and Development Center in Taiwan until May 2008. In June 2008, she began working at the University of Florida as a visiting scholar at the Citrus Research and Education Center. It was during her two years of work on fungal research when Siwy Ling decided to obtain a doctorate degree; in the Fall of 2010 she started her PhD program in the Plant Pathology Department. Under the guidance and support of Dr. Kuang-Ren Chung, she conducted research on the molecular biology of the necrotrophic fungus *Alternaria alternata*, a causal agent of Alternaria brown spot on citrus. Siwy Ling was awarded a Grinter Fellowship from 2010 to 2012 and an A.S. Herlong Sr. Graduate Scholarship in 2013 during her graduate study. Siwy Ling plans to continue her research career in fungal biology after graduation.