ROLE OF PROGRAMMED CELL DEATH IN DISEASE DEVELOPMENT OF *SCLEROTINIA SCLEROTIORUM*

A Dissertation

by

HYO JIN KIM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2010

Major Subject: Plant Pathology

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Approved by:

Chair of Committee,	Martin B. Dickman
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ABSTRACT

Role of Programmed Cell Death in Disease Development of *Sclerotinia sclerotiorum*.

(December 2010)

Hyo Jin Kim, B.S.; M.S., Seoul National University, Korea

Chair of Advisory Committee: Dr. Martin B. Dickman

Plant programmed cell death (PCD) is an essential process in plant-pathogen interactions. Importantly, PCD can have contrasting effects on the outcome depending on context. For example, plant PCD in plant-biotroph interactions is clearly beneficial to plants, whereas it could be detrimental to plants in plant-necrotroph interactions. *Sclerotinia sclerotiorum* is an agriculturally and economically important necrotrophic pathogen. Previous studies have shown that *S. sclerotiorum* secretes oxalic acid (OA) to enhance *Sclerotinia* virulence by various mechanisms including induction of PCD in plants. A recent study has also shown that reactive oxygen species (ROS) generation correlates with induction of PCD during disease development. These studies focus on links between ROS, oxalate, and PCD, and how they impact *S. sclerotiorum* disease development.

I examined the involvement of ROS in pathogenic development of *S. sclerotiorum*. I identified and functionally characterized two predicted *S. sclerotiorum* NADPH oxidases (Nox1 and Nox2) by RNAi. Both *nox* genes appear to have roles in sclerotial development, while only *Nox1*-silenced mutants showed reduced virulence.

Interestingly, the reduced virulence of the *Nox1*-silenced mutant correlated with decreased production of OA in the mutant. This observation suggests that regulation of ROS by *S. sclerotiorum* Nox1 may be linked to OA.

The next study details the phenotype of plants inoculated with an *S. sclerotiorum* oxalate deficient mutant (A2), which showed restricted growth at the infected site. This response resembles the hypersensitive response (HR), and is associated with plant resistance responses including cell wall strengthening, plant oxidative burst, and induction of defensin genes. Conversely, leaves infected with wild type showed unrestricted spreading of cell death and were not associated with these resistant responses. Furthermore, previous work had shown that a *Caenorhabditis elegans* anti-apoptotic gene (*ced-9*) conferred resistance to wild type *S. sclerotiorum*, while this gene had negligible effects on the phenotype of plant leaves inoculated with A2 mutants. These findings suggest that HR-like cell death by A2 and PCD by wild type *S. sclerotiorum* may be regulated by different pathways.

As a whole, these results reveal the importance of ROS, oxalate, and PCD in *Sclerotinia* disease development as well as the significance of interplay between them. These studies contribute to the understanding of the underlying mechanisms of *Sclerotinia* disease.

DEDICATION

To my parents, wife, daughter, and family, for everything you are, and everything you do - thank you.

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I would like to thank my advisor, Dr. Dickman, for his support, encouragement, guidance, and great patience throughout my Ph.D. program. I also thank my committee members, Dr. Ebbole, Dr. Shaw and Dr. Bell-Pedersen for their valuable guidance and advice in the course of this research.

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Thanks also go to my friends and colleagues and the faculty and staff of the Department of Plant Pathology and Microbiology for making my time at Texas A&M University very enjoyable.

Thanks to my father, mother and family for their encouragement and prayer, and to my wife and daughter for their patience and love.

Finally, my biggest gratitude is to YHWH whose name alone is exalted.

NOMENCLATURE

AAL toxin	*Alternaria alternata lycopersici* toxin
DAB	3,3-Diaminobenzidine
DCFDA	2',7'-dichlorodihydrofluorescein diacetate
dpi	Day post inoculation
DPI	Diphenylene iodonium
ETI	Effector-triggered immunity
GEF's	Guanine nucleotide exchange factors
GPCR	G-protein-coupled receptor
HR	Hypersensitive response
JA	Jasmonic acid
LeInh	Tomato proteinase inhibitor
LPS	Lipopolysaccharides
LSD	Lesion simulating disease
NAC	N-acetyl-cysteine
NBT	Nitroblue tetraxolium chloride
NOX	NADPH oxidase
OA	Oxalic acid
OA^-	Oxalate deficient
PAMPs	Pathogen-associated molecular patterns
PCD	Programmed cell death

PKA	cAMP-dependent protein kinase
PR	Pathogenesis-related gene
PTI	PAMP-triggered immunity
R	Resistance
Rap-1	Repressor activator protein-1
RCD	Runaway cell death
REMI	Restriction enzyme mediated integration
Rboh	Respiratory burst oxidase homolog
RNAi	RNA interference
ROS	Reactive oxygen species
SA	Salicylic acid
SOD	Superoxide dismutase

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CHAPTER I

INTRODUCTION

Sclerotinia sclerotiorum; DEVELOPMENT, LIFE CYCLE, SIGNALING, AND PATHOGENESIS

S. sclerotiorum is one of the most successful and devastating fungal pathogens capable of infecting more than 400 species of dicotyledonous plants (Boland and Hall 1994; Dickman 2007). It is a major pathogen of economically important plants including canola, soybean, sunflower, safflower, tomato, potato, tobacco and flax (Hegedus et al. 2005). *S. sclerotiorum* can reduce yield by more than 50% and the estimated loss is over one hundred fifty million dollars per year in the USA (Yajima and Kav 2006). It is found in nearly every country (Steadman 1983). Despite the agronomic importance, there are currently no known successful disease management strategies against *S. sclerotiorum*. For example, the wide host range and the development of environmentally durable sclerotia of *S. sclerotiorum* limit the ability to implement crop rotation for control against this pathogen. Additionally, it is difficult to control *S. sclerotiorum* genetically via breeding, as major genes for resistance have not been found thus far. As a result of economic and agricultural significance, the US Congress has made appropriations via the National *Sclerotinia* Initiative.

This dissertation follows the style and format of *Molecular Plant-Microbe Interactions*.

Life cycle of *S. sclerotiorum*

S. sclerotiorum produces sclerotia, highly melanized multihyphal structures for dispersal, propagation, and long-term survival (Chen et al. 2004). Sclerotia facilitate survival under severe condition and may remain viable for at least 8 years in soil (Adams

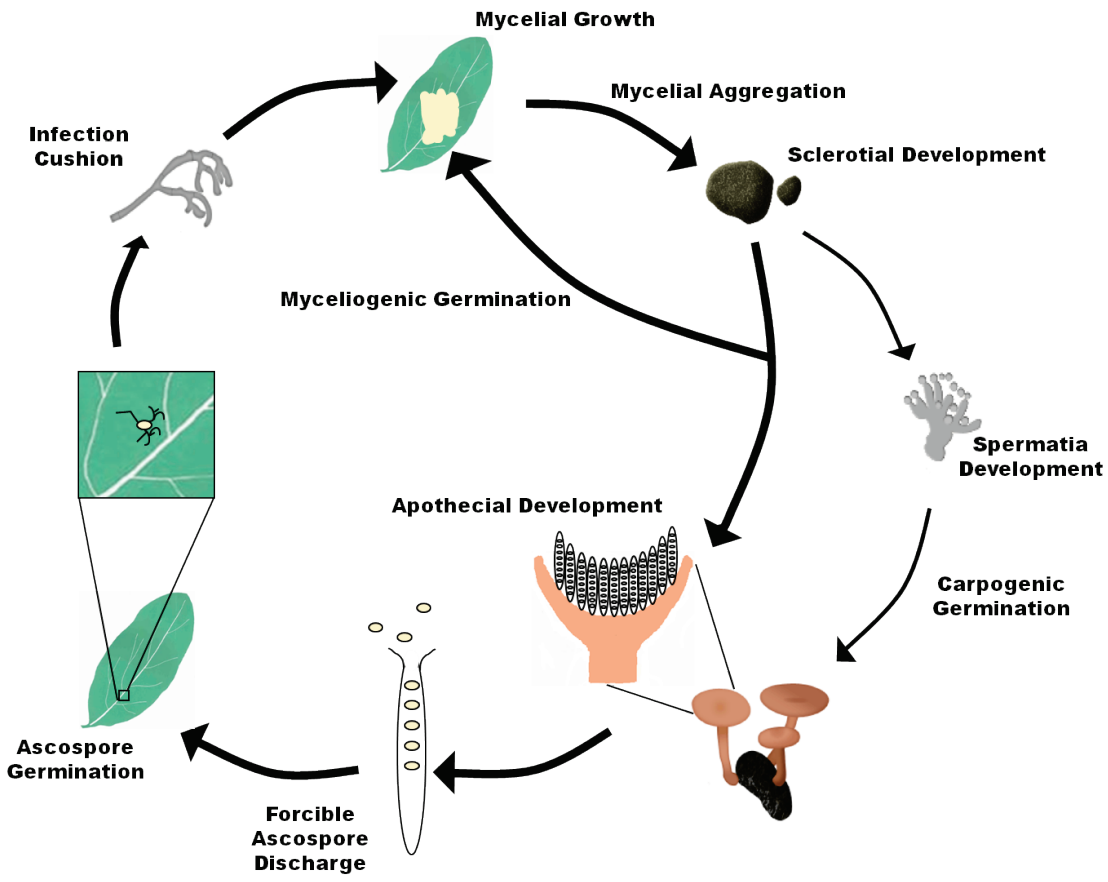


Fig. 1.1. Life cycle of *S. sclerotiorum*.

and Ayers 1979). Sclerotia also play a pivotal role in disease propagation acting as dispersal propagules when transferred in contaminated seed lots or infested soil (Le Tourneau 1979). From a sclerotium, the sexual fruiting bodies (called apothecia) are produced, and ascospores are forcibly discharged from apothecial surface. Ascospores are the primary source of inoculum in most *Sclerotinia* diseases, and are also implicated in disease propagation. Following ascospore germination on the host plant, the fungus grows vegetatively out of the host tissue. When unfavorable environmental conditions occur, sclerotial development is promoted. Mycelia from these germinated sclerotia can infect new host plants (myceliogenic germination), or apothecia from these sclerotia can develop and produce ascospores. Ascospores from apothecia can propagate and also infect new host plants, continuing the lifecycle (see Fig. 1.1).

Three stages of sclerotial development have been distinguished and characterized (Townsend and Willetts 1954): (i) initiation (hyphal aggregation of white mass, sclerotial initials); (ii) development (further hyphal growth and aggregation for augmentation in size), and (iii) maturation (demarcation of sclerotial surface, melanin sedimentation in peripheral rind cells, and internal amalgamation). There are a great number of factors that can impact sclerotia formation. Nutritional factors (carbon and nitrogen sources, phosphorus- and sulfur-containing compounds, K^+, Zn^{2+} and Al^{3+}) and non-nutritional factors (light, temperature, substrate pH, organic acid and stale product accumulation, phenolics, polyphenoloxidase activity, contact with mechanical barriers, -SH group modifiers, and osmotic potential) can influence sclerotial development (Chet

and Henis 1975). Extensive studies also have characterized the structural components and developmental regulation of sclerotia.

Oxalic acid: Pathogenicity factor in *S. sclerotiorum* disease

In *S. sclerotiorum*, the non-host specific phytotoxin oxalic acid is one of the most important factors of pathogenesis (Godoy et al. 1990; Cessna et al. 2000). In addition to *S. sclerotiorum*, there are several fungi including *Aspergillus niger*, *Aspergillus fumigatus*, *Botrytis cinerea*, and many brown-rot and white-rot basidiomycetes that are able to secrete oxalic acid/oxalate (OA) (Han et al. 2007). Three potential pathways for oxalate production in fungi have been proposed; oxidation of glyoxylate, oxidation of glycoaldehyde, and hydrolysis of oxaloacetate (Han et al. 2007). Hydrolysis of oxaloacetate has been suggested as a major pathway of oxalate production in *A. niger* and *B. cinerea* (Han et al. 2007). While considerable effort has been made to identify the major route of oxalate production in *S. sclerotiorum*, it is unclear as yet whether this fungus uses any of these pathways.

Previously, Dickman and colleagues demonstrated the importance of OA in fungal pathogenicity using a genetic approach (Godoy et al. 1990). Mutations were induced in *S. sclerotiorum* by UV irradiation of ascospores, and oxalate deficient mutants were screened by means of a pH indicator plate assay. If mutants were unable to produce OA, they could not sufficiently acidify the media to change the color of the indicator dye. Furthermore, deficiency of OA in selected mutants was also confirmed by gas chromatography and high performance liquid chromatography (HPLC) (Godoy et al.

1990). Importantly, all stable mutants deficient in producing oxalate were unable to cause disease, although production of cell-wall degrading enzymes was normal and even higher than wild type in some cases. In addition, such mutants were unable to produce sclerotia (Godoy et al. 1990; Chen et al. 2004). A revertant to OA^+ was restored in sclerotia formation and virulence. Therefore, these data strongly suggest that oxalate is linked with pathogenesis and fungal development (sclerotia formation).

Further experiments regarding the role of oxalate in sclerogenesis indicated a greater degree of complexity than expected for this simple dicarboxylic acid. Oxalate accumulation led to an ambient acidic condition which was favorable for sclerotial development (Rollins and Dickman 2001). In addition to the role of oxalate in fungal development, several possible roles for oxalic acid in pathogenesis have been proposed: 1) Secretion of oxalate reduces pH, which can maximize the activity of certain fungal cell wall degrading enzymes (Bateman and Beer 1965); 2) Chelation of cell wall Ca^{2+} may impair Ca^{2+}-dependent defenses and diminish the strength of the host cell wall (Bateman and Beer 1965); 3) OA crystals physically cause vascular plugging; 4) OA impacts the function of guard cells, causing foliar dehydration (Guimarães and Stotz, 2004); 5) OA suppresses the oxidative burst of host plant (Cessna et al. 2000), and 6) OA induces programmed cell death of the host plant (Kim et al. 2008b). However, mechanistic details for *Sclerotinia* infection have not been fully validated thus far (Dickman 2007). Therefore, the Dickman lab has been studying *S. sclerotiorum* and OA to understand underlying mechanisms of pathogenic success.

Signaling in development and disease of *S. sclerotiorum*

Previous studies demonstrated that *S. sclerotiorum* rapidly synthesizes oxalic acid in neutral or alkaline pH, leading to acidification and sclerotial development. Additionally, sclerotial development was inhibited under neutral ambient pH conditions, indicating that oxalate may function as a signal for pH-dependent sclerotial development. As a key regulator of gene expression in response to ambient pH, Dr. Rollins and Dr. Dickman (2001) cloned the *S. sclerotiorum* pac1 gene, a pacC homolog that encodes a pH responsive transcription factor PacC of *A. nidulans*. The *S. sclerotiorum* pac1 knock-out mutant was compromised in virulence and was altered in the pattern of oxalate accumulation (Rollins 2003).

In another study, PD98059, a highly selective inhibitor of MEK1 activation and the MAP kinase cascade in animals, blocked sclerotia formation in *S. sclerotiorum*, suggesting that *S. sclerotiorum* MAPK may be involved in sclerotial development (Chen et al. 2004). In addition, transcript levels and activity of Smk1 (a homolog of Fus3 and Kss1 mitogen-activated protein kinase in *S. sclerotiorum*) were increased dramatically during sclerogenesis (Chen et al. 2004). Smk1 transcription also was maximized under acidic pH conditions and was augmented by addition of OA. Furthermore, cAMP blocked expression and activation of Smk1, and elevated production of oxalate in *S. sclerotiorum* (Chen et al. 2004; Chen and Dickman 2005a). These results together suggest that Smk1 modulates a pH-dependent signaling pathway involved in OA production and sclerogenesis. In line with this, cyclic AMP (cAMP) was shown to be an important for regulation of sclerotial development. Increased levels of exogenous and

endogenous cAMP inhibited sclerotial development, and elevated oxalate accumulation in *S. sclerotiorum* (Rollins and Dickman 1998). Therefore, it was hypothesized that cAMP inhibition of sclerotial development was mediated by protein kinase A (PKA) since PKA is the major intracellular receptor of cAMP (Jurick et al. 2004; Rollins and Dickman 1998). However, the effect of cAMP on sclerotial development appeared to be independent of the protein kinase (PKA) pathway. Two potent and selective PKA inhibitors (KT5720 and H89) had no effect on the inhibition of Smk1 and sclerotia formation by cAMP, although PKA activity was significantly inhibited when sclerotial initials of the wild type strains were treated with these inhibitors (Chen and Dickman 2005a). Additionally, a knock-out mutant of the PKA catalytic subunit had negligible effects on sclerogenesis. However, the possibility that another PKA may be present cannot be ruled out (Jurick et al. 2004).

Besides PKA, there are several proteins that bind to and are affected by cAMP (Jurick et al. 2004). Importantly, expressing dominant negative Ras, an upstream activator of the MAPK pathway, in *S. sclerotiorum* inhibited sclerotial development and MAPK activation. Not unexpectedly, an inhibitor of Ras protein in animals (FTI-277, a farnesyltransferase inhibitor) also inhibited sclerotial development. These data suggest that cAMP negatively regulates sclerotial development by a Ras/MAPK (ERK) pathway. The use of bacterial toxins that selectively inhibit the activity of small GTPases, suggested that Rap-1 (a suppressor protein of Ras-induced oncogenic transformation) or Ras possibly mediated the MAPK inhibition by cAMP. Consequently, a potential inhibitor of Rap-1 in animals (GGTI-298, a geranylgeranyltransferase inhibitor) restored

MAPK activation and sclerotia formation that was blocked by cAMP. These results suggest that Ras and MAPK are required for PKA-independent cAMP signaling pathway involved in sclerotial development of *S. sclerotiorum*. Rap-1 may control cross-talk between these two pathways (Chen and Dickman 2005a).

Function of oxalate in disease and development of *S. sclerotiorum*

A potentially important function of oxalate is the suppression of the host oxidative burst, which plays a vital role in the host defense responses to pathogens (Cessna et al. 2000). A previous study demonstrated that wild type *S. sclerotiorum* and ~4 to 5 mM oxalate, a concentration less than that found in diseased tissue, is able to suppress plant produced superoxide, whereas non-pathogenic OA deficient mutants were unable to inhibit plant ROS induction (Cessna et al. 2000). Although it is plausible that low pH suppresses the oxidative burst, oxalate inhibited the oxidative burst at neutral pH, indicating that oxalate is a potent suppressor of the oxidative burst independent of acidification.

A recent study has shown that oxalate is able to induce apoptotic-like programmed cell death (PCD) in the host plant (Kim et al. 2008b). At the beginning of study, it was shown that the fungus-induced cell death in plant cells was accompanied by DNA laddering, a marker for apoptotic PCD, suggesting that plant PCD induced by necrotrophs is reminiscent of apoptosis in animals. Boiled or autoclaved fungal extracts still induced DNA laddering in plants. This result suggests that a protein is likely not responsible for PCD activity although the presence of unknown heat-stable

proteinaceous factors cannot be ruled out. Several lines of evidence are consistent with the idea that oxalate is an elicitor of plant PCD: 1) Oxalic acid and potassium oxalate induced DNA laddering in a time-and dose-dependent manner; other organic acids at similar concentrations were unable to induce ladders, and 2) OA deficient non-pathogenic mutants did not induce laddering, whereas laddering was restored by exogenous addition of OA. Moreover, OA-induced PCD does not occur at acidic pH 3 and 4 but rather at pH 5 and 6, indicating that OA-induced PCD is not due to acidic pH signaling; however, a low pH did induce a necrotic type of cell death. Consistent with this data, potassium oxalate treated leaf discs were heavily stained by 3,3-diaminobenzidine (DAB), a detector of hydrogen peroxide. ROS accumulation was clearly increased at pHs (pH 5 and 6) where DNA laddering concurrently occurred, indicating a relationship between the ROS and PCD. Thus, oxalate appears to modulate plant machinery to induce PCD.

This data has provided an underlying framework for sclerotial development in *S. sclerotiorum*: sclerotial development requires the Ras/MAPK (ERK) pathway which is negatively regulated by a PKA-independent cAMP signaling pathway. This pathway is modulated via the small G-protein Rap-1. Previous studies also have shown an important function of oxalate in fungal disease: suppression of the host oxidative burst and elicitation of programmed cell death in the host plant.

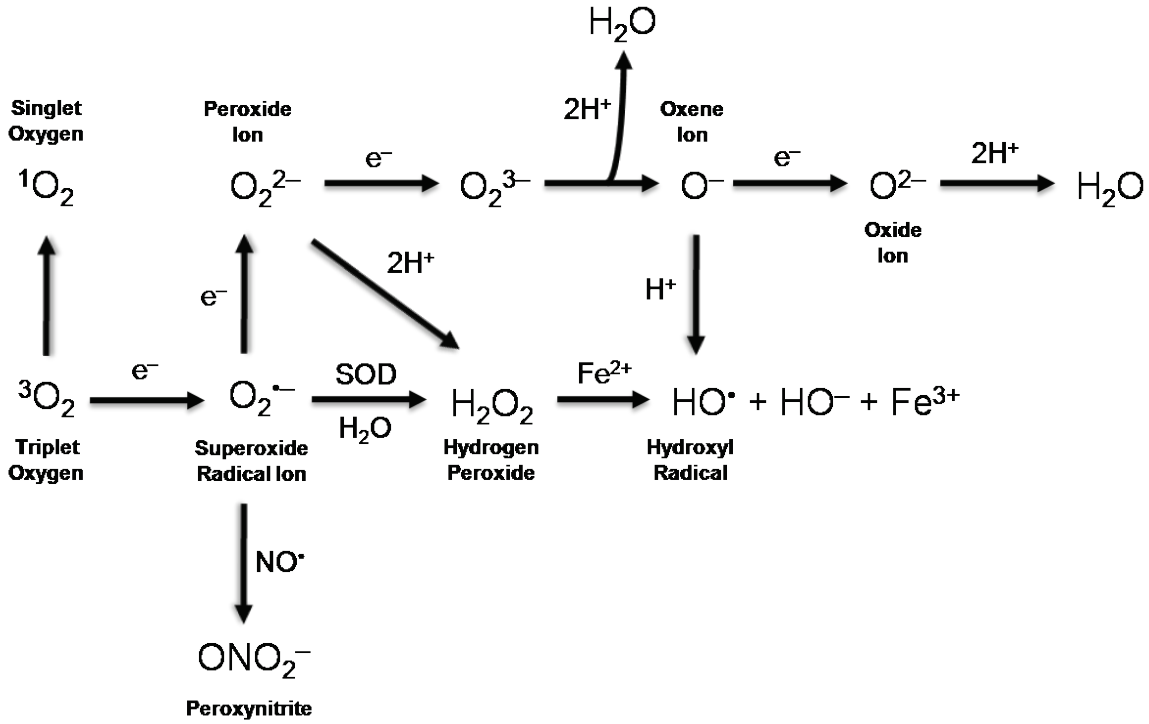


Fig. 1.2. ROS generation by energy transfer, sequential reduction, oxidation, and enzyme reaction in cells.

IMPORTANCE OF REACTIVE OXYGEN SPECIES (ROS) IN PATHOGENESIS AND DEVELOPMENT

Reactive oxygen species are small molecules containing an unpaired electron (Gutteridge 1994). Although reactive oxygen species are produced through several

pathways, the starting material of ROS generation is generally superoxide ($O_2^{\cdot -}$). Reacting with the hydrogen ion, superoxide rapidly dismutates to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) (Fig. 1.2). Diverse reactive oxygen species from superoxide are also generated spontaneously or in the presence of an appropriate partner via several reactions. The hydroxyl radical ($HO^{\cdot}$) can be generated by sequential reduction from superoxide (Fig. 1.2). Superoxide also reacts with nitric oxide ($NO^{\cdot}$) to produce peroxynitrite (ONO_2^-), a highly reactive oxidizing molecule (Fig. 1.2). In the presence of a reduced metal ion, hydrogen peroxide generates a hydroxyl radical and a hydroxide ion via the Fenton reaction (Fig. 1.2). Singlet oxygen ($^1O_2$) is produced by excitation of triplet oxygen (O_2) (Fig. 1.2). Triplet oxygen is actually a biradical; however, it is a stable molecule in nature. Triplet oxygen forms singlet oxygen which is a highly reactive molecule.

All aerobes must balance oxidative metabolism and the generation of unwanted reactive oxygen species particularly in mitochondria, chloroplasts, and peroxisomes (Apel and Hirt 2004). Several oxidative enzymes also unintentionally produce small amounts of superoxide. These ROS can attack proteins, lipids, DNA and carbohydrates in the cell, causing serious problems including DNA mutation, lipid peroxidation and protein oxidation. Numerous studies have shown that ROS are controlled and detoxified by enzymes including SOD, peroxidase, and catalase in plants (Apel and Hirt 2004; Lambeth 2004). Non-enzymatic ROS scavenging mechanisms such as the ascobate-glutathione cycle and antioxidants including tocopherol, flavonoids, alkaloids, and carotenoids also protect cells from the oxidative damage (Apel and Hirt 2004). Emerging

research has shown that ROS is necessary for proper growth and development in animals, plants and fungi (Aguirre et al. 2005; Scott and Eaton 2008).

ROS as a signal molecule

Numerous proteins have been found to be regulated by ROS. Histidine kinases of two component signal transduction systems, which consists of a histidine kinase and a response regulator, are induced in response to ROS signal in prokaryotes, fungi and plants (Apel and Hirt 2004; Singh 2000). In fungi and plants, these signals eventually lead to activation of MAP kinase pathway (e.g. Hog pathway in yeast, and MPK3 and MPK6 in *Arabidopsis*), whereas the action of enzymes such as protein phosphatases is inhibited by ROS (Gupta and Luan 2003; van Montfort et al. 2003). Additionally, the yeast transcription factor Yap1 cooperates with glutathione peroxidase (Gpx3) and regulates expression of genes involved in detoxification of ROS as well as drug and heavy metal resistance (Delaunay et al. 2000; Lin et al. 2009). Although most fungal homologs of the Yap1 transcription factor are ROS-responsive, thus far, only the biotrophic fungus, *Ustilago maydis*, and the necrotrophic fungus, *Alternaria alternata*, have AP1-like transcription factors that are responsive to ROS and impact virulence (Lessing et al. 2007; Lev et al. 2005; Lin et al. 2009; Temme and Tudzynski 2009; Zhang et al. 2000).

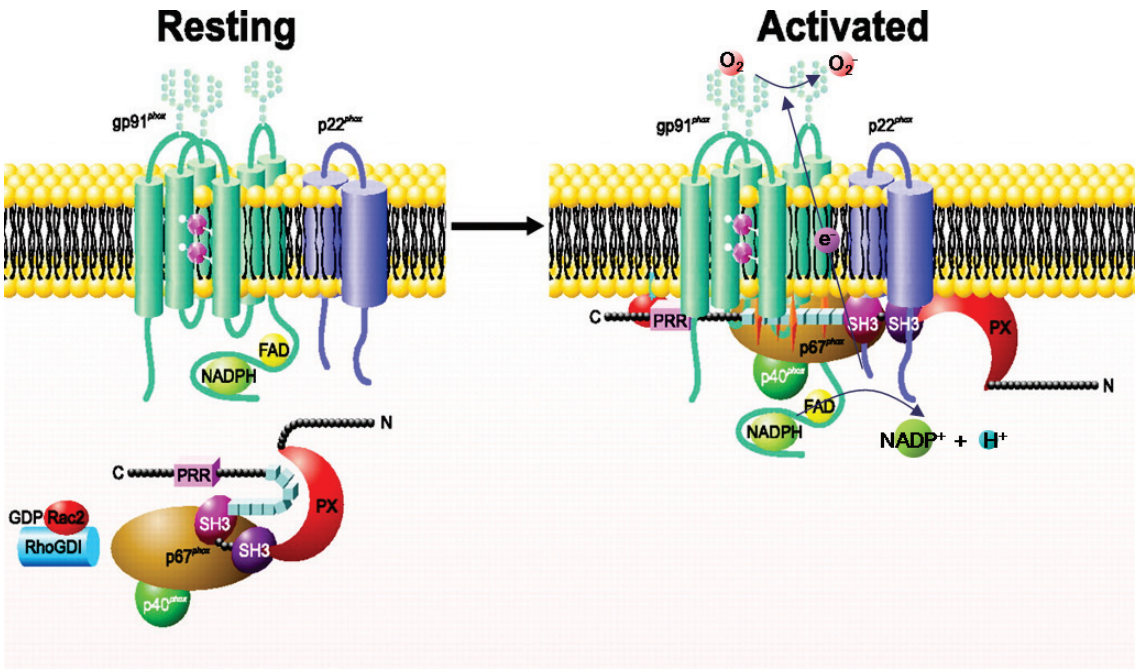


Fig. 1.3. Assembly and activation of the phagocyte NADPH oxidase. See text details. PX: Phox homology domain, SH3: Src homology 3 regions, PRR: proline-rich regions. (adapted from Nauseef 2008)

Mechanism of NADPH activation

Superoxide-generating NADPH oxidase is a major source for generation of ROS in animals, plants and likely in fungi. In animals, the most well-characterized NADPH oxidase (also designated Nox2) is involved in chronic granulomatous, many other

diseases and physiological functions (Segal 2005; Scott and Eaton 2008). The Nox enzyme consists of a multi-subunit complex: the cytosolic regulatory components Rac, $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$, and the integral membrane protein flavocytochrome b_{558} (Scott and Eaton 2008, Fig. 1.3). Heterodimer flavocytochrome b_{558} is the core protein that is composed of the catalytic subunits $gp91^{phox}$ and $p22^{phox}$. Electrons transferred from the electron donor NADPH are translocated to the cytosolic side of the cellular membrane through flavocytochrome b_{558}. These electrons react with the electron acceptor, oxygen, and produce superoxide. From this superoxide as starting material, a large variety of ROS is produced, including oxidized halogens, free radicals, and singlet oxygen (Apel and Hirt 2004). The regulation of Nox protein complex in the mammalian phagocytes is well characterized. In the resting phagocytes, the complex of $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ is in the cytosol, whereas heterodimeric $p22^{phox}$ and $gp91^{phox}$ are already localized in membranes (Fig. 1.3). Stimulating the resting cells, the autoinhibitory domain of $p47^{phox}$ becomes heavily phosphorylated and the entire cytosolic complex is recruited to the membrane (Fig. 1.3). Three cytosolic complexes associate with the two membrane-bound components to generate superoxide from the active oxidase and they are regulated by small GTP proteins such as Rac and RhoGDI (Segal 2005; Lambeth 2004; Nauseef 2008; Apel and Hirt 2004).

Plant NADPH oxidase

Plants induce the production of ROS to kill pathogens and strengthen cell walls via oxidative cross-linking of cell wall glycoproteins (Bradley et al. 1992). ROS also

have been shown to be a signaling molecule (Miller et al. 2009; Torres et al. 2006). Due to the biological importance, plant biologists have attempted to understand the source(s) of ROS in plants. Using genetics and inhibitor studies, two main sources of ROS generation have been shown (Torres et al. 2006). One is the plant Rboh (respiratory burst oxidase homolog) protein, a homolog of human $gp91^{phox}$, which generates superoxide (Grant et al. 2000; Torres et al. 2002). A cell wall-bound peroxidase has also been proposed as a source of ROS (Bindschedler et al. 2006; Bolwell et al. 2002). In relation to this, ROS from the NADPH oxidase of plants are involved in host defense via the hypersensitive response (Torres and Dangl 2005), in development via regulation of plant cell expansion through the activation of Ca^{2+} channels (Foreman et al. 2003), and in several other physiologies (Kwak et al. 2003). Furthermore, Torres and his colleagues (2005) showed that activation of NADPH oxidase inhibited runaway cell death (RCD), an uncontrolled spreading of cell death to uninfected cells surrounding the HR sites. They showed that the *Arabidopsis* double knock-out mutants of NADPH oxidase (AtrbohD) and the LSD (lesion simulating disease) zinc-finger protein, a negative regulator of RCD, resulted in the acceleration of RCD (Torres et al. 2005). However, overexpression of AtrbohD in the *lsd1* mutant reduced RCD compared to *lsd1* mutants (Torres et al. 2005). This data showed that Atrboh and LSD1 were involved in cell death pathway depending on a salicylic acid that induce cell death spread in cells surrounding infection sites (Torres et al. 2005).

Fungal NADPH oxidase

In fungi, ROS studies are increasing, with a significant number of studies into the role of the NADPH oxidase in fungal development. Three different fungal Nox subfamilies have been found thus far: NoxA (Nox1), NoxB (Nox2) and NoxC (Nox3). NoxA and NoxB are fungal homologs of $gp91^{phox}$, and NoxC has putative calcium binding EF-hand motifs, which are found in the NH_2-terminal ends of animal Nox5 and plant Rboh proteins. NoxA (Nox1) is required for the development of sexual fruiting body in *A. nidulans*, *Podospora anserina* and *Neurospora crassa* (Scott and Eaton 2008). Aguirre and his colleagues (Lara-Oritz et al. 2003) demonstrated that deletion of NoxA is responsible for inhibition of cleistothecial differentiation in *A. nidulans*. However, this gene was not implicated in hyphal growth and asexual development. Consistently, Nox1, a homolog of NoxA, of *N. crassa* and *P. anserina* is required for sexual development, although Nox1 of *N. crassa* also regulates asexual development and hyphal growth (Cano-Dominguez et al. 2008; Malagnac et al. 2004). Conversely, NoxB appeared to be responsible for regulation of ascospore germination in those three fungi (Lara-Oritz et al. 2003; Cano-Dominguez et al. 2008; Malagnac et al. 2004). Notably, several fungi including *Fusarium* spp., *M. grisea*, *P. anserina*, *Aspergillus terreus* and *Phaeosphaeria nodorum* have the third *nox* gene, *noxC* (*nox3*), although its function is unclear (Takemoto et al. 2007).

The regulation of the Nox complex is not well characterized in fungi. NoxR, a homologue of $p67^{phox}$, and Rac protein, a small GTPase of the Rho subfamily, are required for the regulation of Nox1 (NoxA) and Nox2 (NoxB) in fungi (Takemoto et al.

2006; Scott and Eaton 2008). In many cases, inactivation or elimination of NoxR or Rac showed similar phenotypes to mutants that are disrupted in Nox1/NoxA or Nox2/NoxB enzyme (Cano-dominguez et al. 2008; Segmüller et al. 2008; Tanaka et al. 2008; Takemoto et al. 2006). This suggests that fungal NoxR and Rac play a pivotal role in regulation of ROS in fungus. For activation of fungal NADPH oxidases, therefore, it is speculated that fungal Rac and NoxR may be recruited to the plasma membrane and interact with Nox1 (NoxA) or Nox2 (NoxB), leading to production of superoxide. Interestingly, a search of fungal genomes for homologs of the mammalian Nox regulatory components revealed the apparent absence of $p47^{phox}$ and $p40^{phox}$ homologs in fungi (Takemoto et al. 2007). Furthermore, the protein-protein interaction domain found in mammalian $p67^{phox}$ for interaction with $p47^{phox}$ and $p40^{phox}$ is absent in C-terminus of fungal NoxR. These data suggest that only NoxR and Rac are major regulators for fungal NADPH oxidase complexes compared to animal Nox complexes or fungi may use novel regulators. In animals, it has been demonstrated that p38 MAPK and p21-activating kinase are also involved in regulation of Nox enzyme complex (Takemoto et al. 2006; Scott and Eaton 2008). In accordance, SakA (stress-activating MAP kinase), a p38 MAPK homolog, of *A. nidulans* and *Epichloë festucae* is associated with expression or modification of noxA (Lara-Oritz et al. 2003; Eaton et al. 2008). In *C. purpurea*, Cla4, a homolog of p21-activating kinase (Pak), suppressed expression of *nox1* gene (Rolke et al. 2008). Increasing information regarding NADPH oxidase in several fungi supports

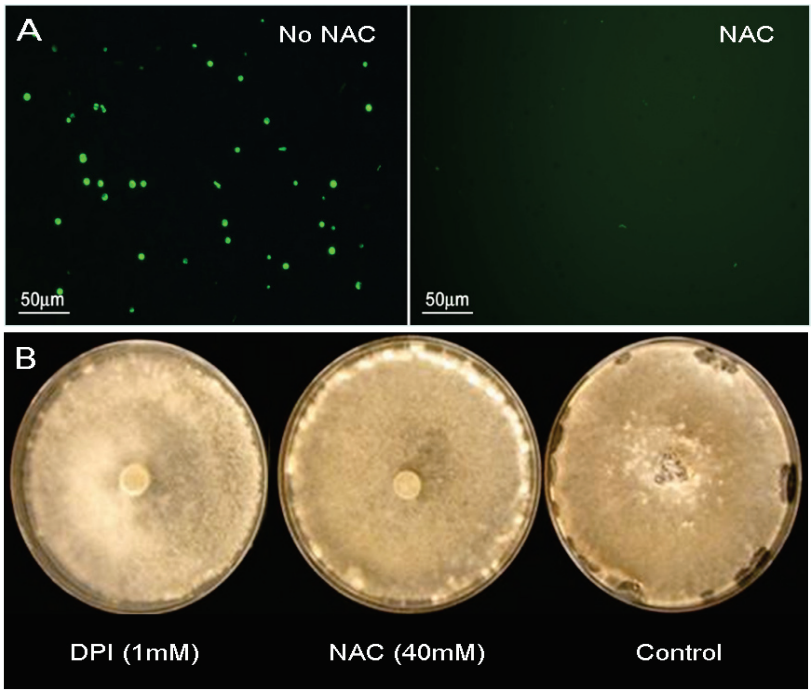


Fig. 1.4. Antioxidants impair sclerotial development in *S. sclerotiorum.* A, Sclerotial initials generate high amounts of ROS. An agar-mycelium plug of the wild type isolate 1980 was inoculated onto potato dextrose agar (PDA) and grown until the appearance of sclerotial initials. Aliquots of protoplasts generated from sclerotial initials were then stained with 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA) to visualize ROS within the cells, as described in Materials and Methods. Figures shown are representatives of three independent experiments. Panel a: wild type; Panel b: wild type treated with the antioxidant N-acetyl-cysteine (NAC). **B,** An agar-mycelium plug of the wild type isolate 1980 was inoculated on a fresh PDA plate supplemented or not with 40mM N-acetyl-cysteine (NAC) or 1mM diphenylene iodonium (DPI). Sclerotial development was monitored and photographed after 14 days growth at room temperature. Courtesy of Changbin Chen.

the idea that fungal NADPH oxidases are required for development; however, the underlying mechanism and pathway for the regulation awaits future study.

Involvement of ROS in development of *S. sclerotiorum*

A previous study in *S. sclerotiorum* has shown the role of ROS during sclerotial development (Chen et al. unpublished data). Intracellular ROS production from sclerotial initials was monitored by the ROS indicator 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA). Fluorescence emission was higher in sclerotial initials, suggesting that sclerotial initiation includes the generation of intracellular ROS (Fig. 1.4A). Wild type *S. sclerotiorum* was treated with either NAC or diphenylene iodonium (DPI), an inhibitor of flavoenzymes such as NADPH oxidase, to further examine ROS involvement in sclerotia formation. NAC (40mM) and DPI (1mM) treatment of the fungus hindered sclerotia formation and prevented sclerotia maturation. There were no significant differences in radial growth of *S. sclerotiorum* in these treatments (Fig. 1.4B). Together, these experiments suggest that the generation of ROS is required for sclerotial development and an NADPH oxidase may generate ROS of *S. sclerotiorum*. In line with this, I show that ROS from *S. sclerotiorum* NADPH oxidase is required for development in Chapter II.

Role of fungal NADPH oxidase in plant-microbe interactions

Accumulating evidence strongly suggests that ROS play a pivotal role in plant-microbe symbiosis and pathogenesis. *E. festucae* is a fungal mutualist providing stress

protection to the host; the host provides a suitable niche for the fungus. A restriction enzyme-mediated integration (REMI) screen for mutualistic mutants identified NoxA, a *nox* gene. When NoxA was deleted, the fungus unexpectedly exhibited a pathogenic phenotype (Tanaka et al. 2006). Elimination of NoxR and RacA led to a similar phenotype in the plant-*noxA* mutant association (Tanaka et al. 2006; Tanaka et al. 2008; Takemoto et al. 2006). This suggests that ROS production via fungal NADPH oxidase is necessary for maintenance of plant-fungus mutualism. Additionally, Talbot and his colleagues (Egan et al. 2007) showed that an NADPH oxidase of *Magnaporthe grisea* plays an essential pivotal role in differentiation of the infection cell, the appressorium: the *Δnox1*, *Δnox2,* and *Δnox1Δnox2* mutants were unable to infect. Consistent with these studies, *Claviceps purpurea* required Cpnox1 activity for full pathogenicity and germination of conidia. Cpnox1 is also involved in fungal growth, vegetative differentiation, and formation of sclerotia (Giesbert et al. 2008). In contrast to Cpnox1, Cpnox2 had no effect on virulence (Giesbert et al. 2008). Consistent with NADPH oxidases of *M. grisea*, however, *Botrytis cinerea* required both *bcnoxA* and *bcnocB* genes for pathogenesis (Segmüller et al. 2008). Deletion of *bcnoxA* led to slow colonization in the host tissue, although *bcnoxA* mutants were able to penetrate the host tissue. The *bcnoxB* and double knock-out mutants (*bcnoxAB*) were unable to penetrate epidermal cells; moreover double knock-out mutants colonized more slowly than *bcnoxA* and *bcnoxB* mutants, and were thus weakly pathogenic. In Chapter II, involvement of ROS in disease of *S. sclerotiorum* was investigated.

PROGRAMMED CELL DEATH IN DEVELOPMENT, STRESS RESPONSE AND PATHOGENESIS

Programmed cell death (PCD) is an inevitable cellular process to eliminate unwanted or damaged cells, and occurs in all multicellular organisms. This process must be tightly regulated since improper control of cell death can obviously provoke deleterious effects on the organisms. Human diseases such as cancer, retenosis, and autoimmunity are caused by uncontrolled excessive accumulation of cells, while inappropriately accelerated PCD and leads to diseases such as stroke, myocardial infarction, inflammation, AIDS, Alzheimer's disease (reviewed in Williams and Dickman 2008). Notably, many pathogens such as viruses, bacteria, and fungi subvert the cell death machinery of a host, in order to influence the infection process (Navarre and Wolpert 1999; Roshal et al. 2001; Roulston et al. 1999; Williams and Dickman 2008).

Features of cell death mechanism in mammalian cell; apoptosis, autophagy and oncosis/necrosis

Mammalian cell death is categorized in several forms: apoptosis, autophagy, necrosis/oncosis, pyroptosis, etc (Labbé and Saleh 2008). Apoptosis at one end of the programmed cell death spectrum plays a pivotal role in development and physiology in animal cells. Specific physiological signals initiate activation of the endogenous cell suicide program, which leads to defined morphological and biochemical changes, and finally death (Kabbage and Dickman 2008; Williams and Dickman 2008). Characteristic

hallmarks of morphological and biochemical changes indicating apoptotic PCD include cell shrinkage, surface blebbing, chromatin condensation, and DNA fragmentation, among others (Heath 1998; Kabbage and Dickman, 2008; Mur et al. 2008; Williams and Dickman 2008). The caspases, families of intracellular cysteine proteases, play an important role as executioners in apoptotic PCD (Williams and Dickman 2008).

Autophagy, a form of non-apoptotic programmed cell death, is required for the degradation of long-lived proteins and cytoplasmic organelles (Levine and Klionsky 2004). Autophagy is involved in turnover of cytoplasmic cell components for homeostasis and also triggered by environmental and physiological changes such as starvation, cellular and tissue remodeling, and cell death (Levine and Klionsky 2004; Shitani and Klionsky 2004; Williams and Dickman 2008). The characteristic hallmark of autophagy is the accumulation of a double-membrane vesicle, an autophagosome, via lysosomal machinery in the cytoplasm. Importantly, autophagy is associated with both death and survival with different stimuli and under different conditions (Levine and Klionsky 2004; Shitani and Klionsky 2004; Williams and Dickman 2008). In addition, autophagy shares some of the same gene products that mediate apoptotic PCD. Compared to apoptosis and autophagic cell death, oncosis/necrosis is poorly described. It has been considered as an accidental process. However, recent studies suggest that this cell death may be controlled and programmed (Festjens et al. 2006).

Although our knowledge of cell death in animals is still incomplete, studies of animal cell death have provided a steady stream of fundamental knowledge to investigate cell death of other organisms including plants and fungi.

Plant PCDs in development and stress response

In plants, numerous physiological processes including development, biotic and abiotic stress have been shown to require programmed cell death (Pennell and Lamb 1997). These processes often have representative cytological features reminiscent of mammalian programmed cell death, including shrinkage of the cytoplasm, chromatin condensation, DNA cleavage, DNA fragmentation, caspase-like protease activity, and the formation of apoptotic bodies (Li and Dickman 2004; Navarre and Wolpert 1999; Ryerson and Heath 1996; Wang et al. 1996b; Williams and Dickman 2008). Despite this similarity, the identification of plant genes homologous to those in mammalian PCD pathway awaits discovery (Heath 1998).

Several developmental processes involve in PCD: embryogenesis; seed germination; the differentiation of tracheary elements; formation of aerenchyma; leaf shape remodeling; senescence of leaf and reproductive organ; trichome development, and PCD in the root cap (Gadjev et al. 2008). These developmental processes are consummated with PCD (Gadjev et al. 2008). Developmental PCDs of plants have several characteristic hallmarks in common with animal apoptosis although there are several features unique in PCDs of plants because of the presence of chloroplasts and a prominent vacuole and the cell wall in plant PCDs (Williams and Dickman 2008). For example, an increase in vacuolization has been observed during developmental PCDs such as differentiation of floral nectary tissue (Gaffal et al. 2007), and development of a megagametophyte (Yadegari and Drews 2004) and male sequal organs (Rogers 2006).

One of the best-characterized developmental PCDs in plants occurs in formation of tracheary elements in the xylem of vascular plants (Fukuda 2000). In contrast to other plant PCDs, this PCD does not show typical apoptotic features such as chromatin condensation. Instead, degradative enzymes accumulate in the vacuole (Lam 2004; Nakashima et al. 2000), followed by the destruction of vacuole and by nuclear and organelle DNA fragmentation at the end of this process (Lam 2004). Notably, this vacuole-mediated cell death may be functionally similar to the animal phagocytosis system, which engulfs and degrades apoptotic cells in a non-inflammatory manner (Hatsugai et al. 2006; Williams and Dickman 2008). Since plants do not have phagocytes, plants may utilize an alternative combination of vacuole and autophagy to remove dying cells in plant PCD (Hatsugai et al. 2006; Williams and Dickman 2008).

PCD is also observed during abiotic stress responses: cold, heat, drought, salt, and pollutants such as ozone and excessive UV radiation (Lam 2004; Williams and Dickman 2008). PCD induced by exposure to abiotic stress also have shown typical features of apoptotic PCD such as DNA fragmentation, and nuclei and chromatin condensation (Huh et al. 2002; Koukalova et al. 1997; Lin et al. 2006; Overmyer et al. 2005). This PCD is typically accompanied by the generation of ROS (Coffeen and Wolpert 2004; Gao et al. 2008; Garnier et al. 2006; Huh et al. 2002; Mahalingam et al. 2006; Shabala et al. 2007; Vacca et al. 2007). Importantly, some of the PCDs triggered by abiotic stress were prevented in transgenic plants, which carry anti-apoptotic genes such as *ced-9* and *bcl-xL* (Shabala et al. 2007; Xu et al. 2004). This suggests that

regulators of PCD are functionally conserved between plants and animals (Dickman et al. 2001).

The hypersensitive response (HR) in plant-pathogen interactions

The HR is a well-documented form of plant PCD in response to pathogen invasion. Plants rapidly induce the HR to prevent the ingress of a pathogen. The HR is defined as a rapid localized cell death at the infected site (Agrios 1988; Mur et al. 2008) and delimits pathogen spread (Greenberg and Yao 2004). The HR is generally elicited by interaction between resistance (R) protein-avirulence (Avr) proteins. This interaction initiates resistance responses such as generation of ROS, cell wall strengthening, and activation of defense-related genes (Apel et al. 2004; Delannoy et al. 2005; Hamiduzzaman et al. 2005; Kuc 1995; Lamb and Dixon 1997; Rinaldi et al. 2007; Stone and Clarke, 1992). In the resistant hosts, rapid cell death with these various responses halts the growth of pathogens, and the plant survives (Greenberg and Yao 2004; Heath 2000; Liu et al., 2005; Mur et al. 2008). However, defense responses associated with the HR do not always accompany cell death.

Because of its importance, many studies have attempted to understand underlying mechanism of HR. Similar to plant developmental and abiotic stress-related PCD, studies have shown that the HR shares some features with mammalian apoptosis (reviewed in Mur et al. 2008). Recent studies have reported that *BECLIN1/ATG6* (an ortholog of the yeast and mammalian autophagy gene *ATG6/VPS30/beclin 1*) silenced lines of *N* gene-containing *Nicotiana benthamiana* were unable to restrict PCD in an

incompatible pathogen challenge during *N*-mediated (tobacco mosaic virus), R genes-mediated (*Cf9* with *Avr9*, *Pto* with *AvrPto* and *Pseudomonas syringae pv. tomato* DC3000) and elicitors-mediated (*Phytophthora infestans* elicitor INF1) HR-PCD (Liu et al. 2005). The silenced lines also did not show features of autophagy such as autolysosome-like structure and autophagic body, which was observed in the HR-PCD (Mur et al. 2008; Liu et al. 2005; Patel et al. 2008).

Programmed cell death in disease of a necrotroph pathogen, *S. sclerotiorum*

Plant pathogens are broadly divided into two groups depending on their life styles: biotrophs and necrotrophs. Biotrophs must obtain nutrition from living tissue, whereas necrotrophs obtain nutrition from dead tissue (Glazebrook 2005). Since the HR prevents access of pathogen to the host tissue, the host plant can control biotrophic pathogens by the HR. In contrast, host programmed cell death induced by necrotroph might be beneficial to the pathogen (Williams and Dickman 2008).

The expression of animal anti-apoptotic cytoprotective genes conferred the resistance against the necrotrophic pathogen, *S. sclerotiorum* (Dickman et al. 2001). When the host plant was challenged with the wild type fungus, fungi grew vegetatively on the leaf surfaces of transgenic plants carrying antiapoptotic genes but did not cause disease, suggesting these genes are not fungicidal but presumably autoprotective. Additionally, these transgenic host plants were resistant to *Botrytis cinerea*, another broad-host-range necrotrophic fungus, and *Cercospora nicotianae*, which produces non-host-specific toxin, cercosporin. These results strongly suggest that necrotrophic fungi

like *S. sclerotiorum* might induce cell death in the host plant to facilitate pathogenesis. Furthermore, plants inoculated with *S. sclerotiorum* exhibited DNA laddering, a marker for apoptosis, whereas oxalate deficient (OA^-) mutants were unable to generate DNA laddering and were non-pathogenic. As discussed, it was later shown that oxalate specifically induced programmed cell death of host plants (Kim et al. 2008b). OA elicited DNA laddering and generation of ROS. Thus, OA from wild type *S. sclerotiorum* appears to function as a modulator of plant PCD possibly via regulation of the ROS signaling pathway. These studies together strongly support that oxalate produced by *S. sclerotiorum* elicits programmed cell death in the host plant.

Consistently, studies with *Cochliobolus victoriae* which produces host selective toxin, victorin, demonstrated that this fungus elicits programmed cell death, which shares features of apoptosis such as DNA fragmentation and formation of apoptotic bodies (Coffeen and Wolpert 2004; Curtis and Wolpert 2002; Lorang et al. 2007; Navarre and Wolpert 1999). In addition, research on mycotoxins including *Alternaria alternata lycopersici* (AAL) toxins and fumonisin B1 also showed induction of apoptotic-like marker. It was demonstrated that this metabolic perturbation leads to host cell death, and involves some apoptotic features such as DNA fragmentation, nuclear condensation, and the requirement of *de novo* protein synthesis (Asai et al. 2000; Stone et al. 2000; Wang et al. 1996a). Collectively, these studies suggest that necrotroph fungi generate metabolites that can trigger host PCD.

It is unclear whether PCD induced by necrotroph is analogous to the HR, though Govrin and Levine (2000) suggested that necrotrophs such as *Botrytis* and *Sclerotinia*

induce the HR to infect the host plant. My recent data showed that cell death induced by the OA^- mutant of *S. sclerotiorum* was restricted, similar to the cell death during an incompatible interaction (See Chapter III). Furthermore, this HR-like cell death was accompanied by various defense responses such as generation of ROS, cell wall strengthening, and activation of defense genes. Considering the importance of oxalate in pathogenesis, the underlying mechanism for this phenotype is of interest.

OBJECTIVES OF THIS WORK

Considerable effort has been made to understand underlying mechanisms responsible for *Sclerotinia* disease. Increased knowledge may lead to alternative strategies for disease control. As mentioned, there is evidence showing the importance of ROS in plant-*Sclerotinia* interaction. In addition, accumulating data showed that oxalate is important in the development and pathogenicity of *S. sclerotiorum*. Therefore, I have focused on role of ROS and oxalate as the key modulators in plant-*Sclerotinia* interaction. I have examined following hypotheses:

1. NADPH oxidases of *S. sclerotiorum* are important for development and pathogenicity of *Sclerotinia* (Chapter II). To test this hypothesis, I characterized the two predicted NADPH oxidases from *S. sclerotiorum* employing RNAi.

2. The host plant response against the oxalate deficient mutant is mechanistically similar to the hypersensitive response (Chapter III). To address this hypothesis, I characterized the cell death response against both wild type and OA deficient mutants.

CHAPTER II

IDENTIFICATION AND CHARACTERIZATION OF *Sclerotinia sclerotiorum nox* GENES

OVERVIEW

Numerous studies have shown both the detrimental and beneficial effects of reactive oxygen species (ROS) in animals, plants and fungi. These organisms utilize controlled generation of ROS for various aspects of cellular regulation, signaling, and development. In addition, recent studies highlight the significance of ROS in host-microbe interactions. Here, I show that ROS are essential for the pathogenic development of *Sclerotinia sclerotiorum*. I identified two *S. sclerotiorum* NADPH oxidases (Nox1 and Nox2), which are presumably involved in ROS generation. RNA interference (RNAi) was used to functionally analyze Nox1 and Nox2. This study showed that *nox1* has a crucial role in both pathogenesis and sclerotial development, while *nox2* plays only a limited role in pathogenesis. Inhibition of *nox1* also correlated with a reduction of ROS generation, oxalate synthesis, and virulence. In addition, *Nox1*-silenced mutants resulted in the induction of the plant oxidative burst. These results indicate that NADPH oxidases are involved in pathogenicity and development of *S. sclerotiorum*.

INTRODUCTION

Sclerotinia sclerotiorum is an economically important necrotrophic fungal pathogen, which is able to infect the dicotyledonous plants of more than 400 species (Yajima and Kav 2006). Among these hosts, several agriculturally important dicot plants can be devastated (Hegedus et al. 2005). It is a particularly effective pathogen because of the difficulty to control this fungus both culturally and chemically. Crop rotation is generally ineffective due to the ability of the fungus to overwinter as well as the broad host range via sclerotia. Spray regimes also have been largely unsuccessful, and breeding programs have had limited success. Considerable effort has been made to control *Sclerotinia* diseases, yet there are currently no effective control regimes. A previous study have shown that oxalate is important for pathogenicity and recently was shown to be involved in increasing plant ROS levels and the induction of host plant programmed cell death (PCD) during *Sclerotinia* infection (Kim et al. 2008b). In this chapter, fungal NADPH oxidases which generate ROS were examined in more detail.

NADPH oxidases in animals are a primary source of superoxide, an important precursor of several reactive oxygen species (ROS) including hydrogen peroxide. For the activation of Nox enzymes, cytosolic regulatory components (Rac, $p67^{phox}$, $p47^{phox}$, and $p40^{phox}$) are recruited into the integral membrane protein flavocytochrome b_{558} consisting of the catalytic subunits $gp91^{phox}$ and $p22^{phox}$ (Nauseef 2008; Scott and Eaton 2008). The activated Nox enzyme complex generates superoxide from oxygen utilizing NADPH as an electron donor, thereby leading to the formation of other ROS. Among NADPH oxidases in animals, the most well-known is Nox2 ($gp91^{phox}$), which is

associated with chronic granulomatous disease (CGD) (Babior 2004; Segal 2005; Scott and Eaton 2008). As in animals, the plant NADPH oxidase (respiratory burst oxidase homolog, Rboh), the $gp91^{phox}$ homolog, is also considered to be a primary, but not the only source of ROS (Torres et al. 2002). ROS from the *Arabidopsis* NADPH oxidases (AtrbohD and AtrbohF) are associated with host defense via the hypersensitive response (Torres et al. 2002; Torres et al. 2005). ROS-dependent abscisic acid (ABA) signaling by these enzymes also regulate cytosolic Ca^{2+} levels, thereby effecting stomatal closure, seed germination, and root elongation (Kwak et al. 2003). In addition, an *Arabidopsis* NADPH oxidase (AtrbohC) regulates plant cell expansion through the activation of Ca^{2+} channels during root hair development (Foreman et al. 2003).

NADPH oxidases have been found in most fungal species, excluding the hemiascomycete yeasts and other unicellular fungi (Takemoto et al. 2007). Interest in fungal NADPH oxidases has recently increased. Several studies have shown the importance of fungal NADPH oxidases in sexual and asexual development (Cano-Dominguez et al. 2008; Lara-Oritz et al. 2003; Malagnac et al. 2004). In plant pathogenic fungi, deletion of *nox* genes resulted in developmental defects in structures such as appressoria and sclerotia that are associated with pathogenesis (Egan et al. 2006; Segmüller et al. 2008). It has also been shown in the symbiotic interaction between the endophytic fungus *E. festucae* and perennial ryegrass, that ROS generated from fungal NADPH oxidases functions as a signal molecule to modulate the plant-fungus symbiotic interaction (Tanaka et al. 2006).

This study examined the contribution of *S. sclerotiorum* NADPH oxidases to fungal pathogenesis, growth and development. I identified two predicted NADPH oxidases in the *S. sclerotiorum* genome sequence (http://www.broadinstitute.org/annotation/genome/sclerotinia_sclerotiorum/MultiHome.html). Silencing the *nox1* (SS1G_05661.1) and *nox2* (SS1G_11172.1) genes by RNAi revealed that they are required for sclerotial development. In addition, *Nox1*-silenced mutants (SsNOX1-RNAi mutant) were severely compromised in their ability to cause disease. I also noticed a significant reduction in production of oxalate (a pathogenicity factor) in the *Nox1*-silenced mutant. Tomato plants challenged with *Nox1*-silenced mutants showed an increased plant oxidative burst, suggesting that the decreased virulence of *Nox1*-silenced mutants may be a result of active defense response in host plants. SsNOX2-RNAi mutant-infected plants showed negligible increases in generation of superoxide in the plant and were still pathogenic. Hence, Nox1 of *S. sclerotiorum* is important for both virulence possibly affecting oxalate production and fungal development.

RESULTS

Transcript levels of *S. sclerotiorum* Nox1 increased *in planta* and during sclerotial development

From the available genomic sequence of *S. sclerotiorum* (http://www.broadinstitute.org/annotation/genome/sclerotinia_sclerotiorum/MultiHome.html), I indentified two predicted *nox* genes that harbored significant homology to human

1.

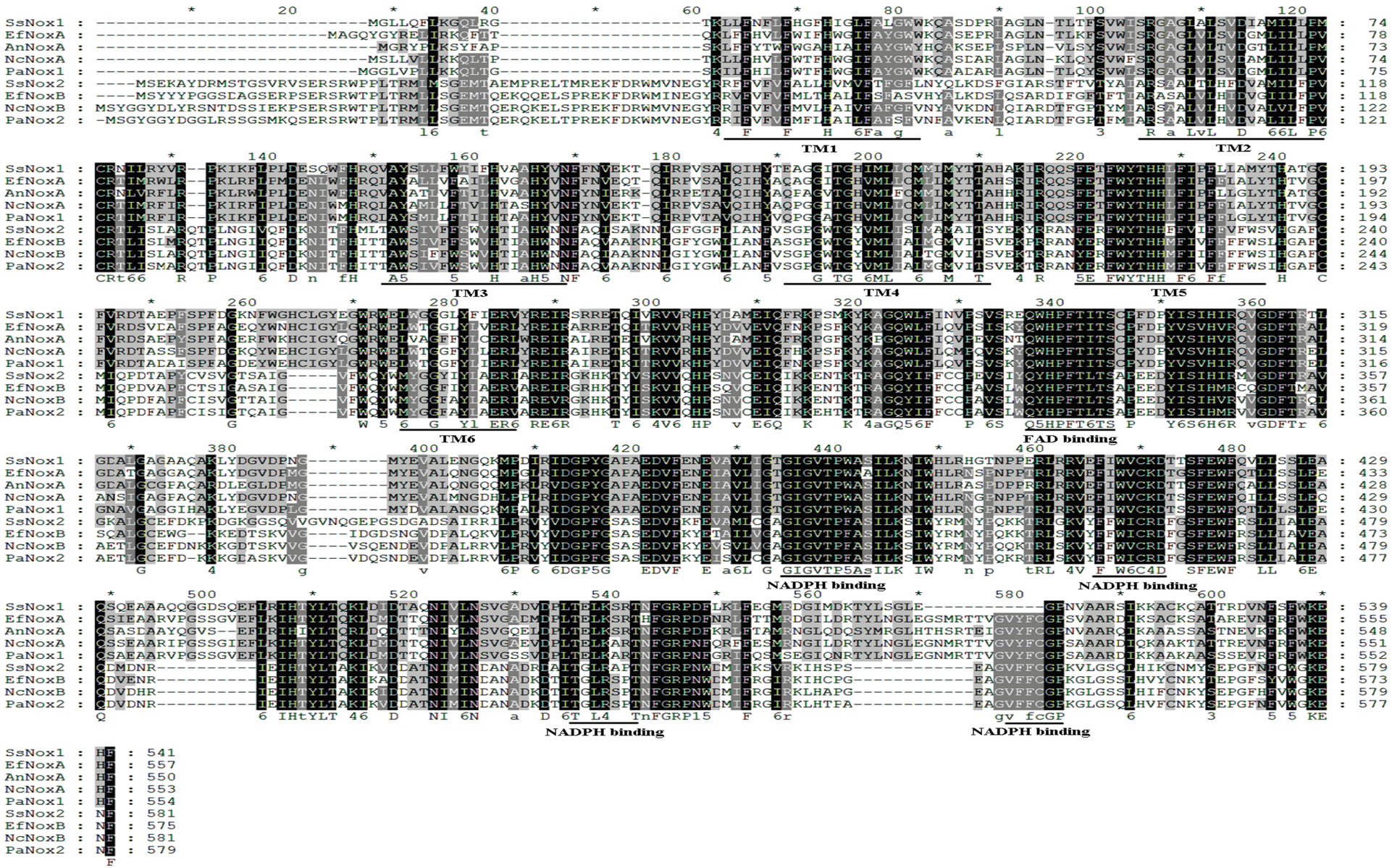


Fig. 2.1. Multiple sequence alignment of NADPH oxidase from *S. sclerotiorum* NADPH oxidases and other fungi. Six different entries were aligned using ClustalW. GeneDoc version 2.7.000 was used to shade the residues in the following manner: residues within a black background are 100% identical among all listed proteins, dark gray are identical in 80% of listed proteins, light gray are identical in 50% of listed proteins. Important conserved motifs of NADPH oxidase were denoted: transmembrane (TM) region, FAD binding motif, and NADPH-binding motif. Each entry is annotated with a two-letter abbreviation for the genus and species name in italics followed by the corresponding gene name. Ss, *Sclerotinia sclerotiorum*; Ef, *Epichloë. festucae*; Nc, *Neurospora crassa*; As, *Aspergillus nidulans*; Pa *Podospora anserine.*

gp91^{phox} and Nox homologs in several filamentous fungi. These two *nox* genes were amplified from the genomic DNA of *S. sclerotiorum*. The predicted amino acid sequences of *S. sclerotiorum* Nox1 (541aa) and Nox2 (581aa) were aligned with *Epichloë festucae* NoxA (AB236860.1), *E. festucae* NoxB (AB236861.1), *Aspergillus nidulans* NoxA (AY174088.1), *Podospora anserina* Nox1 (AF364817.1), *P. anserina* Nox2 (AY372210.1), *Neurospora crassa* NoxA (AABX02000003.1), and *N. crassa* NoxB (AABX02000010.1). *S. sclerotiorum* Nox1 is 74%, 69%, 73%, and 72% identical to *E. festucae* NoxA, *A. nidulans* NoxA, *N. crassa* NoxA, and *P. anserine* Nox1. *S. sclerotiorum* Nox2 is 77%, 78%, and 79% identical to *E. festucae* Nox2, *N. crassa* NoxB, and *P. anserine* Nox2. *S. sclerotiorum* Nox1 is 36% identical to *S. sclerotiorum* Nox2. Both *S. sclerotiorum* Nox coding regions contain hallmark features of Nox including a six-transmembrane (TM) region, a putative FAD binding motif, and four NADPH-binding motifs (Fig. 2.1). Since these motifs are highly conserved regions among multicellular organisms, it is expected that the two NADPH oxidases of *S. sclerotiorum* function similarly to other fungal homologs.

Semi-quantitative RT-PCR was used to examine the expression levels of *S. sclerotiorum nox* genes during disease development in tomato leaves and during sclerotial development *in vitro*. *nox1* and *nox2* showed distinct patterns of expression during both processes. *nox2* was constitutively expressed not only during growth and sclerotia formation but also during plant infection (Fig. 2.2). However, transcript levels of *nox1* were low during hyphal growth, and dramatically increased during the induction of sclerotial initials and developing sclerotia (Fig. 2.2). Furthermore, transcript levels of

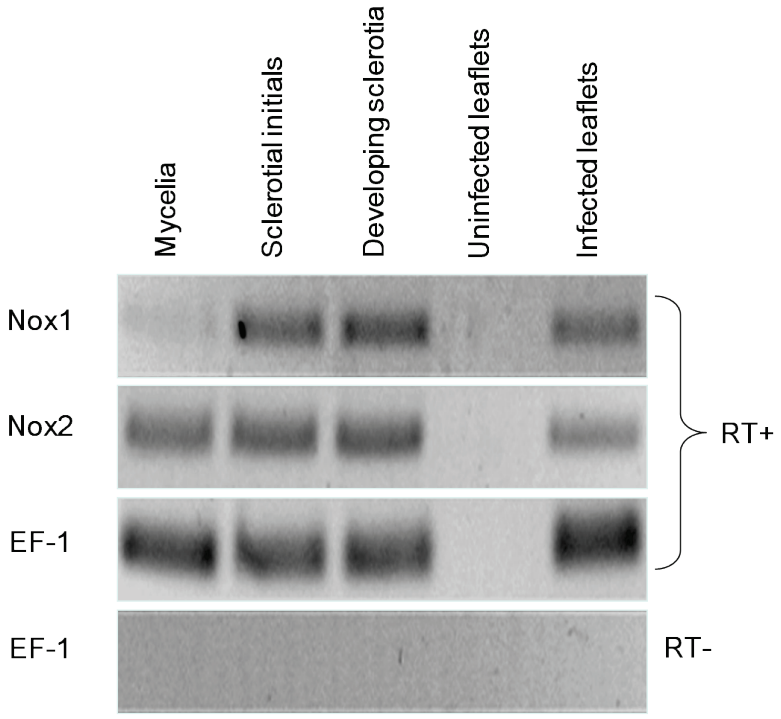


Fig. 2.2. Expression analysis of *nox* genes of *S. sclerotiorum* during development and plant infection. RNA was extracted from fungal tissues in the indicated stages and also from tobacco leaves, 4 days after inoculation with an agar plug (5 mm in diameter) embedded with *S. sclerotiorum* (infected) or uncolonized PDA (uninfected). RT-PCR was performed with gene-specific primers for *S. sclerotiorum* nox1, nox2, or elongation factor 1α (EF-1) as described in Materials and Methods.

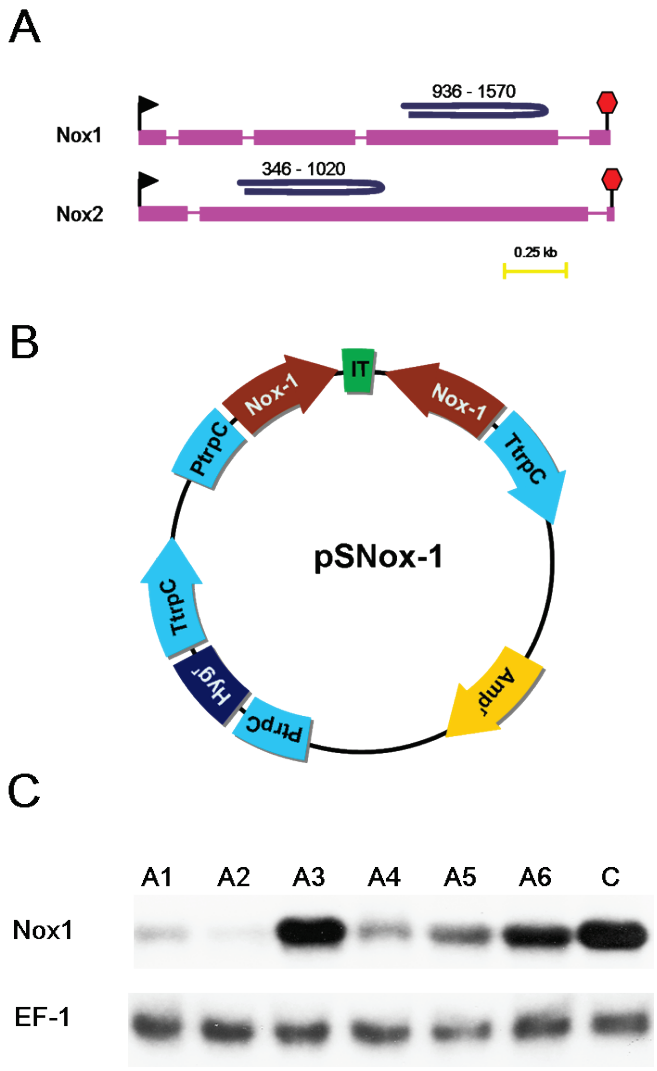


Fig. 2.3. Silencing of *S. sclerotiorum nox1*. A, Schematic maps of Nox1 and Nox2. Pink boxes represent exons. Dark blue hairpin structures indicate the target of silencing for Nox genes. **B,** Nox1 RNAi vector. Targeted sense and anti-sense of Nox1 as shown in panel A were inserted into pSilent-1 vector (Nakayashiki et al. 2005). Two sequences were separated by intron 2 of the cutinase gene from *Magnaporthe oryzae* (IT). Amp^r: ampicillin-resistant gene, Hyg^r: hygromycin-resistant gene, PtrpC: *A. nidulans* trpC promoter, and TtrpC: *A. nidulans* trpC terminator. **C**, Northern blot of Nox1 mRNA expression was analyzed in the *Nox1*-silenced transformants. Elongation factor-1 (EF-1) served as an internal control. A1, A2, A4, and A5 (silenced transformants) show reduced RNA expression compared to wild type control and non-silenced transformants (A3 and A6).

nox1 also increased when strains were inoculated to the plant (Fig. 2.2). These data show that *nox1* and *nox2* expression patterns are not redundant; therefore, *nox1* and *nox2* have distinct but overlapping functions. In addition, Nox1 may play a more important role than Nox2 in sclerotial development and disease progression, although the *Nox2*-silenced mutant showed that Nox2 has a role in sclerotial development as shown later.

Sclerotinia nox-silenced mutants

RNA-mediated gene silencing (RNAi) has proven to be an efficient method to inhibit expression of genes in many eukaryotes including fungi (Nakayashiki et al. 2005). To genetically investigate the role of Nox1 and Nox2 in development and pathogenesis, the RNAi silencing vector, pSilent-1 (Nakayashiki et al. 2005), was used to express the hairpin structures of Nox1 and Nox2. Dr. Kyou-Su Kim and I constructed two silencing vectors (pSNOX1 and pSNOX2, Fig. 2.3B and Fig. 2.4A) carrying the hygromycin B resistance gene as the selectable marker. As a control, an empty vector (pSilent-1) was employed. These constructs were introduced into wild type *S. sclerotiorum* via protoplast transformation (Rollins 2003). RNA blots (Fig. 2.3C) or semi-quantitative RT-PCR analyses (Fig. 2.4B) confirmed silencing. Four *Nox1*-silenced mutants and three *Nox2*-silenced mutants were selected (Fig. 2.3C and Fig. 2.4B).

To examine the role of *nox* genes in hyphal growth of *S. sclerotiorum*, *Nox1*- and *Nox2*-silenced mutants, the empty vector stains, and wild type strains were grown in potato dextrose agar (PDA). Hyphal growth of the mutants was not significantly

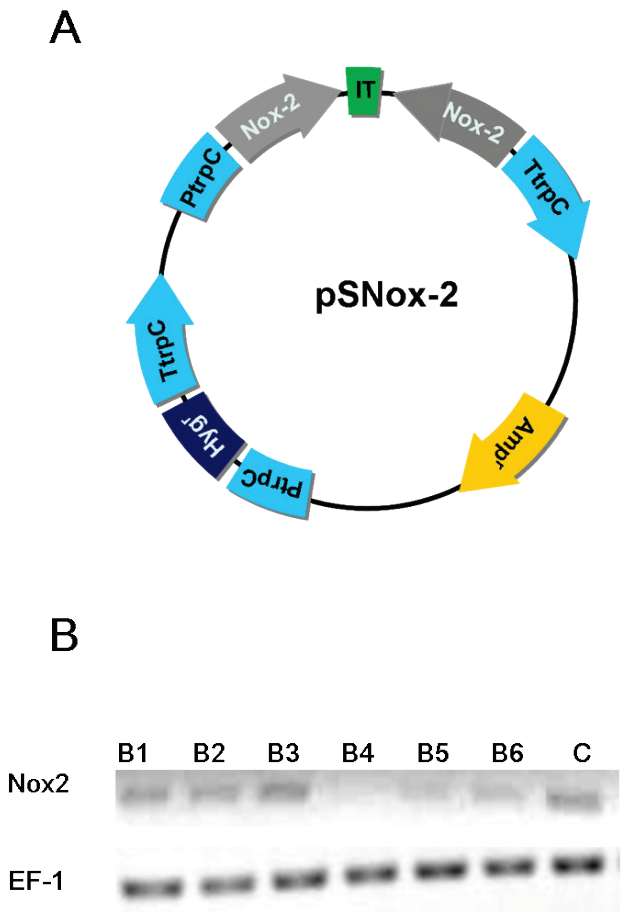


Fig. 2.4. Silencing of *S. sclerotiorum nox2*. A, Nox2 RNAi vector. For the pSNox-1 construct, sense and anti-sense as shown in panel A were inserted into pSilent-1 vector as Nox1 RNAi vector. **C,** RT-PCR analysis of Nox1 mRNA expression was examined for the *Nox2*-silenced transformants. Elongation factor-1 (EF-1) served as an internal control. B1~3 are silenced transformants and B4~6 are non-silenced transformants. C represents wild type control.

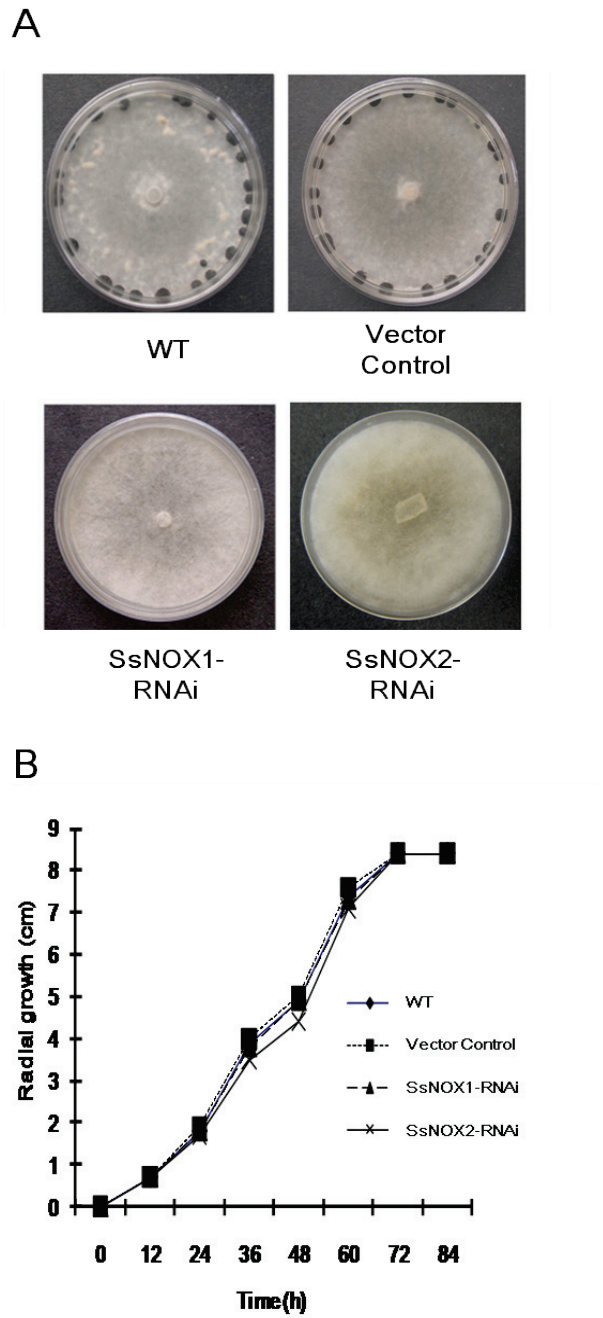


Fig. 2.5. Phenotypes of *nox* knock-down mutants of *S. sclerotiorum*. A, Sclerotial development of *Nox*-silenced mutants (SsNOX1-RNAi and SsNOX2-RNAi) on PDA. Complete defect in sclerotial development was observed in SsNOX1-RNAi and SsNOX2-RNAi mutants while WT and a transformant (Control) containing empty vector only developed sclerotia. Photographs were taken 3-weeks post-inoculation. **B,** Comparison of hyphal growth rate on PDA. Agar plugs (5 mm in diameter) obtained from growing hyphal tips of each tested strains were inoculated on the center of Petri dish plates. Colony diameters were measured at 12 h intervals. Data were obtained from three independent assays; error bars are omitted for clarity.

different in all strains (Fig. 2.5B). Careful examination revealed that hyphae of the SsNOX2-RNAi mutant were more densely packed than those of wild type controls, whereas the SsNOX1-RNAi mutant was identical in vegetative growth morphology to the wild type. Interestingly, the phenotype of the SsNOX2-RNAi mutant was similar to what was reported in an *E. festucae noxA* deletion mutant, where an increase of fungal biomass *in planta* was observed (Tanaka et al. 2006). In addition, inactivation of the small G protein Rac, a key component of the NADPH oxidase complex, consistently resulted in increased hyphal branching of *E. festucae* (Tanaka et al. 2008). This suggests that NADPH oxidase may be involved in regulation of hyphal branching. In accordance with these studies, I observed that the hyphae of SsNOX2-RNAi mutants had more frequent branching than wild type and *Nox1*-silenced mutants (Fig. 2.6). Thus, *S. sclerotiorum* Nox2 may be involved in hyphal hyperbranching.

To determine whether sclerotial development was affected in mutant stains, I monitored sclerotia formation of wild type and *nox* RNAi mutant strains on PDA. As with the pharmacological study, SsNOX1-RNAi mutants were unable to produce sclerotia, and few to no sclerotia were developed in SsNOX2-RNAi mutants (Fig. 2.5A). The more prominent effect on SsNOX1-RNAi mutants may be due to the importance of Nox1 in sclerotial development, corresponding to results from RT-PCR which showed significant induction of Nox1 during sclerotial development. In contrast, wild type *S. sclerotiorum* and strains carrying the empty vector control showed normal sclerotial development. The defect in sclerotial development of *Nox*-silenced strains was not due

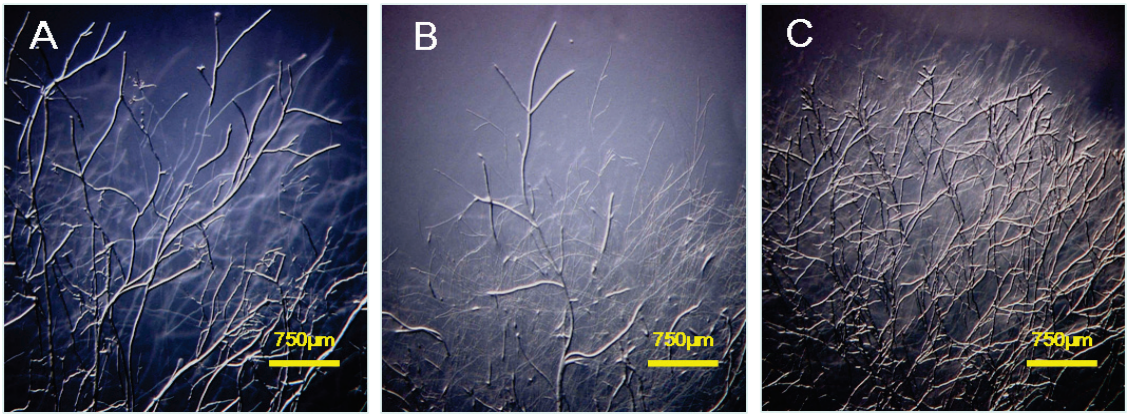


Fig. 2.6. *In vitro* hyphal development of SsNOX1-RNAi and SsNOX2-RNAi mutants. Two *Nox*-silenced mutants and wild type strains cultured on PDA for 2 days. Vegetatively growing hyphae were observed under dissecting microscope. Panel A: wild type, Panel B: SsNOX1-RNAi, Panel C: SsNOX2-RNAi. Bar = 750 ㎛.

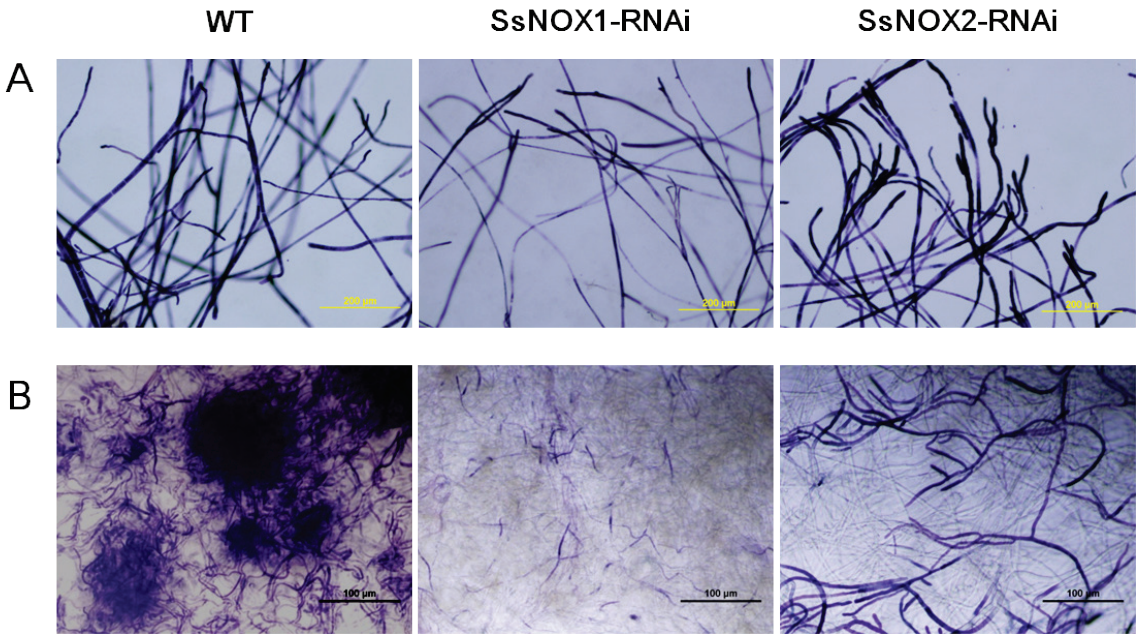


Fig. 2.7. Superoxide accumulation in *Nox*-silenced mutants. Strains were inoculated on PDA. Superoxide accumulation was observed at **A,** 2 days post-inoculation (hyphal growth) and **B,** 4 days post-inoculation (sclerotial initial). For detection, NBT staining was used and monitored under the light microscope. Blue staining represents accumulation of superoxide. For panel A, growing hyphal tips of wild type (WT) and mutants (SsNOX1-RNAi and SsNOX2-RNAi mutants) denoted on the top were collected before staining. For panel B, the edge of the medium was stained using NBT. Notably, wild type in the panel B shows the extensive precipitation of blue formazan that was changed from white sclerotial initials. However, SsNOX1-RNAi and SsNOX2-RNAi mutants show lesser accumulations of blue formazan. (A) Bar = 200 ㎛, (B) Bar = 100 ㎛.

to aberrant physiological growth defects since the radial growth of all strains were essentially identical (Fig. 2.5B). These data, coupled with the result that DPI treatment inhibited sclerotia formation, support the idea that NADPH oxidases are essential for sclerotial development.

Production of superoxide in SsNOX1-RNAi mutants is impaired

NADPH oxidases generate superoxide. Therefore, I reasoned that accumulation of superoxide might be reduced in *Nox*-silenced mutants. To determine whether inactivation of Nox1 and Nox2 leads to altered ROS generation, I examined ROS production during hyphal growth and sclerotia formation in both mutants. Nitroblue tetrazolium (NBT) staining was used to detect superoxide (Egan et al. 2007). Precipitation of blue formazan results following accumulation of superoxide. During hyphal growth, no significant differences in superoxide production were observed between *Nox2*-silenced mutants and the wild type, whereas the *Nox1*-silenced mutant had a slightly reduced accumulation of superoxide, suggesting the presence of residual ROS after inactivation of NADPH oxidases (Fig. 2.7A). This result suggests that superoxide generated by NADPH oxidases may have a minor role during hyphal growth, and an alternative source of ROS by other oxidases or non-enzymatic manner such as the "leakage" of the respiratory chain reaction in mitochondria may play a major role during this stage (Djordjević 2004; Egan et al. 2007; Tanaka et al. 2006). However, ROS levels

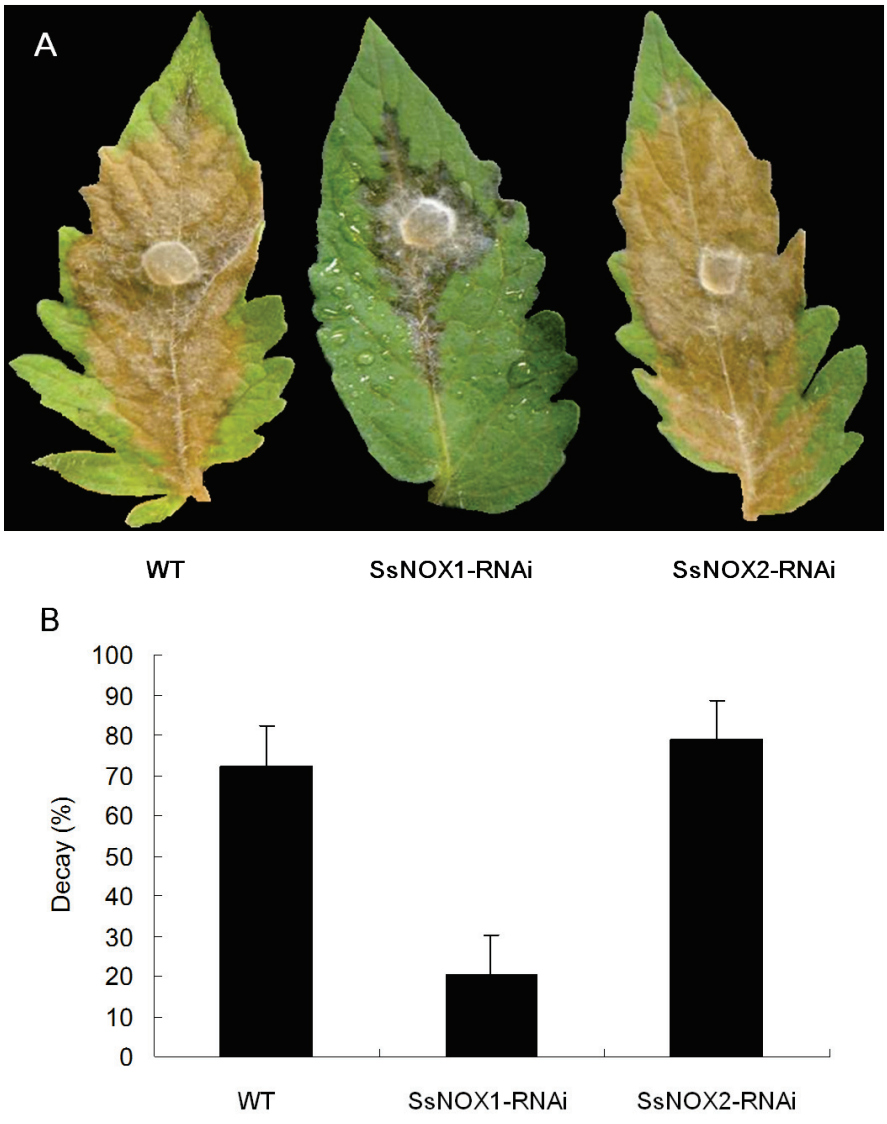


Fig. 2.8. Pathogenicity of SsNOX1-RNAi and SsNOX2-RNAi mutants. A, Lesion development on tomato leaves. The leaves were inoculated with an agar plug (5 mm in diameter) of each strain. Inoculation with wild type fungus was served as controls. Photographs were taken at 3 days after inoculation. **B,** Relative lesion area on tomato leaves. Lesion areas in the panel A were quantitatively analyzed with ImageJ 1.38x software.

of SsNOX1-RNAi mutants could be affected during sclerotial development since transcript levels of *nox1* were induced during this stage of growth. Thus, superoxide levels generated during sclerotial development of both wild type and mutant strains were monitored. Wild type and two *Nox*-silenced mutant strains were cultured on PDA, and samples were stained with NBT at 4 days after inoculation, when sclerotia formation initiates. Dark-blue formazan precipitates were observed after staining the wild type fungus with NBT during sclerotial initiation (Fig. 2.7B). The SsNOX1-RNAi mutant showed significantly reduced staining compared to wild type indicating that SsNOX1-RNAi mutants were impaired in superoxide production after 4 days of growth. Generation of superoxide in SsNOX2-RNAi mutants was also reduced, but not to the extent observed in SsNOX1-RNAi mutants (Fig. 2.7B). This suggests that Nox1 produces more ROS during sclerotial development than Nox2.

SsNOX1-RNAi mutants compromise virulence on *S. lycopersicum* and reduced oxalate production

I was interested in determining the phenotype of these mutant strains with respect to plant disease. NADPH oxidase mutants in *Magnaporthe grisea* and *Botrytis cinerea* were less pathogenic than the corresponding wild type fungal strains (Egan et al. 2006; Segmüller et al. 2008). The virulence of the SsNOX1-RNAi mutant in tomato plants was found to be significantly reduced. However, the SsNOX2-RNAi mutant was as virulent as the wild type (Fig. 2.8A and B). This correlated with the results from the RT-PCR analysis of *nox* genes showing that *nox1* expression was induced during infection, and

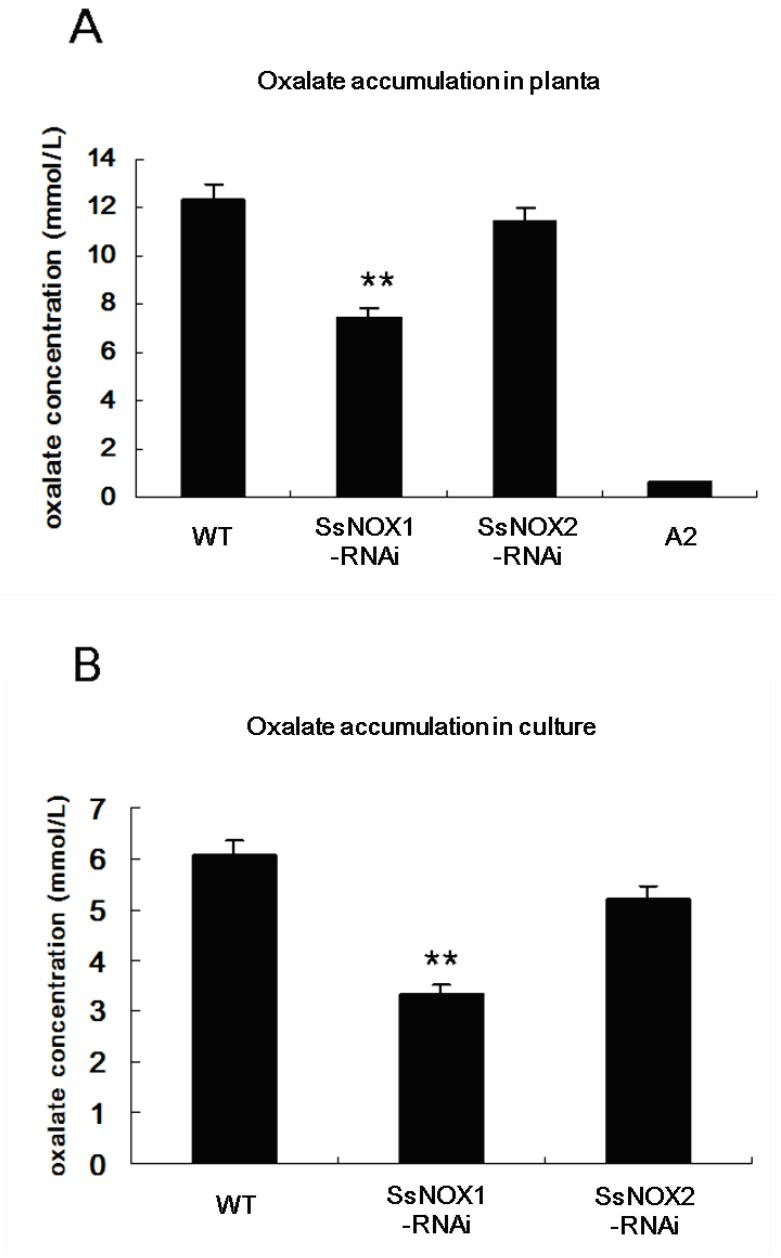


Fig. 2.9. Oxalate accumulation in SsNOX1-RNAi and SsNOX2-RNAi mutants. **A,** Oxalic acid accumulation *in planta*. Leaves were inoculated with a PDA plug (5 mm in diameter) of each strain. These agar plugs were obtained 2 days after inoculation and was utilized for analysis of oxalate accumulation. Wild type fungus was served as positive control and A2, an oxalate deficient mutant, was used for negative control. **B,** Oxalic acid accumulation in potato dextrose broth (PDB). Each strain was cultured for 3 days and the broth was analyzed for oxalate accumulation.

nox2 was constitutively expressed (Fig. 2.2). Therefore, *nox1* but not *nox2* in *S. sclerotiorum* appears to play a more prominent role during the infection process.

Recent studies in *Aspergillus parasiticus* and *Aspergillus flavus* showed that the accumulation of ROS was linked to the production of the secondary metabolite, aflatoxin (Reverberi et al. 2008; Kim et al. 2008a). Based on this result, I asked whether reduced production of ROS in the SsNOX1-RNAi mutant affects production of oxalate, a secondary metabolite and phytotoxin. I measured exogenous oxalate accumulation in both mutant and wild type strains after culturing fungi in potato dextrose broth (PDB) for 3 days. The SsNOX1-RNAi mutant produced about half as much oxalate as wild type *S. sclerotiorum* (Fig. 2.9B). However, no significant reduction of oxalate production was detected in the SsNOX2-RNAi mutant, which correlated with previous data (Fig. 2.9A). Next, I examined oxalate production in fungi when grown on the plant. At 2dpi, PDA plugs were collected and analyzed for oxalate accumulation. Consistent with data from *in vitro* culturing in PDB, production of oxalate was significantly reduced in the SsNOX1-RNAi mutant compared to production of oxalate in wild type during infection, whereas the SsNOX2-RNAi mutant showed no difference in production of oxalate (Fig. 2.9A). More importantly, when supplemented with potassium oxalate (K-OX, pH 7.0) *Nox1*-silenced mutants increased in virulence while there was no effect on the virulence of wild type strains (Fig. 2. 10), suggesting that this correlation might have functional consequences. In accordance, K-OX, which elicits apoptotic-like cell death (≈ pH 6), can also restore virulence of oxalate deficient A2 mutants (Godoy et al. 1990; Williams et al.

1. WT + water
2. WT + K-OX (pH 7.0)
3. SsNOX1-RNAi mutant + water
4. SsNOX1-RNAi mutant + K-OX (pH 7.0)

Fig. 2.10. OA partially restores virulence of the *Nox1*-silenced mutant.
For inoculation, each strain was cultured for 3 days on PDA. *Nicotiana benthamiana* leaves were infiltrated with designated solutions and inoculated with an agar plug (5 mm in diameter) of each strain. Pictures were taken 1.5 days post-inoculation.

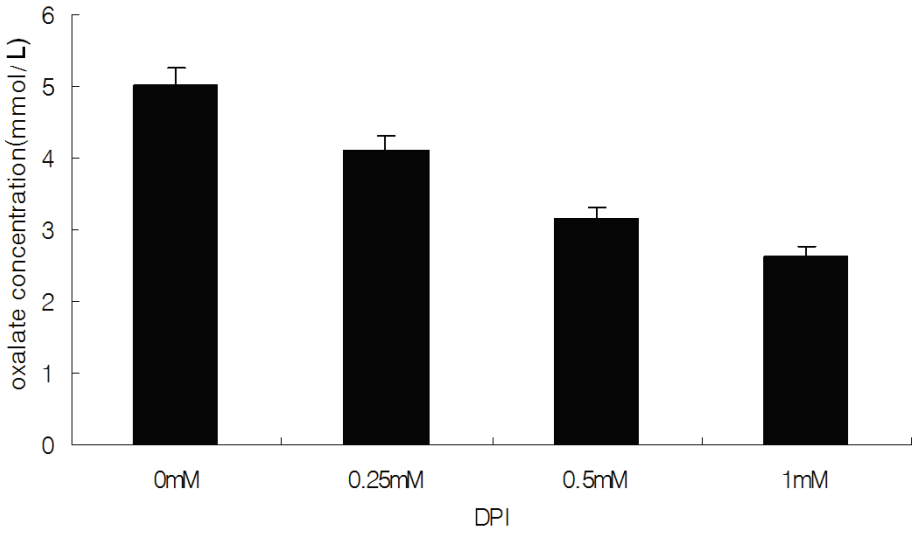


Fig. 2.11. Oxalate accumulation and ROS inhibition following DPI treatment. *S. sclerotiorum* wild type fungi were cultured in PDB treated with 0mM - 1mM DPI and analyzed for oxalate accumulation.

submitted). This suggests that decreased levels of oxalate production may impact the reduced virulence of Nox1 silenced mutants. Since DPI blocks sclerotia formation (Chen et al. unpublished data), I examined the production of oxalate by growing fungi in PDB treated with DPI (0~1mM) (Fig. 2.11). Results showed that DPI treatment correlated with the production of oxalate in a dose-dependent manner, when levels of ROS decreased levels of oxalate also decreased. This suggests that NADPH oxidase may be linked to oxalate production; however, it is also plausible that reduced oxalate production in the SsNOX1-RNAi mutant and DPI-treated wild type *S. sclerotiorum* is due to unknown indirect effects of Nox1.

Impaired oxalate production in Nox1 may affect ROS production in plant tissue

The oxidative burst is among the earliest and most universal defense responses in plants (Apel and Hirt 2004; Bolwell 1999; Bolwell et al. 1999). Since oxalate can suppress the oxidative burst (Cessna et al. 2000), reduction of oxalate production in Nox1 could lead to a decrease in oxalate-induced inhibition of the oxidative burst. I performed NBT staining with the leaves of tomato plants (2 dpi) infected with the various fungal genotypes. Tomato leaves infected with the SsNOX1-RNAi mutant showed more staining than those with wild type and the SsNOX2-RNAi mutant strains (Fig. 2.12). This observation shows that reduced levels of ROS in *Nox1*-silenced mutants correlated with reduced virulence and increased resistance of host plants. The SsNOX2-RNAi-infected leaf also showed slightly increased NBT staining, though the SsNOX2-

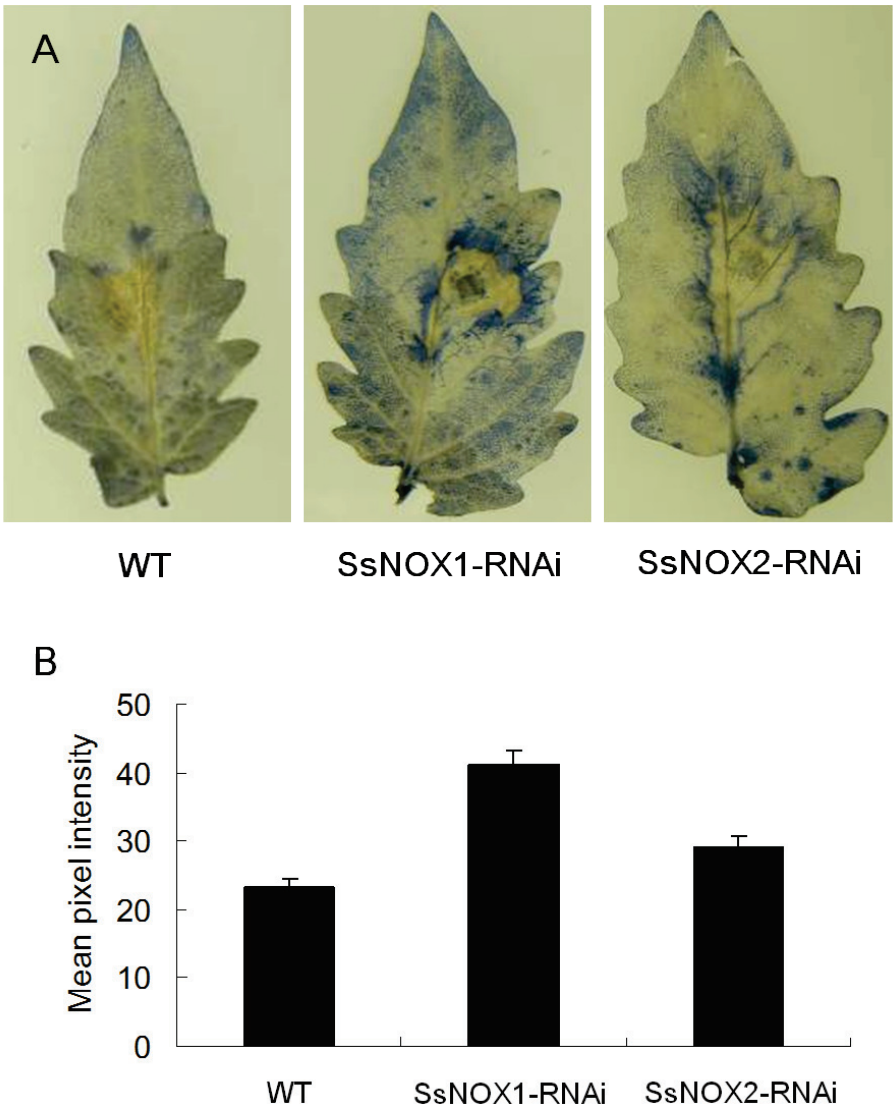


Fig. 2.12. Superoxide production in leaves infected with *Nox*-silenced mutants. A, Leaves were inoculated with a PDA plug (5 mm in diameter) of each strain. Inoculation with wild type fungus served as a control. Localized superoxide was observed after NBT staining was carried out at 2 days post-inoculation. **B,** ImageJ 1.38x software was used to evaluate relative pixel intensity reflecting accumulation of superoxide. Pictures were inverted and measured mean pixel intensity (n=5).

RNAi mutant was not affected in virulence. This suggests that Nox2 may have a minor role in virulence which impacts host oxidative burst. Taken together, these observations show that both NADPH oxidases of *S. sclerotiorum* affect the host oxidative burst, while Nox1 has a more significant impact on the plant oxidative burst.

DISCUSSION

Originally, studies with ROS focused on the toxic effects of ROS including mutations, lipid peroxidation, and protein oxidation in cells (Lambeth 2004). Emerging evidence has now clearly established that ROS also has beneficial functions. In fungi, there is an increasing number of studies documenting the importance of ROS in development and pathogenesis (Cano-Dominguez et al. 2008; Egan et al. 2007; Giesbert et al. 2008; Lara-Oritz et al. 2003; Malagnac et al. 2004; Segmüller et al. 2008; Takemoto et al. 2006; Tanaka et al. 2006). Here, I have identified and functionally characterized the two NADPH oxidases of *S. sclerotiorum*.

Role of NADPH oxidase in development of *Sclerotinia*

Increase of ROS was observed in fungal developmental structures including cleistothecia, appressoria and hyphal tips (Egan et al. 2007; Lara-Oritz et al. 2003; Malagnac et al. 2004; Takemoto et al. 2006). In accordance, generation of ROS was observed in sclerotial initials and infection cushions in *S. sclerotiorum* (Chen et al. unpublished data). Furthermore, protoplasts generated from sclerotial initials were shown to oxidize non-fluorescent DCFH (2′,7′-dichlorofluorescin) to fluorescent DCF

(2′,7′-dichlorofluorescein), indicating ROS production (mainly H_2O_2 but also other ROS such as hydroxyl radicals and nitric oxide) (Chen et al., unpublished data; Eagan et al, 2007). These observations suggest involvement of ROS in development of *S. sclerotiorum*. As an explanation for the role of ROS in fungal development, it was proposed that a burst of ROS may influence mobilization of nutrients from cells to cells (Lalucque and Silar 2003; Scott and Eaton 2008). In relation to this, the ROS scavenger NAC inhibited DCF fluorescence from sclerotial initials. Exogenous treatment with NAC or DPI also strongly inhibited sclerotia formation. Georgiou and colleagues (2006) have also shown several hydroxyl radical scavengers and antioxidants inhibit sclerotial development of several fungi including *S. sclerotiorum*. Antioxidants including N,N'diphenyl-1,4-phenylene diamide, 1,3-dimethyl-2-thiourea, ammonium pyrrolinedimethyl-dithiocarbamate, and NAC blocked chemiluminescence enhanced by lucigenin (indicating the formation of ROS) during the differentiation process and conidiation of *N. crassa*, similar to observations in this study (Hansberg et al. 1993). Exposure to the antioxidant ascorbic acid and the catalase mimic Mn(III) tetrakis[1-methy-4-pyridyl]porphyrin (MnTMPyP) also inhibited germination and appressorium formation of *M. grisea* (Eagan et al. 2007). These studies indicate ROS plays a key signaling role in fungal development.

I identified two homologs of the human $gp91^{phox}$ in *S. sclerotiorum*. Most of fungal genomes sequenced thus far also have two Nox subfamilies (NoxA/Nox1 and NoxB/Nox2), while several fungi including *Fusarium* spp., *M. grisea*, *P. anserina*, *Aspergillus terreus* and *Phaeosphaeria nodorum* have a third Nox subfamily member

(NoxC/Nox3). NoxA/Nox1 and NoxB/Nox2 are homologous to human gp91^{phox}, whereas NoxC/Nox3 has a distinct putative calcium binding EF-hand motif observed in the NH_2-terminal ends of animal Nox5 and plant Rboh proteins (Scott and Eaton 2008). In this study, *S. sclerotiorum* Nox1 and Nox2 were cloned. Both *S. sclerotiorum* Nox coding regions contain a six-transmembrane (TM) region, a putative FAD binding motif, and four NADPH-binding motifs similar to plant and animal NADPH oxidases. However, neither NADPH oxidase has a putative calcium binding EF-hand motif in the NH_2-terminal ends, suggesting that their features are similar to human gp91^{phox} (Nox2) rather than animal Nox5 or plant Rboh.

To functionally examine the two *S. sclerotiorum nox* genes, Dr. Kyung-Su Kim and I obtained *Nox1*- and *Nox2*-silenced mutants using RNAi. Silencing of Nox1 resulted in strains unable to form sclerotia. In other fungi, NoxA/Nox1 is involved in development of sexual structures. In *A. nidulans*, knocking out NoxA inhibits differentiation of cleistothecia, although hyphal growth and asexual development was normal (Lara-Oritz et al. 2003). Studies with *N. crassa* and *Podospora anserina* also showed a block in sexual development when the Nox1 homologs of NoxA were deleted. In contrast to the NoxA of *A. nidulans*, Nox1 of *N. crassa* and *P. anserina* is involved in asexual development and hyphal growth (Cano-Dominguez et al. 2008; Malagnac et al. 2004). For example, *N. crassa* Nox1 affects conidiation, vegetative growth and development of aerial hyphae (Cano-Dominguez et al. 2008). Inactivation of *P. anserina* Nox1 leads to a defect in aerial hyphae differentiation and pigment accumulation in mycelium (Malagnac et al. 2004). In *S. sclerotiorum*, sclerotia formation is required for

generation of the apothecium, a fungal sexual fruiting body. Thus, SsNOX1-RNAi mutants are unable to develop apothecia. In contrast to NoxA/Nox1, NoxB/Nox2 is required for ascospore germination in *N. crassa* and *P. anserina* (Cano-Dominguez et al. 2008; Malagnac et al. 2004). Similarly, ascospores of SsNOX2-RNAi mutants cannot be generated in *S. sclerotiorum*. Importantly, this result is analogous to what was reported in *Botrytis cinerea*, where both *nox* genes were involved in sclerotia formation and *Δbcnoxb* mutants produced few small and abnormal sclerotia (Segmüller et al. 2008).Thus, Segmüller and colleagues (2008) also could not monitor apothecia or ascospore formation in *Δbcnox* mutants. These results suggest that the function of *S. sclerotiorum* NADPH oxidases in development is similar to that of other fungal homologs.

In *N. crassa*, *nox1* and *nox2* genes were differentially expressed (Cano-Dominguez et al. 2008). Transcript levels of *N. crassa nox1* were induced during protoperithecium (sexual structure) development. Transcript levels of *N. crassa nox2* were negligible during sexual differentiation, while *N. crassa nox2* mRNA was detected in conidia. Consistently, *Δnox1* were unable to differentiate mature fruiting bodies or perithecia (Cano-Dominguez et al. 2008). In sclerotial initials and developing sclerotia, transcript levels of *S. sclerotiorum nox1* were dramatically increased relative to *nox1* transcript levels in growing hyphae, coinciding with formation of sclerotia in SsNOX1-RNAi mutants. In contrast, transcript levels of the *nox2* gene were constitutive during both processes, suggesting that *S. sclerotiorum nox2* may play a partial role in sclerotial development. Transcript levels of NADPH oxidases also correlated with ROS generation

in *Nox*-silenced mutants. Generation of ROS in the SsNOX1-RNAi and SsNOX2-RNAi mutants was significantly reduced during the time of sclerotial development in wild type. However, levels of ROS in the SsNOX1-RNAi are lower than in the SsNOX2-RNAi mutants. These results suggest that *S. sclerotiorum* Nox1 plays a pivotal role in the generation of ROS in sclerotial initials. Nox2 is also involved in accumulation of ROS during sclerotial development but its role appears to be less effective than Nox1.

To summarize, it was shown that 1) *nox1* is induced during sclerotial development, 2) DPI and NAC blocks sclerotial development, 3) sclerotia formation is completely inhibited in the SsNOX1-RNAi mutant. Sclerotia are also strongly inhibited and delayed in the SsNOX2-RNAi mutant but not to the extent as in the SsNOX1-RNAi mutant, and 4) there is a drastic reduction in ROS generation in the SsNOX1-RNAi mutants. Based on these results, I conclude that Nox1 and Nox2 play key roles in sclerotial development, but Nox1 appears to have a more important function than Nox2.

NADPH oxidase activity is associated with virulence

Recent studies involving *Epichloë festucae* NADPH oxidases provided interesting and perhaps unexpected evidence for involvement of ROS in a plant-fungal mutualistic relationship (Tanaka et al. 2006; Takemoto et al. 2006). A restriction enzyme-mediated integration (REMI) screen revealed that one of the two fungal *nox* genes (NoxA) in *E. festucae* is responsible for converting the normal mutualism phenotype to one of pathogenesis (Tanaka et al. 2006). Wild type *E. festucae* maintained a symbiotic association with the perennial ryegrass, whereas inactivation of *E. festucae*

NoxA resulted in increased fungal biomass and disease symptoms, thereby causing disruption of symbiosis and provoking precocious senescence (Tanaka et al. 2006). Importantly, ROS accumulation was detected at the interface between the extracellular matrix and host cell walls of meristematic tissue in ryegrass-*E. festucae* wild type interaction (Tanaka et al. 2006). However, impairment of NoxA led to a significant decrease in ROS accumulation at the interface between ryegrass and the *noxA* mutant (Tanaka et al. 2006). The pathogenesis-related (PR) genes PR1 and PR5 were also more highly expressed in the *noxA* mutant than the wild type (Tanaka et al. 2006). These observations suggest that wild type *E. festucae* may utilize ROS (from NADPH oxidase) as a secondary messenger to induce signaling pathways in the host plant (Tanaka et al. 2006). This ROS signal from wild type *E. festucae* may moderate the plant defense responses to maintain the mutualistic interaction. This is in agreement with observations in the animal NADPH oxidases (Nox1, Nox3, and Nox4) where relatively low levels of superoxide activate pathways to control cell proliferation (Suh et al. 1999; Lambeth et al. 2000). Therefore, the low concentrations of ROS generated from NADPH oxidases may play a role in intercellular signaling. In contrast to *E. festucae*, Talbot and colleagues demonstrated that the *Magnaporthe grisea nox1* and *nox2* genes play a pivotal role in infection-related appressorium differentiation, a specialized infection structure that builds up mechanical and enzymatic force to rupture the cuticle and gain access to the host plant (Egan et al. 2007). They hypothesized that *Nox*-generated ROS within appressorium may facilitate oxidative cross-linking of proteins, thereby strengthening the appressorium cell wall to withstand enormous turgor pressure generated by the

uptake of water following the accumulation of glycerol (Thines et al. 2000). Consequently, deletion of *nox* genes appeared to result in weakened cell walls of the appressorium that was unable to cause infection (Egan et al. 2007). Consistent with the finding in studies with *M. grisea*, Nox2 is also required in the appressorium-like structure to penetrate the plant cuticle in *B. cinerea* (Segmüller et al. 2008). In this study, I showed that NADPH oxidase (Nox1) silencing compromised virulence in *S. sclerotiorum*, while *Nox2*-silenced mutants showed no defect in virulence. This suggests that Nox1 plays a crucial role in signaling between plants and fungi. This scenario is analogous to that shown in the studies with *E. festucae*, where only NoxA (homolog of *S. sclerotiorum* Nox1) but not NoxB (homolog of *S. sclerotiorum* Nox2) appears to have an important role in signaling between plants and fungi (Tanaka et al. 2006). Despite this functional similarity, impairment of *nox1* genes in *S. sclerotiorum* and *E. festucae* leads to contrasting phenotypes. Silencing of *nox1* from *S. sclerotiorum* reduced virulence whereas *E. festucae noxA* knock-out mutants showed increased virulence. This implies that ROS produced by *S. sclerotiorum* Nox1 and *E. festucae* NoxA may activate different molecular targets. In mammalian systems, sub-cellular localization of ROS activates specific redox signaling pathways (Ushio-Fukai 2006; Ushio-Fukai and Urao 2009). In addition to this, several studies have also shown cooperation between ROS and other signaling molecules such as cytosolic Ca^{2+} (Zhu et al. 2008), tumor necrosis factor α (TNFα) (Nadif et al. 2003), and cytokines (Xie et al. 2004), among others. In *Arabidopsis*, different combinations between ROS and other signaling molecules (phytohormones) appear to result in different degrees of cell death (Overmyer et al.

2005). Therefore, different combinations with different signaling molecules and/or specific localization of NADPH oxidase homolog may lead to contrasting phenotypes in the NADPH oxidase mutants in different organisms.

NADPH oxidase is linked to oxalate production

A key pathogenicity factor in *S. sclerotiorum* is oxalic acid (OA) (Cessna et al. 2000; Godoy et al. 1990). Here, I provide evidence that Nox1 is linked with oxalate production. First, levels of oxalate in the SsNOX1-RNAi mutant were significantly lower both in culture and importantly in the plant, compared to wild type and the SsNOX2-RNAi mutant strains. Second, DPI, an inhibitor of NADPH oxidase, reduced production of oxalate. In *A. parasiticus* and *A. flavus*, generation of ROS correlates with production of the secondary metabolite, aflatoxin (Reverberi et al. 2008; Scott and Eaton 2008). Aflatoxin and ROS production in an ApyapA deletion mutant of *Aspergillus parasiticus* were concurrently increased compared to wild type, suggesting a link between ROS generation and secondary metabolite production (Reverberi et al. 2008). Moreover, exogenous application of the antioxidant caffeic acid significantly reduced production of aflatoxin (Kim et al. 2008a). Interestingly, these observations are consistent with a previous study showing that caffeic acid blocked sclerotia formation by up-regulating cyclic AMP levels (Rollins and Dickman 1998). In accordance, a superoxide dismutase (SOD) mutant of *S. sclerotiorum* also had reduced levels of oxalate production (Selvakumar et al. unpublished data). This is not surprising since SOD and NADPH oxidase activities are coupled (Scott and Eaton 2008). Superoxide

generated from NADPH oxidases is converted to hydrogen peroxide via SOD. Findings in this study suggest that the reduction of ROS generation is possibly associated with the decrease of oxalate accumulation in *S. sclerotiorum*.

Recently, I noted two bicupin proteins in the genome sequence of *S. sclerotiorum* (SS1G_10796.1: 47% identical to csOxo1 and SS1G_08814.1: 46% identical to csOxo2). It has been suggested that cupins function as oxalate oxidases in *Ceriporiopsis subvermispora* (Escutia et al. 2005). Oxalate oxidase (also known as germin in plants) catalyzes the generation of ROS from oxalate in *C. subvermispora*. Although it is yet to be shown whether *S. sclerotiorum* bicupin proteins have oxalate oxidase activity, they could possibly be associated with ROS generation. A previous study has shown that oxalate induced hydrogen peroxide in plant tissue is involved in programmed cell death (Kim et al. 2008b). This observation suggests that fungus-produced OA may impact ROS generation in the plants, though this study conversely shows that an increase in ROS generation can also lead to an increase in OA production. While mechanistic details are elusive, this and previous studies show a consistent correlation between ROS generation and production of OA. Therefore, an imbalance in redox environment through the impairment of *S. sclerotiorum* Nox1 may negatively regulate the production of OA.

There are several potential routes for oxalate biosynthesis. In *Sclerotium rolfsii*, the oxidation of glyoxylate by glyoxylate dehydrogenase was suggested as a major pathway for oxalate biosynthesis (Balmforth and Thomson 1984). In addition, exogenous application of $^{14}CO_2$ in *A. niger* revealed the biosynthesis of oxalate by

hydrolysis of oxaloacetate (Kubicek et al. 1988). Hammel and colleagues (1994) also showed oxalate production via oxidation of glycolaldehyde by *Phanerochaete chrysosporium* glyoxal oxidase. Several cofactors such as NAD^+ are necessary to maintain these pathways. For example, the TCA cycle, considered one of the main pools for production of secondary metabolites including oxalate and glyoxylate, has three rate-limiting enzymes dependent on NAD^+ (McCormack and Denton 1987), and glyoxylate dehydrogenase also uses one NAD^+ to produce one oxalate molecule. Therefore, recycling of cofactors for a steady-state supply of NAD^+ and consumption of NADH could be required for production of oxalate. Transhydrogenase which converts ($NADP^+$ + NADH) to (NADPH + NAD^+) can contribute to this recycling. For example, $NADP^+$ from NADPH oxidase and NADH from the TCA cycle and oxidation of glyoxylate can be transformed into NADPH and NAD^+ respectively by transhydrogenase, thereby providing cofactors for ROS generation and oxalate production. Hence, conversion of NADPH to NADP by NADPH oxidase may impact production of secondary metabolites as noted by Lalucque and Silar (2003), and transhydrogenase may contribute to this process. In line with this, *S. sclerotiorum* has a putative transhydrogenase (SS1G_09058.1) which has the characteristic NAD(P) transhydrogenase beta subunit. Thus, this transhydrogenase may play a role in the generation of NADPH and NAD^+ from $NADP^+$ and NADH respectively. Interestingly, transhydrogenase homologs have not been observed in available genomes of the hemiascomycetes yeasts and other unicellular fungi (Tarrío et al. 2006), where *nox* genes are also absent (Takemoto et al. 2007). Moreover, most of the filamentous fungi for which NADPH oxidases have been

reported also contain transhydrogenase homologs, suggesting a functional relationship between transhydrogenases and NADPH oxidases in these fungi. Therefore, future work regarding transhydrogenase in *S. sclerotiorum* may be informative.

Inactivation of Nox1 and exogenous addition of the NADPH oxidase inhibitor DPI both resulted in reduction of oxalate levels, correlating with reduced virulence of Nox1. Moreover, oxalate treatment restored virulence of *Nox1*-silenced mutants to similar levels as wild type, suggesting a functional link between NADPH oxidase and the production of oxalate. The decrease in oxalate production in the *Nox1*-silenced mutant also compromised suppression of the host oxidative burst, leading to strong localized ROS production in plant tissues when inoculated with the SsNOX1-RNAi mutant. Therefore, addition of oxalate should restore suppression of the host oxidative burst in *Nox1*-silenced mutants. Mechanistic studies regarding the effects of Nox1 on oxalate production will give valuable insight into how fungal NADPH oxidases contribute to plant-fungus interactions.

MATERIALS AND METHODS

Fungal strains, growth conditions and media

S. sclerotiorum wild type (1980) and *Nox*-silenced mutants were incubated on potato dextrose agar (PDA) or broth (PDB) at 25 °C. For infection assays, fungi were infected on leaves detached from *Solanum lycopersicum* (tomato) cultivar Rutgers and

Motelle were grown in the green house for 4-6 weeks at 18°C-25 °C as described (Chen et al. 2004; Sagi et al. 2004).

Pharmacological studies

Wild type *S. sclerotiorum* was incubated on the PDA for 2-3 days; agar plugs with fungi were transferred and incubated to PDA containing 40mM NAC or 1mM DPI.

RNAi constructs and fungal transformation

The fungal RNAi vector pSilent-1 was obtained from Dr. Oded Yarden (Erental et al. 2007). Sequences of *S. sclerotiorum* Nox1 and Nox2 were obtained by aligning human $gp91^{phox}$ protein with *S. sclerotiorum* genome (http://www.broadinstitute.org/ annotation/genome/sclerotinia_sclerotiorum/MultiHome.html). *nox1* and *nox2* were amplified from *S. sclerotiorum* genomic DNA and cloned into pGEM-T. To silence *nox1*, sense and antisense fragment by following two set of primers (XhoINOX1F: AACTCG-AGCGAAAGCCATCGATGA AG and HindIIINOX1R: AAAAGCTTCGACATCGG-CTCCTACAC) and (KpnINOX1F: AAGGTACCCGAAAGCCATCGATGAG and BglIINOX1R: AAAGATCTCGACATCGGCTCCTACAC) were amplified with genomic DNA from *S. sclerotiorum* genome and introduced into pSilent-1 with a spacer fragment to make hairpin RNA structure. For *nox2*, (XhoINOX2F: AACTCGAGTGCT-AGATCAGCTGCCTTGA and HinIIINOX2R: AAAAGCTTCCTCGGGACAACAAA-AGAAA) and (KpnINOX2F:AAGGTACCTGCTAGATCAGCAGCCTTGA and BglIINOX2R: AAAGATCTCCTCGGGACAACAAAAGAAA) PCR sets were used to

construct a silencing vector (confirmed by DNA sequencing and PCR). Two silencing vectors for *nox1* and *nox2*, and pSilent-1 vector for control were used to transform *S. sclerotiorum*. Generation of protoplast and fungal transformation was performed as described by Rollins (2003).

RNA blots

Total RNA was extracted with TriZOL solution according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, U.S.A.). RNA blots were performed as described (Chen et al. 2004).

Semi-quantitative RT-PCR analyses

For semi-quantitative RT-PCR, total RNA was extracted in TriZOL by following the manufacturer's instructions (Invitrogen, Carlsbad, CA, U.S.A.). Total RNA was then treated with Dnase and subjected to first-strand DNA synthesis using M-MLV Reverse Transcriptase (1 ug total RNA/reaction). Semi-quantitative RT-PCR was performed as described previously (Sagi et al. 2004). A no RT control was performed without reverse transcriptase. SsNOX1F (CGAAAGCCATCGATGAAG) and SsNOX1R (CGACATC-GGCTCCTACAC) primers were used for amplification of *S. sclerotiorum nox1*, and SsNOX2F (TGCTAGATCAGCTGCCTTGA) and SsNOX2R (CCTCGGGACAACAA-AAGAAA) for *S. sclerotiorum nox2*. For internal control, SsEF-1F (TCCTATCTCCG-

GTTTCAACG) and SsEF-1R (GCAAGCAATGTGAGCAGTGT) were used. PCR amplification of each reverse transcription products (1 uL) was carried out using *Taq* DNA polymerase from Invitrogen.

ROS detection assay

In order to detect superoxide in tomato leaves (1-2 days after fungal inoculation), leaves were placed in 0.5 mg/ml NBT (10 mM potassium phosphate buffer, pH 7.5) aqueous solution for 2 hour. After incubation, leaves were rinsed in 70 % ethanol. Samples were mounted in 50 % glycerol.

For detection of superoxide in fungal structures, tissues were incubated in 0.5 mg/ml NBT (10 mM potassium phosphate buffer, pH 7.5) aqueous solution for 2 hour. After incubation, fungal tissues were observed with light microscopy. For confirmation, the colorimetric NBT assay also was performed. After incubation with NBT, fungi were ground with liquid nitrogen. The reduced NBT was solubilized with the same volume of 2M KOH and volume of 1.3 times DMSO for 30 min.

For DCF staining, an agar-mycelium plug of the wild type isolate 1980 was inoculated on PDA plate and incubated until sclerotial initials were observed. Aliquots of protoplasts generated from sclerotial initials were then stained with 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA) to visualize H_20_2.

Oxalic acid assay

S. sclerotiorum strains were grown on the PDA or PDB with the designated treatment. After incubation, 10 mM EDTA (pH 7.6) was added to the agar plug and the mixture was melted at 95 °C. Samples in the PDB were directly analyzed. Oxalic acid was quantified using Oxalate Detection Kit according to the manufacturer's instruction (Trinity Biotech, Wicklow, Ireland).

Microscopy

Microscopic observations were made using an Olympus IX-81 microscope with differential interference contrast optics (10× UPlanFLN objective, 0.30 N.A.). Digital images were captured with an Olympus DP70. All images were collected using Olympus DP controller and manager software using the same conditions (image size: 1360×1024, exposure time: auto, ISO sensitivity: ISO200, exposure mode: auto), and were saved as TIF files.

CHAPTER III

NON-PATHOGENIC OXALATE DEFICIENT MUTANTS OF *Sclerotinia sclerotiorum* TRIGGER A HYPERSENSITIVE RESPONSE (HR)-LIKE CELL DEATH IN THE PLANT

OVERVIEW

Cell death regulation can be key to the outcome of many plant-pathogen interactions. In biotrophic interactions, incompatibility is generally associated with a hypersensitive response (HR) believed to delimit pathogen ingress, and contribute to host resistance. Conversely, necrotrophic pathogens appear to exploit plant cell death for their benefit and, thus, the consequences for cell death are life style dependent. Coupled with previous studies, I have shown that *Sclerotinia sclerotiorum* subverts host cell death pathways to promote programmed cell death for disease development (Kim et al. 2008b). Interestingly, a non-pathogenic oxalate deficient mutant (A2) triggers an HR-like response resulting in restricted cell death that is associated with impeded growth of the mutant. In accordance, accumulating evidence indicates that several host responses associated with the HR correlate with defense.

INTRODUCTION

Plant pathogens are broadly divided as biotrophs and necrotrophs depending on pathogenic lifestyle (Glazebrook 2005). These lifestyle differences may lead to contrasting consequences in plant-pathogen interactions, including the effectiveness of

many plant defense responses. The hypersensitive response (HR) is a type of programmed cell death (PCD) that is believed to play an important defense role in certain plant-biotroph interactions by delimiting pathogen growth and spread. As biotrophs by definition require living cells for growth and colonization, cell death is clearly associated with plant defense. The HR in plant-biotroph interactions frequently correlates with other responses including antimicrobial activity, cell wall strengthening, ROS generation, phenolic accumulation, induction of defense-related genes, and hormone activation, all of which may contribute to confinement of the pathogen (Chisholm et al. 2006; Glazebrook 2005; Greenberg and Yao 2004; Morel and Dangl 1997; Mur et al. 2008; Heasth 2000). In gene for gene interactions, response is mediated by plant resistance (R) gene products that directly or indirectly recognize pathogen effectors (previously known as avirulence (Avr) proteins) to trigger cell death (Glazebrook 2005; Greenberg and Yao 2004). The recognition of effectors by R proteins is also accompanied by the expression of ROS and other cytological and biochemical markers (Morel and Dangl 1997). Collectively, these defense responses, coupled with cell death, are detrimental to biotrophic pathogens and culminate in plant resistance.

Plant PCD in response to necrotrophic pathogens is theoretically advantageous to the pathogen. Studies involving PCD in plant-necrotroph interactions demonstrated that several necrotrophic toxin producing fungi, including *Cochliobolus victoriae* (victorin) and *Alternaria alternata lycopersici* (AAL), trigger an apoptotic-like PCD during disease development (Curtis and Wolpert 2002; Navarre and Wolpert 1999; Wang et al. 1996a). This apoptotic-like PCD shares characteristic features of animal apoptosis

including DNA fragmentation, formation of apoptotic bodies, nuclear condensation and the requirement of *de novo* protein synthesis (Asai et al. 2000; Coffeen and Wolpert, 2004; Curtis and Wolpert 2002; Lorang et al. 2007; Navarre and Wolpert 1999; Stone et al. 2000; Wang et al. 1996a). In accordance with these results, a previous study showed that transgenic plants carrying animal anti-apoptotic genes suppressed plant disease caused by several necrotrophic pathogens including *S. sclerotiorum*, *B. cinerea*, and *C. nicotianae* (Dickman et al. 2001). This study revealed that wild type *S. sclerotiorum* and its non-selective toxin, oxalic acid (OA), induced hallmark features of PCD in host plants that were suppressed by the expression of anti-apoptotic genes. Nonpathogenic oxalate deficient (OA^-) mutants of *S. sclerotiorum* induced neither disease nor apoptotic-like PCD features (Dickman et al. 2001; Kim et al. 2008b). Thus, these data suggest that oxalate from *S. sclerotiorum*, at least in part, is an elicitor that triggers an apoptotic-like PCD in host plants.

Govrin and Levine (2000) proposed that *B. cinerea* and *S. sclerotiorum* induced an HR to obtain nutrition from the dead tissues. However, it is still unclear whether these necrotrophs induce cell death in a manner consistent with an HR. I noticed that plant response to the non-pathogenic oxalate deficient mutant of *S. sclerotiorum* (A2) was reminiscent of a classical HR. The A2 mutants showed constrained growth at the infected site. I also observed callose deposition, lignin formation, and cell wall cross-linking, all of which correlated with the HR and plant defense. While ROS generation was also suppressed in plant leaves infected with wild

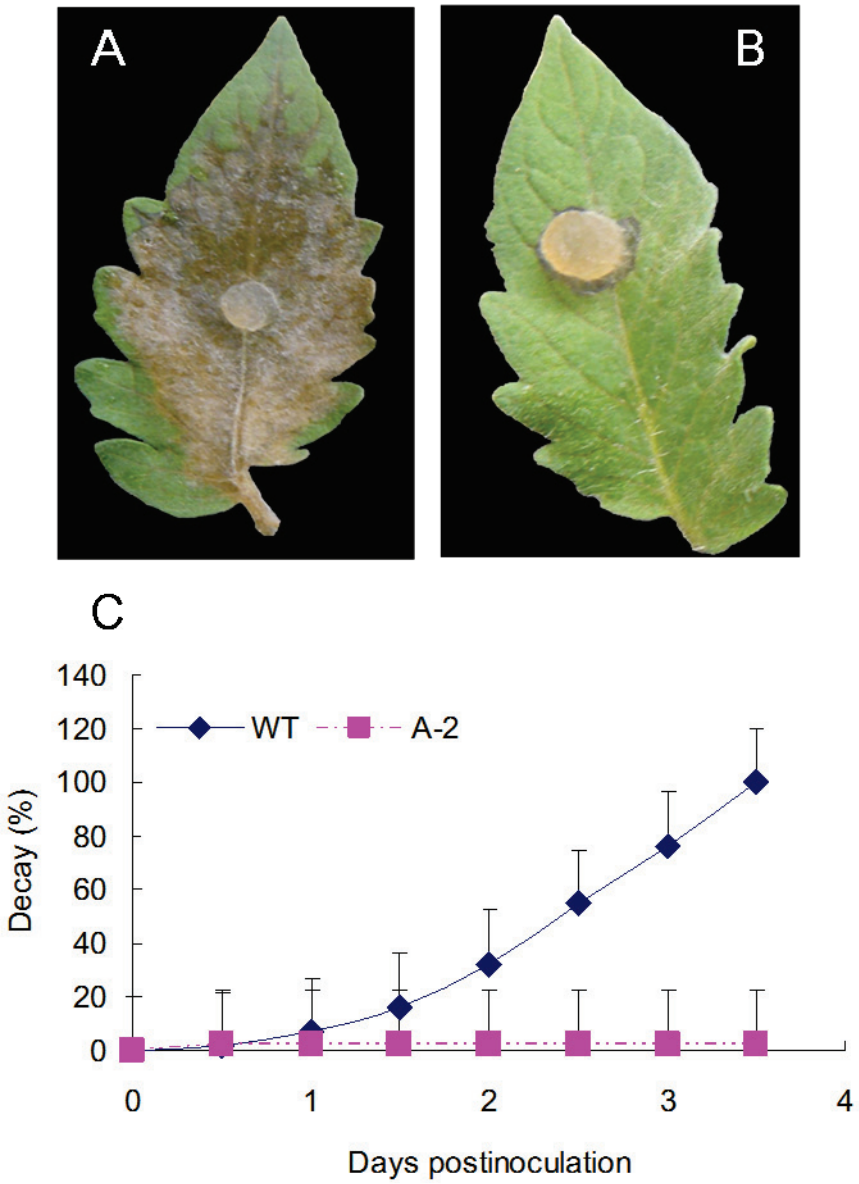


Fig. 3.1. HR-like response of the oxalate deficient mutant (A2). Tomato leaves were inoculated with agar plugs (5 mm in diameter) containing wild type (**A**) and the oxalate deficient A2 mutant (**B**) strains and monitored during infection. **A** and **B** panels were taken at 3 days post-inoculation. **C,** Percentage of cell death and HR-like lesion (decayed area/total area) was quantified with Image J 1.38x software after capturing images at different time point (0-3.5 days after inoculation). A2 growth was restricted within 1 day after inoculation.

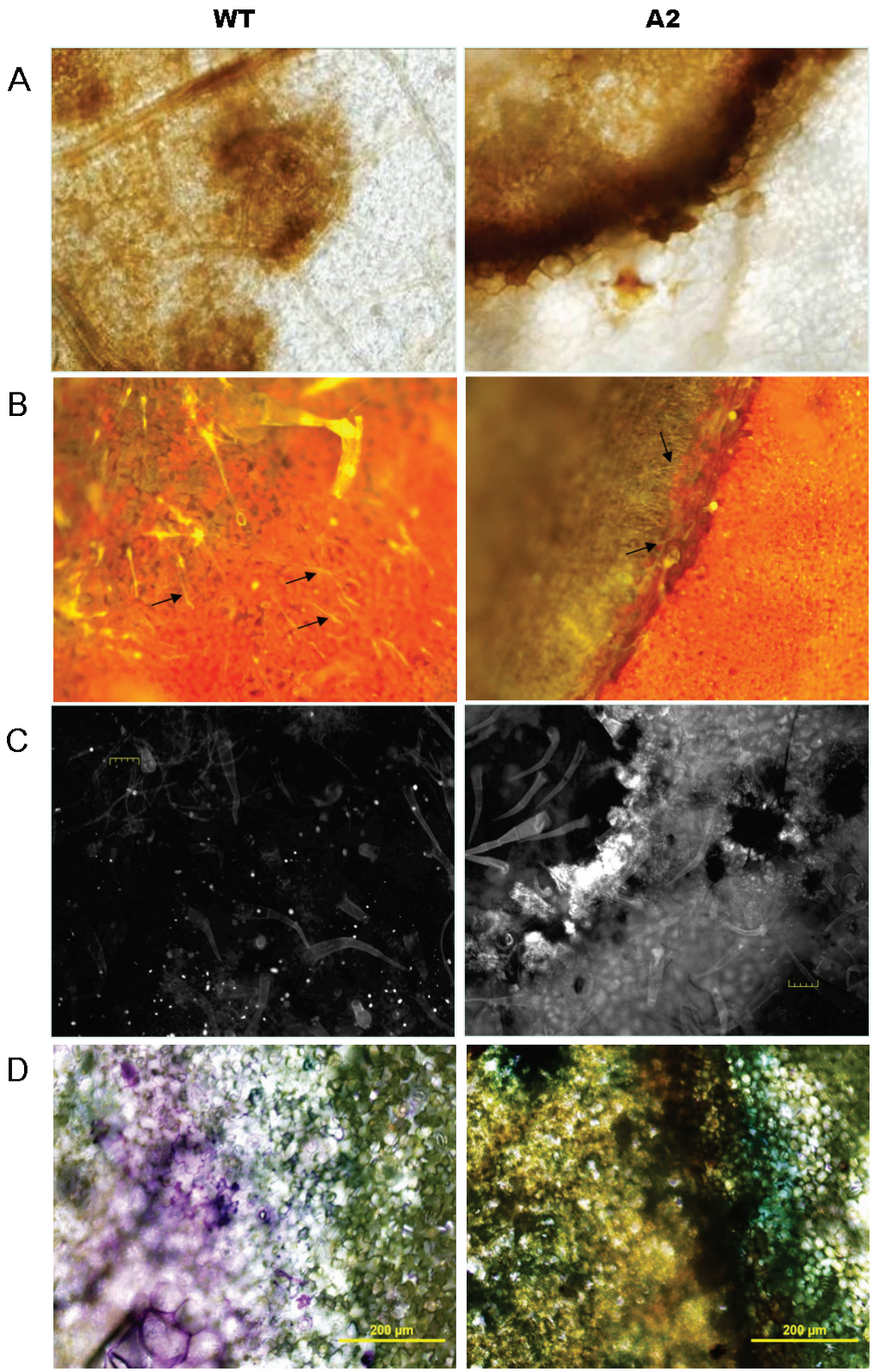
type fungi, ROS was significantly up-regulated following inoculation with non-pathogenic mutants. Among defense-related genes that I examined, defensin and proteinase inhibitor1 genes were up-regulated by A2 mutants and suppressed by wild type fungi. Furthermore, *ced-9*, a nematode anti-apoptotic gene, conferred resistance to wild type *S. sclerotiorum*, but had negligible effects the HR-like response induced by A2 mutants. These findings suggest that the oxalate deficient A2 mutant triggers a distinct response to that of the wild type response, although both induce a programmed cell death.

RESULTS

An oxalate deficient mutant induces a host cell death

A previous study showed that oxalate deficient *S. sclerotiorum* mutants were non-pathogenic (Godoy et al. 1990). Subsequent studies also showed that wild type *S. sclerotiorum* or oxalate alone triggered an apoptotic-like cell death in plants (Kim et al. 2008b). I examined the phenotype of leaves inoculated with the oxalate deficient mutant (A2) in more detail. In contrast to apoptotic-like cell death observed in wild type infected tomato leaves, A2-inoculated leaves revealed that plant-A2 interaction led to cell death that dramatically limited growth of A2 (Fig 3.1A and B). The wild type fungi colonized the entire tomato leaf within 3-4 days, whereas A2 mutants (Godoy et al. 1990) ceased to grow within 2 days post-inoculation (dpi) (Fig 3.1). These observations suggest that the A2 mutant phenotype is at least somewhat analogous to the HR; 'the rapid death of plant cells in association with the restriction of pathogen growth'

Fig. 3.2. Characterization of the HR-like response of the oxalate deficient mutant. **A,** Wild type- and A2 mutant-infected tomato leaves were bleached with 95 % ethanol at 2 days post-inoculation and observed under light microscopy. The wild type fungus grew through the tissue, but the A2 mutant (right panel) showed restricted growth at the dark brown line. **B**, WT- and A2-infected plant leaves (2dpi) were observed under UV. Arrows indicate fungal hyphae. **C**, Callose deposition correlated to the HR-like response following inoculation of the A2 mutant (right panel). Leaves were inoculated with wild type (left panel) and the A2 mutant, and stained with aniline blue at 2 days post-inoculation. **D**, Lignification of plant leaf tissues. At 2 days after inoculation, tomato leaves were stained using toluidine blue. Accumulation of lignin in leaf tissues was visualized with color alteration from blue to green. No color change was monitored in the wild type fungus-infected leaf (left panel), but color change from blue to green was observed and overlap with the brown line in the leaf inoculated with A2 (right panel). Bar = 200 μm.


WT
A2
A
B
C
D
200 µm
200 µm

(Goodman and Novacky 1994; Greenberg and Yao 2004; Heath 1998). Another interesting observation in the HR-like phenotype was the delimited necrotic line on the border of the lesion (Fig. 3.2A). This line appeared to separate dead and living cells and was not observed in the leaf inoculated with wild type fungi. Importantly, the necrotic line was observed at the edge of the A2 mutant colony (Fig. 3.2B). Examination of fungal-infected leaves under a fluorescent microscope shows that A2 mutants (yellow autoflorescence) appear to be unable to gain access into living cells (red fluorescence), while wild type *S. sclerotiorum* hyphae are found in both living and necrotic cells (Fig. 3.2B). Taken together, the phenotype of tomato leaves infected with A2 mutants is opposed with an apoptotic-like PCD induced by wild type *S. sclerotiorum* as reported (Dickman et al. 2001), and resembles an HR.

A2-induced HR-like cell death correlates with defense responses

As mentioned above, the HR is usually associated with plant resistance including the restricted growth of pathogens in plant-biotroph interactions (Greenberg and Yao 2004). However, Heath (2000) suggested that growth of non-biotrophic pathogens (such as necrotrophs) would not be limited by host cell death. Thus, I was interested in examining this HR-like cell death response in more detail. There are many responses associated with plant defense. For example, localized callose deposition creates a physical barrier to prevent pathogen ingress (Ton and Mauch-Mani 2004; Roetschi et al. 2001; Yun et al. 2006; Zimmerli et al. 2000). I monitored callose deposition by staining with Aniline blue after inoculation of wild type and A2 mutant strains on tomato

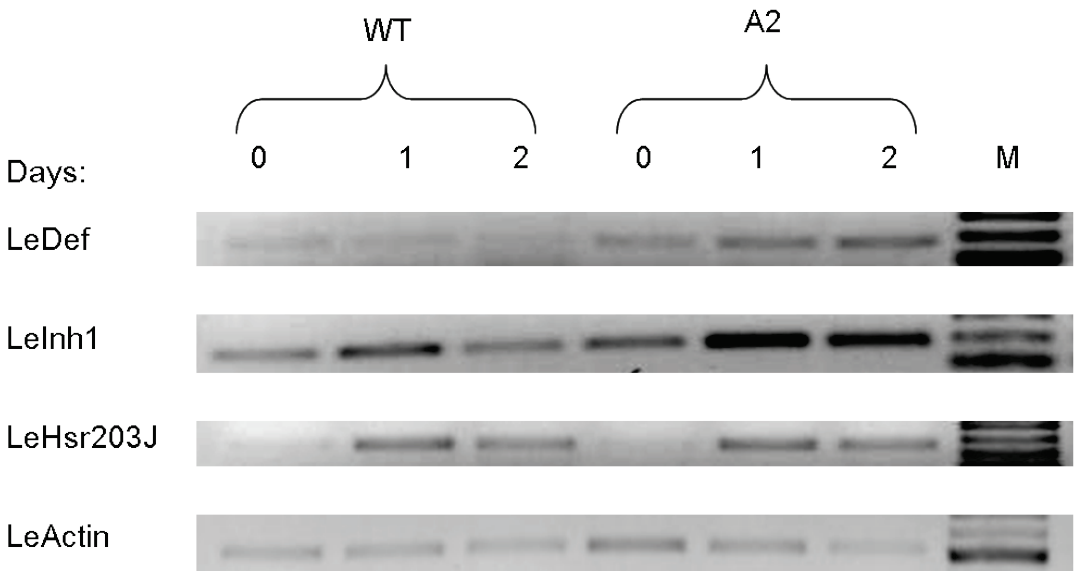


Fig. 3.3. Suppression of defense-related tomato gene after infection. After inoculation with wild type and A2 mutant strains, tomato leaves were collected at different time points (0, 1 day, 2 days after inoculation). Total RNA was extracted for RT-PCR. Tomato actin gene was used as internal control. RT-PCR for LeDef, LeInh1, LeHsr203J and LeActin was performed. M indicates 100bp DNA ladder. LeDef, tomato defensin; LeInh1, tomato proteinase inhibitor1; LeHsr203J, tomato Hsr203J; LeActin, tomato actin.

plants (2dpi). Leaves inoculated with the OA^- mutants had significantly higher levels of callose deposition localized to the interface where inoculation occurred and pathogen growth was halted (Fig. 3.2C). The presence of lignin, a complex polymer which strengthens the cell wall and also functions as a physical barrier, was also observed (Baldridge et al. 1998; Hatfield and Vermerris 2001; Jaeck et al. 1992; Lawton and Lamb 1987; Ni et al. 1996; Sutela et al. 2009). Toluidine blue staining for lignin showed that plant tissues inoculated with A2 mutants have a distinct alteration of blue to green indicating accumulation of lignin (O'Brien et al. 1965). This alteration was not observed in the plants infected with wild type *S. sclerotiorum* (Fig. 3.2D). These results suggest that cell wall strengthening including callose and lignin deposition may be associated with the HR-like response triggered by OA^- mutants. Further, cell wall strengthening could contribute to the prevention of the pathogen growth.

I also examined the expression of selected defense-related tomato genes and found a mixed pattern of results. The expression pattern of the tomato defensin gene, which encodes a small antimicrobial peptide that inhibits the *in vitro* growth of a broad range of fungi (reviewed at Bart et al. 2002), was induced in tomato plants inoculated with A2, but suppressed by wild type *Sclerotinia* (Fig. 3.3). This result correlated with studies showing that *Botrytis cinerea*, a related necrotrophic fungus, was inhibited by defensin genes of several solanaceous plants (Lay et al. 2003). The expression of defensin also correlated with callose deposition. In addition, a wound (insect)-inducible tomato proteinase inhibitor I (LeInh1) gene was also up-regulated in the leaves infected with OA^- mutants but not with the wild type fungi (Fig. 3.3). Consistently, the

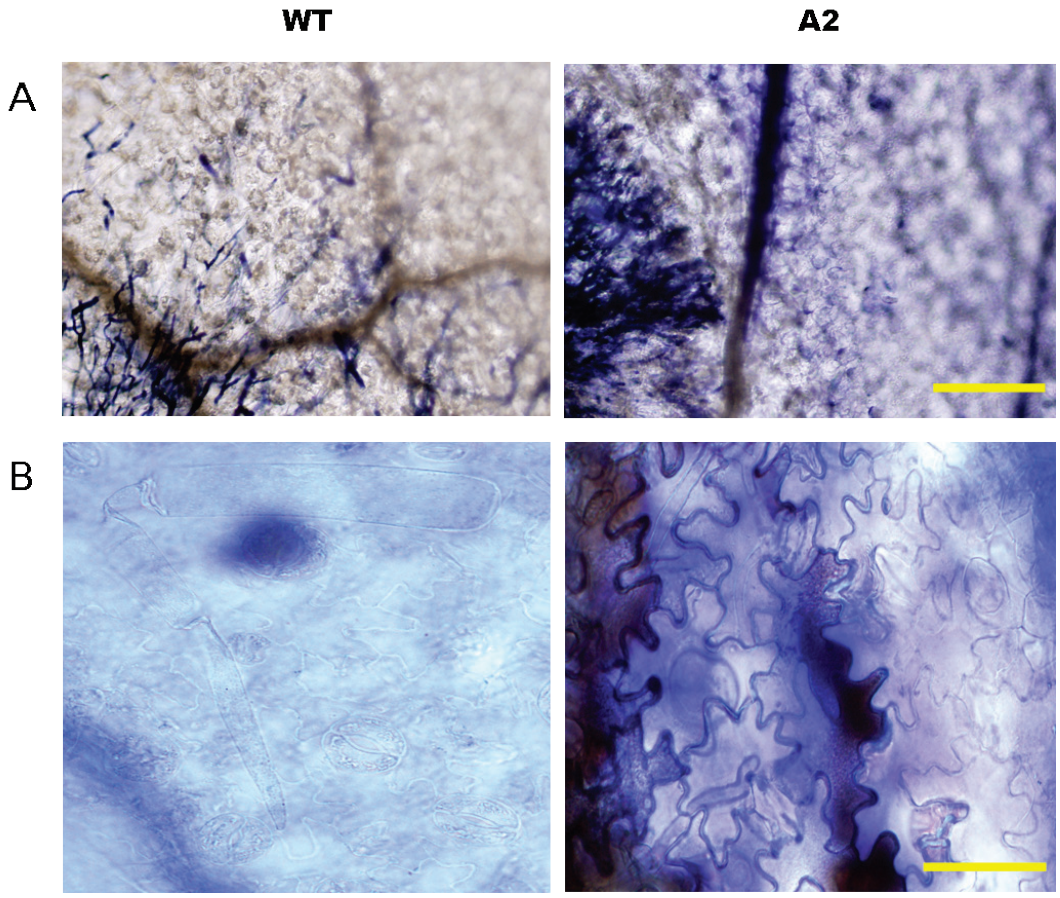


Fig. 3.4. Generation of superoxide (A) and cell wall cross-linking (B) in tomato leaves. A. Tomato leaves were inoculated by wild type and A2 mutant strains, and stained with NBT as described in Materials and Methods. Blue color indicates accumulation of superoxide. Fungal mycelia also show blue color suggesting that both fungi produce superoxide. Bar = 100 μm **B** To detect cell wall cross-linking in wild type tomato leaves induced by wild type (left panel) and A2 mutant (right panel) strains, Coomassie blue staining was used at 2 days after inoculation. Bar = 50 μm.

proteinase inhibitor II gene was also induced by *B. cinerea*, suggesting that *Botrytis* may induce a wound-like response that is similar to responses to insects (Abuqamar et al. 2008). These data suggest that tomato defensin and proteinase inhibitor gene expression are correlated and could contribute to the defense response against A2 mutants. I also examined the expression of *hsr203J*, a gene that is associated with the HR (Pontier et al. 1998; Takahashi et al. 2004; Tronchet et al. 2001). However, transcript levels of *hsr203J* genes measured from the leaves challenged with wild type and A2 mutant strains were not significantly different (Fig. 3.3), suggesting that the *hsr203J* gene has a limited role, if any, in defense response against A2.

ROS induction in plant-A2 interaction

A universal characteristic of the HR is the generation of reactive oxygen species (ROS). ROS including superoxide and hydrogen peroxide are extensively accumulated in many plant interactions that induce the HR as a defense response. A previous study showed that production of ROS in tobacco can be suppressed by oxalate (Cessna et al. 2000). To determine if oxidative burst is involved in the HR-like cell death, we compared ROS generation in the HR-like cell death to that in PCD induced by wild type *S. sclerotiorum*. Superoxide generation of tomato leaves infected with wild type and A2 mutant strains was detected by nitroblue tetrazolium (NBT) staining. As with previous data, microscopy of tomato leaves consistently revealed that signal intensities of NBT were strong, indicative of an oxidative burst in plant tissues inoculated with A2 while wild type fungi inoculated tissues showed complete absence of staining (Fig. 3.4. A).

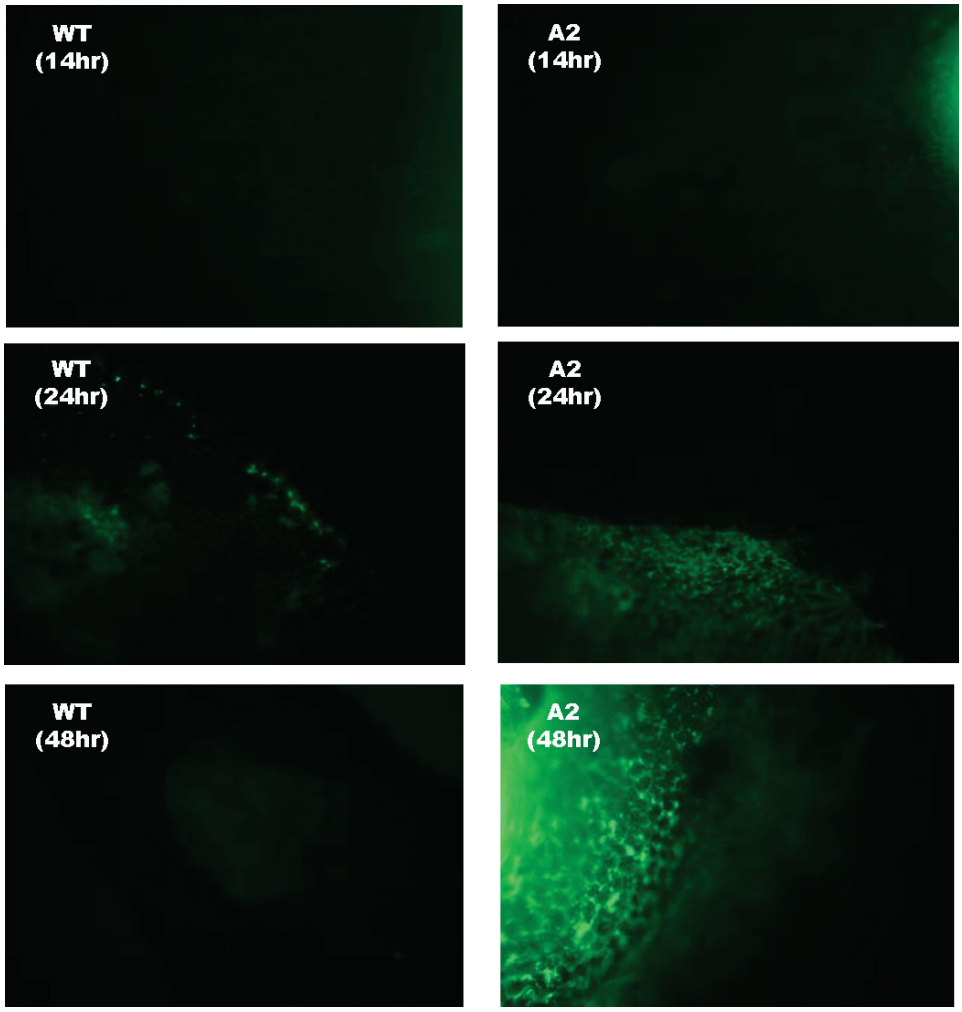


Fig. 3.5. Accumulation of ROS in tomato leaves following inoculation with *S. sclerotiorum* wild type and A2 mutant strains. Tomato leaf tissues were inoculated fungi and were collected at 14, 24, 48 hour post-inoculation. Samples were stained with DCFDA for 30 min.

Importantly, both fungal strains showed strong accumulation of formazan precipitates, indicating the presence of superoxide in vegetative hyphae (Fig. 3.4. A). These data are in agreement with my result using 3,3-diaminobenzidine (DAB) staining of hydrogen peroxide accumulation (Williams et al. submitted). In early infection stages, the oxidative burst was observed in DAB stained leaves challenged with the A2 mutant strain, but not in leaves inoculated with the wild type strain. Since superoxide rapidly dismutases to hydrogen peroxide by superoxide dismutase (SOD), these results are reasonable. The oxidative burst often leads to cell wall cross-linking, which strengthens the cell wall and presumably limits the growth of pathogen (Bradley et al. 1992). I used Coomassie blue staining to examine whether cell wall cross-linking was associated with accumulation of superoxide in the plant-mutant interaction (Mellersh et al. 2002). Protein cross-linking was observed in the plant-A2 interaction but was rarely detected in the plant-wild type interaction (Fig. 3.4B). In accordance with former observations, Coomassie blue staining was prominent at the front of the HR-like lesion.

ROS generation was also monitored during the infection process using the ROS indicator 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA) (see Chapter II). DCFHDA fluorescence microscopy showed that ROS was up-regulated in the interaction between the tomato plants and the OA deficient mutants, whereas ROS was not observed in wild type fungus during infection (Fig. 3.5) (Selvakumar et al, unpublished). NBT staining of *Arabidopsis* leaves infected with both fungi showed similar results to that of DCFHDA fluorescence microscopy (Fig. 3.6).

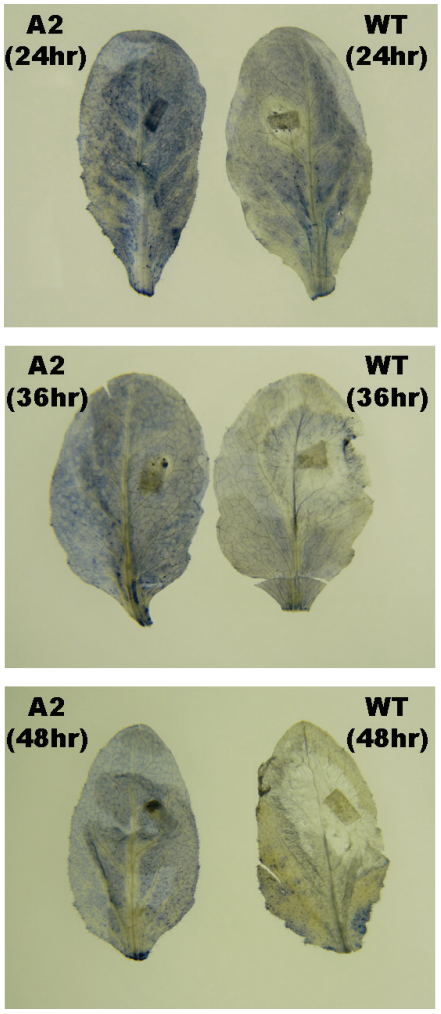
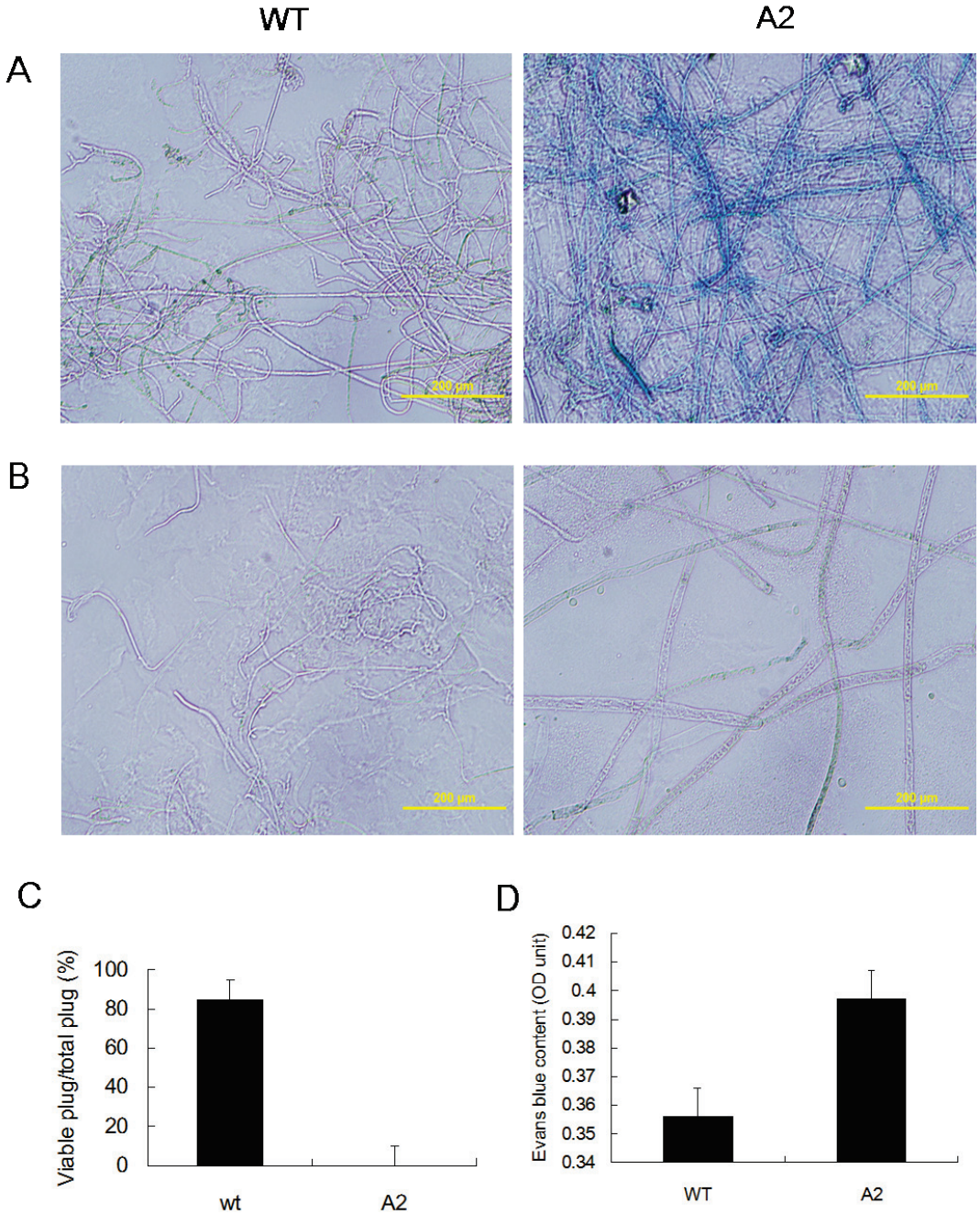


Fig. 3.6. Down-regulation of superoxide in *Arabidopsis* leaves infected with wild type and A2 mutant strains. *Arabidopsis* leaves infected with wild type and mutant strains were collected 24, 36, 48 hour after infection. Samples were stained with NBT for 10 min and clarified with 95 % ethanol.

Fig. 3.7. HR-like response of the oxalate deficient mutant leads to fungal cell death. **A**, PDA plugs containing vegetatively growing fungal strains (WT and A2) inoculated on tomato leaves and collected after 2 days. Agar plugs were stained with Evans blue and monitored under light microscope. Bar = 200 ㎛ **B**, PDA plugs with both fungi which had not been inoculated on plant and had been placed onto empty sterile plate without media for 2 days were collected and stained with Evans blue as a control. Bar = 200 ㎛ **C**, Viability test of a PDA plug embedded with fungi. PDA plugs embedded with both fungal strains inoculated on tomato leaves and collected at 6 days after inoculation. These agar plugs were transferred to and cultured on the PDA. Wild type *S. sclerotiorum* was confirmed by sclerotia formation after one week. **D**, Spectrometric analysis of Evans blue staining confirmed panel A. Same experiments with panel A were performed except using spectrometry. After staining, agar plugs with both fungal strains were washed and incubated in 50 % methanol containing 1 % SDS for 30 min at 50 °C and quantified by absorbance at 600 nm.


WT
A2
A
B
C
Viable plug/total plug (%)
100
80
60
40
20
0
wt
A2
D
Evans blue content (OD unit)
0.42
0.41
0.4
0.39
0.38
0.37
0.36
0.35
0.34
WT
A2

Plant HR-like response triggered by A2 mutants leads to the cell death in the mutants

In plant-biotroph associations, the HR-PCD coupled with other coordinated defense responses, presumably delimit pathogens from growth and nutrition, thus causing the pathogens to starve and eventually die (Glazebook 2005). Since A2 induced an HR-like response, I examined whether this plant response resulted in cell death of the pathogen. If the host defense response to OA^- mutants is successful, HR-like cell death might occur and the fungus would lose viability. Following inoculation, both fungal strains were stained with Evans blue, a stain that is excluded by living fungal cells having an intact cell membrane (Chen and Dickman 2005b; Ponce de León et al. 2007). At 2 days after wild type and A2 mutant strains were inoculated on tomato plant leaves, I stained the agar plugs that were used for inoculation. The A2 agar plugs showed abundant dark bluish hyphae but wild type fungus showed limited blue staining when compared to controls (Fig. 3.7A and B), suggesting that only A2 hyphae are not viable. However, wild type fungi and controls remained unstained indicating the cell membranes were intact. Quantification of these data by spectrophotometric detection at 600nm was consistent with these observations (Fig. 3.7D). To further verify cell viability of these fungi, agar plugs obtained at 6 days after inoculation on tomato leaves were transferred to axenic potato dextrose agar (PDA) plates. A2 mutants were unable to grow in axenic culture following inoculation on the host, whereas wild type fungi grow (Fig. 3.7C). These results indicate that wild type fungi inoculated on tomato leaves were viable, while the corresponding A2 mutants were dead after inoculation on plant leaves.

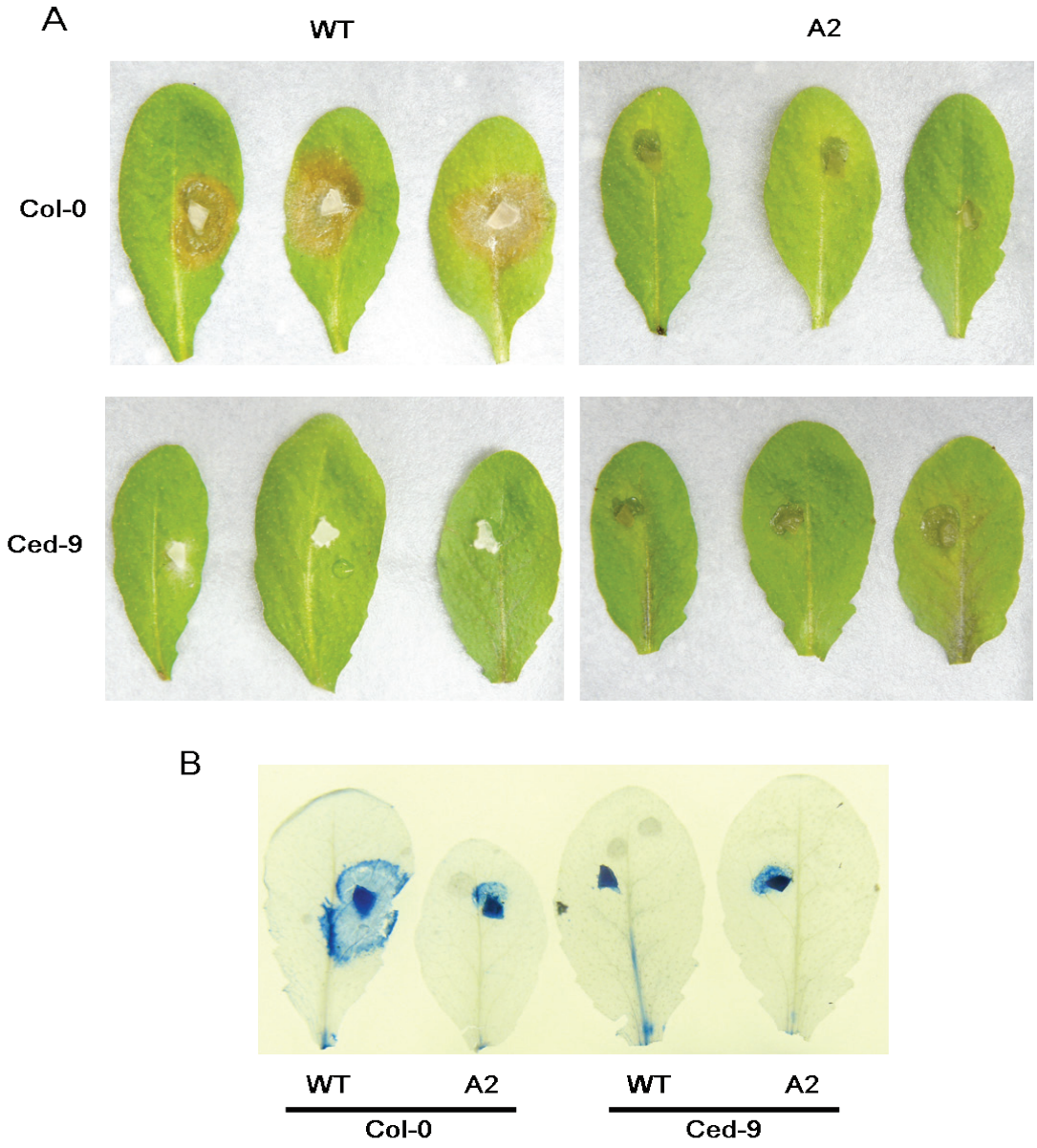


Fig. 3.8. The *ced-9* gene does not prevent HR-like response. A, Lesion development on *Arabidopsis* leaves carrying nematode *ced-9* gene. The leaves were inoculated with an agar plug with wild type and A2 mutant strains. *Arabidopsis* Col-0 was served as controls. Photographs were taken at 2 days post-inoculation. **B,** Cell death was observed by staining with Evans blue. Leaves (2 dpi) were collected and stained with 0.05 % Evans blue for 10 min as described in Materials and Methods. Picture was taken on a transilluminator.

A nematode anti-apoptotic gene (*ced-9*) has no impact on the inhibition of the HR-like response triggered by oxalate deficient mutants

Previous data showed that transgenic plants carrying mammalian anti-apoptotic genes can inhibit programmed cell death (disease) induced by wild type *S. sclerotiorum*. These genes were also able to hinder the N gene-related HR response to the tobacco mosaic virus (TMV) and most other members of the tobamovirus family. This inhibition of N gene-related HR in tobacco plants carrying the anti-apoptotic genes is similar to typical disease symptoms showing unlimited chlorosis as in a susceptible tobacco plant infected with TMV. In this study, I examined whether the HR-like response triggered by A2 mutant strains could be inhibited by anti-apoptotic genes. Detached leaves of *Arabidopsis thaliana* ecotype Columbia-0 (Col-0), serving as a positive control, were destroyed by the wild type strains. Transgenic *Arabidopsis* carrying anti-apoptotic gene *ced-9* inhibited programmed cell death induced by wild type strains to colonize plant tissues (Fig. 3.8A). However, no differences in cell death responses in *ced-9* and Col-0 plants were found when leaves were challenged with A2 mutants (Fig. 3.8A). Evans blue staining of dead plant tissues also confirmed these observations (Fig. 3.8B). These results are consistent with data showing that anti-apoptotic genes inhibit programmed cell death induced by wild type *S. sclerotiorum* (Dickman et al. 2001). However, the anti-apoptotic *ced-9* gene appears to be unable to impede HR-like cell death, suggesting that the HR-like death response may differ from the apoptotic PCD induced by wild type fungus.

DISCUSSION

S. Sclerotiorum oxalate deficient mutant elicits an HR-like response

Perception of a pathogen at the host cell surface initiates signaling pathways that trigger various defense responses including transcriptional induction of defense-related genes, generation of ROS, and deposition of callose to strengthen the cell wall at the site of infection, thereby preventing microbial growth and spread (Chisholm et al. 2006). There are two established host defense schemes when a plant perceives a pathogen. The host response driven by pathogen-associated molecular patterns (PAMPs) such as flagellin and lipopolysaccharides (LPS), which are conserved microbial structures, is collectively known as PAMP-triggered immunity (PTI). In some cases, pathogens can target these signaling pathways using effectors to suppress PTI (Chisholm et al. 2006). Plants, however, adapt to recognize effectors at a more specific level often via R proteins. This type of plant resistance response through effector-R protein recognition is referred to as effector-triggered immunity (ETI). PTI is basal host resistance that could be effective against most of microbial pathogen and PTI is usually durable. In contrast, ETI often includes a strong plant oxidative burst and restricted (programmed) cell death, indicative of a hypersensitive response (Nicaise et al. 2009). Thus, ETI can be more effective than PTI to protect against a specific pathogen containing corresponding R protein.

Previous studies have shown that necrotrophic pathogens such as *S. sclerotiorum, C. victoriae* and *A. alternata* induce programmed cell death (Asai et al. 2000; Coffeen

and Wolpert, 2004; Curtis and Wolpert 2002; Lorang et al. 2007; Navarre and Wolpert 1999; Stone et al. 2000; Wang et al. 1996a; Williams and Dickman 2008). Although PCD induced by these necrotrophs was analogous to a degree to mammalian apoptosis, few attempts have been made to examine the relationship between necrotroph-induced PCD and the HR. Govrin and Levine (2000) suggested that necrotrophs such as *S. sclerotiorum* and *B. cinerea* trigger an HR. Their hypothesis was supported by generation of reactive oxygen species (ROS), induction of an HR-specific gene *hsr203J*, and nuclear condensation in *B. cinerea*. These features are often used as markers for the HR; however they are not universal. For example, the *hsr203J* gene is correlated with the HR, but this gene is also expressed in response to the heavy metals leading to necrosis (Pontier et al. 1998). Thus, induction of this gene could be associated with other cell death or general stress responses besides the HR. In our case, both wild type and A2 mutant strains induced *hsr203J* genes.

This study showed direct evidence that cell death triggered by a non-pathogenic oxalate deficient mutant (A2) was completely limited to the fungal infection site, contrasting with PCD induced by wild type *S. sclerotiorum*. The plant response to the A2 mutant is also associated with several defense responses in addition to the HR. (Chisholm et al. 2006; Glazebrook 2005; Greenberg and Yao 2004; Heath 2000). Defense responses associated with the HR include: (i) Rapid generation of ROS (Apel et al. 2004; Lamb and Dixon 1997). A biphasic ROS is generated in the bacterial incompatible response - weak ROS in the initial phase, followed by a more pronounced oxidative burst in the second phase (Lamb and Dixon 1997). (ii) Induction of physical

barriers (Hamiduzzaman et al. 2005; Kuc 1995; Rinaldi et al. 2007; Stone and Clarke 1992). (iii) Induction of antimicrobial compounds (e.g. phytoalexins) (Delannoy et al. 2005). (iv) Expression of marker genes (Dempsey et al. 1998). Induction of certain pathogenesis related (PR) proteins have been observed in the HR response. However, none are specifically causal to the HR and are probably associated with more general defense on stress response pathways (Pontier et al. 2001). In this study, I observed that infection with A2 resulted in the induction of (restricted) cell death along with several defense responses. Cell wall strengthening through callose deposition, lignification, and cell wall cross-linking are deployed in the HR-like response when tomato plants were infected with A2 mutants. In addition, A2 mutants stopped growing, suggesting that the HR-like response prevents pathogen access into their adjacent living cells. In contrast, wild type *S. sclerotiorum* did not display any of these responses. Notably, wild type fungal hyphae are observed not only in dead cells, but also appear to be access into living neighbor cells, suggesting that cell death in the plant does not inhibit pathogen growth in this case. Therefore, plant defense responses may not impact plant-wild type *S. sclerotiorum* interaction or may be suppressed (or inactivated) by wild type *S. sclerotiorum*.

Oxidative burst in the HR-like response

In gene for gene interactions, plant recognition of pathogen effectors through R proteins triggers an oxidative burst and defense. The plant NADPH oxidase (respiratory burst oxidase homolog, Rboh) is considered a primary source of ROS for the oxidative

burst (Torres et al. 2002). Superoxide generated by NADPH oxidase can be enzymatically or non-enzymatically converted to hydrogen peroxide or other types of ROS (see Chapter I). For example, superoxide is rapidly transformed to hydrogen peroxide via superoxide dismutases (SOD). Since ROS are toxic to cells, the oxidative burst is detrimental to the pathogen (and the plant), and therefore is considered an important defense response (Wu et al. 1995). Hydrogen peroxide and other ROS intermediates are also emerging as systemic signals in defense responses (Orozco-Cárdenas et al. 2001; Davletova et al. 2005). In particular, hydrogen peroxide is suitable for a mobile signal molecule because it is relatively stable and more diffusible than other ROS when spreading from cell to cell. Accordingly, a manipulative plant oxidative burst could be an important target for the survival of the pathogen.

Here, I show that hydrogen peroxide and superoxide generation was suppressed when plant leaves were challenged by wild type *S. sclerotiorum* as previous studies have shown (Cessna et al. 2000; Selvakumar et al. unpublished). However, plants inoculated with oxalate deficient A2 mutants display a massive oxidative burst. These results correlated with other up-regulated defense responses of the host plant inoculated with A2 mutants. Interestingly, extensive accumulation of superoxide and hydrogen peroxide was observed contained in wild type fungal hyphae during infection process, while plant tissue remained clear (unstained). This suggests that wild type fungi suppress the plant oxidative burst while maintaining fungal ROS. This observation also suggests that ROS in hyphae may carry out certain roles such as intra- or inter-cellular signaling. In relation to this, I showed that fungal ROS played an important role in pathogenicity and

development of *S. sclerotiorum* in Chapter II. Two *S. sclerotiorum* NADPH oxidases (Nox1 and Nox2), the primary sources of superoxide, were characterized. Silencing of *nox1* and *nox2* genes and treatment with the NADPH oxidase inhibitor (diphenylene iodonium) led to severe defects in sclerotia formation. I also showed that inhibition of the NADPH oxidase correlated with oxalate production, thereby leading to reduced virulence. In addition, *S. sclerotiorum* superoxide dismutase is also involved in sclerotial development and pathogenicity (Selvakumar et al. unpublished). Coupled with results from Chapter II, observations in this study suggest that ROS from fungal NADPH oxidases may play an important role in the pathogenesis of *S. sclerotiorum* in conjunction with oxalate. Collectively, this study suggests that plants actively recognize the A2 mutant and induce an oxidative burst, while wild type *S. sclerotiorum* appears to suppress, avoid, inactivate or circumvent oxidative burst in the plant tissues, resulting in disease.

Plant active recognition of A2 mutant invasion

In plant-biotroph interactions, it has been speculated that rapid suicide committed by the host cells in the vicinity of pathogens causes deprivation of nutrients and water for pathogens, possibly leading to the starvation and death of pathogens (Glazebrook 2005). In this study, Evans blue staining of wild type and A2 mutant strains on tomato leaves revealed that A2 mutants were dead on plants after inoculation. The cell death of A2 mutants may be due to deprivation of nutrients. By definition, however, necrotrophs do not require living host cells to obtain nutrients, thus the host cell death may not

isolate necrotrophic pathogens from nutrients as in the case of biotrophs. Therefore, plant responses other than cell death may be required to restrict growth of necrotrophic pathogens. Interestingly, mycelia in A2 agar plugs (controls), which were placed on a sterile empty Petri dish, were viable after the same duration that was set for those on the inoculated leaves. This observation suggests that A2 mutants inoculated on plant leaves may be much more likely to die not because of starvation but through active responses by the plants. Therefore, host defense responses may contribute to the restricted growth of the mutants.

There was no growth defect observed in A2 mutants when grown on PDA or even on water agar medium, which has minimal nutrients. Constrained growth of A2 mutants was only observed on plant leaves. Therefore, A2 mutants might be physiologically debilitated when they grow on plant leaves, as observed with *Magnaporthe grisea* mutants that are defective in producing appressoria (Eagan et al. 2007; Xu and Hamer 1996). In addition, previous studies have shown that growth of oxalate deficient mutants on plant leaves can be partially restored by exogenous oxalate treatment (Godoy et al. 1990; Kim et al. 2008b; Williams et al. submitted). This result suggests that oxalate is, at least partially, required for the growth of the oxalate deficient A2 mutant on plant leaves as would be expected. Furthermore, HR-like cell death in plants against A2 mutants is associated with several defense responses including an extensive oxidative burst and cell wall strengthening, which are not observed in plants inoculated with wild type fungi. These results are consistent with the importance of oxalate for *S. sclerotiorum* disease. Oxalate secreted by wild type *S. sclerotiorum*

suppress host defense responses (Cessna et al. 2000), and also promotes plant PCD pathways via ROS (Williams et al. submitted; Kim et al. 2008b). This opposing function of oxalate was supported by a recent study using a real-time plant-based redox sensitive GFP (green fluorescent protein) reporter (ro-GFP) in plant-*Sclerotinia* interaction (Williams et al. submitted). In line with the importance of oxalate in pathogenicity, transgenic sunflowers over-expressing oxalate oxidase (an enzyme that converts oxalate to hydrogen peroxide and water) showed that cell death and defensin expression was induced (Hu et al. 2003). Importantly, these transgenic lines were also resistant to *S. sclerotiorum* (Hu et al. 2003). Oilseed rape (*Brassica napus*) expressing wheat oxalate oxidase also showed increased levels of hydrogen peroxide and enhanced resistance to *S. sclerotiorum* (Dong et al. 2008). Furthermore, overexpression of a gene encoding oxalate decarboxylase, which converts OA into CO_2 and formate, from the basidiomycete *Trametes versicolor* enhanced resistance to *S. sclerotiorum* in tobacco plants (Walz et al. 2007). These studies suggest that removal of oxalate impacts pathogenicity.

The HR-like response induced by oxalate deficient mutants may be distinct from PCD induced by the wild type fungus

Previous research demonstrated that expression of animal anti-apoptotic genes conferred resistance to *Sclerotinia* and prevented PCD (Dickman et al. 2001). Here, I showed that transgenic *Arabidopsis* carrying the nematode anti-apoptotic gene *ced-9* prevented apoptotic-like PCD induced by wild type *Sclerotinia* but not the HR-like

response to OA^- mutants. This result suggests that mechanisms leading to plant PCD may differ as they do in mammals. Work in the Dinesh-Kumar lab showed the involvement of autophagic components in the restriction of the HR induced by tomato mosaic virus (TMV) (Liu et al. 2005). *N* gene-containing *Nicotiana benthamiana* plants infected with GFP-tagged TMV (TMV-GFP) showed restricted HR as expected. However, plant *BECLIN 1* (an ortholog of the yeast and mammalian autophagy gene *ATG6/VPS30/beclin 1*) deficient leaves infected with TMV-GFP underwent runaway PCD. This result suggests that plant autophagy may have a role in confining HR-PCD to infection sites (Liu et al. 2005). *BECLIN 1* and associated components may limit the spread of cell death in the HR-PCD; however, autophagic components may not be involved in limiting apoptotic-like PCD of plants. Based on the observation in *ced-9* overexpressing *Arabidopsis* infected with A2 mutants, it appears that antiapoptotic gene *ced-9* has a limited role in inhibiting HR-like cell death. This suggests that apoptotic cell death may not be involved in the HR-like cell death, whereas autophagic cell death may play an important role in restricting the HR-like response. In accordance, my preliminary data using Lysotracker, a marker of autophagic vacuoles (Biederbick et al. 1995), showed a high level of fluorescence observed in A2-infected plant tissues but not in wild type-infected plant tissues. This result suggests the possible involvement of autophagic-like activity in restricting the HR-like response. Since Lysotracker primarily detects lysosomes and acidic vesicles, these results are quite preliminary. Further studies regarding autophagy in plant-A2 interaction may clarify our understanding of the underlying mechanism of this HR-like response.

MATERIALS AND METHODS

Plant growth and fungal treatments

Wild type *S. sclerotiorum* isolate 1980 and an oxalate-deficient (A2) mutant strains were cultured on potato dextrose agar as previously described by Godoy et al. (1990). Wild type *Solanum lycopersicum* (tomato) cv Motelle, Pearson, Garden peach, Porter's pride plants were grown from seed and maintained under greenhouse conditions. Newly emerging leaves were inoculated with growing fungus and incubated under high humidity conditions at 25 °C.

Histochemical analyses

Calcofluor staining

Tomato leaves were infected with wild type and A2 mutant strains for 2 days and stained with calcofluor white stain (ENG scientific, Clifton, NJ, USA) solution for 30 sec. 10 % KOH was added to samples and observed under fluorescent microscopy (UV).

Toluidine blue staining

Two days post-inoculation leaves were incubated in a solution of 0.05 % toluidine blue in citrate/citric acid buffer (50 mM, pH 3.5) for 5 min (Asselbergh et al, 2007). Following incubation, samples were destained with 70 % ethanol and were mounted for microscope analysis.

Coomassie blue staining

Forty eight hours post-inoculation leaves were decolorized by boiling in 95 % ethanol for 10 min. Following de-staining samples were immersed in 1 % SDS for 24 hrs at 80 °C and stained with 0.1 % Coomassie blue in 40 % ethanol / 10 % acetic acid for 5 min at room temperature. Excess stain was removed by rinsing in 40 % ethanol / 10 % acetic acid. Samples were mounted in 50 % glycerol.

Detection of ROS

To detect H_2O_2, leaves were stained in 2 mg / ml 3'3-diaminobenzidine-tetrahydrochloride (DAB) for 2-4 hrs followed by de-staining in 70 % ethanol at 70 °C until totally cleared.

To visualize hydrogen peroxide, 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA) was used. Tomato leaves were stained with 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA) in PBS buffer for 30 min after infection with wild type and A2 mutant strains, and washed with PBS. Samples were observed using fluorescence microscopy (Zeiss M^2BIO Fluorescence Combination Zoom Stereo/Compound microscope), as described (Sunilkumar et al. 2002).

To monitor superoxide production, wild type *S. sclerotiorum*- and A2-infected leaves were stained with 0.05 % nitroblue tetrazolium and clarified with 95 % enthanol.

An Olympus IX-81 microscope with differential interference contrast optics (10X UPlanFLN objective, 0.30 N.A.; digital images were captured with an Olympus

DP70) was used for this work. All images were collected by using Olympus DP controller and manager as Chapter II.

Semi-quantitative RT-PCR analyses

For semi-quantitative RT-PCR, total RNA was extracted in TriZOL (Invitrogen, Carlsbad, CA, USA) and treated with Dnase according to the manufacturer's instruction. First-strand cDNA was synthesized using oligo dT primers and M-MLV Reverse Transcriptase (1 ug total RNA/reaction). Semi-quantitative RT-PCR was performed as described previously (van den Burg et al, 2008). The primers used for semi-quantitative PCR were as follows: LeHsr203J (LeHsr203J-5, 5'-TCCCGTCATTCTTCACTTCC-3'; LeHsr203J-3, 5'-GTTGAAATCGGCGTATTCGT-3'), LeDef (LeDef-5, 5'-TGTCATG-GCTACTGGACCAA -3'; LeDef-3, 5'-ATGGCCTAGTGCAAAAGCAA-3'), LeInh1, (LeInh1-5, 5'- CCGGTTCCTTCACTCTTTACA-3'; LeInh1-3, 5'- CTGGCCACATTT-GTTTTCCT-3'). No RT control was performed without reverse transcriptase. As a control, tomato Actin transcript was amplified using ApActin-5 (5'-GAGAAGATGAC-CCAGATCATGTTTG-3') and ApActin-3 (5'-TCCTAATATCCAC-GTCGCACTTCA-T-3'). PCR amplification was conducted using 1 uL of reverse transcription products (Tm = 55 °C, 27cycles for LeInh1; Tm = 56 °C, 27cycles for LeDef; Tm = 56 °C, 27 cycles for LeHsr203J; Tm = 55 °C, 25 cycles for actin). As a negative control, PCR was performed with RNA that had not been subjected to cDNA synthesis.

Evans blue staining

To detect cell death of fungi, newly emerging tomato leaves excised from 4-6 week old plants were inoculated with an agar plug (5 mm) embedded with *S. sclerotiorum* wild type and A2 mutant strains. Four days post-inoculation, agar plugs removed from the leaves were stained with 0.05 % Evans blue for 45 min at 25 °C and washed with PBS. For controls, agar plugs embedded with both strains but not inoculated were stained after 4 days.

For light microscopy, samples were observed immediately after staining. Samples for spectrophotometry analysis were incubated in 50 % methanol containing 1 % SDS for 30 min at 50 °C and quantified by absorbance at 600 nm (Delledonne et al. 2001).

To detect cell death of plant tissues, Evans blue staining was also used. Fungus-infected *Arabidopsis* leaves were stained with 0.05 % Evans blue for 10 min at 25 °C. The leaves were repeatedly destained with 95 % ethanol until samples were completely clarified. Photographs of samples were taken on the transilluminator.

Callose staining

Aniline blue staining was performed as described by Asselbergh and Höfte (2007). Briefly, 2 days post-inoculation tomato leaves were incubated in lactophenol for 60 min at 65 °C. Samples were replaced with a fresh solution of lactophenol after 30 min, and then transferred to room temperature. Following incubation for a further 12 hrs, samples were washed in 50 % ethanol for 5 min. After fixation and clarification, samples

were stained for 30 min in the dark with 0.01 % aniline blue in 150mM K2HPO4 (pH 9.5). Stained samples were observed under Olympus IX-81 confocal microscope.

CHAPTER IV

CONCLUSION

Programmed cell death (PCD) is indispensable for development, stress response and pathogenesis in plants. Dysregulation of PCD of these physiological processes may cause a lethal or at least detrimental effect on plants. Particularly, PCD as a defense mechanism against multiple pathogens must be tightly regulated to permit proper response depending on the pathogen's lifestyle. For example, plant PCD contributes to resistance to biotrophic pathogens. In this case, successful biotrophic pathogens may have mechanisms to prevent PCD in the host plant. In contrast to biotrophs, accumulating evidence has indicated that plant PCD could be beneficial to necrotrophic pathogens that obtain nutrition in dead cells. In plant-necrotroph interactions, therefore, pathogens and host plants require opposite strategies. The bottom line is that regulation of PCD in plants is critical in both cases, and the organism that controls plant PCD is in an advantageous position to win the battle.

The Dickman lab has been studying pathogenic development of the necrotrophic pathogen *S. sclerotiorum* because this is an agriculturally and economically important fungus. Previous works in the Dickman lab have shown that wild type *S. sclerotiorum* and oxalic acid (OA) induce PCD in host plants. Interestingly, OA suppresses the oxidative burst in the process of infection. Recent work also suggests that reactive oxygen species (ROS) generation correlates with the induction of PCD in *S. sclerotiorum* disease (Kim et al. 2008b). Therefore, to further understand the link and

cooperation between ROS, oxalate and PCD in *Sclerotinia* disease was the main goal in this study.

Dr. Chen showed the importance of ROS in the pathogenic development of *S. sclerotiorum* (unpublished data). In addition, recent studies have indicated that ROS generation is relevant to plant-fungal interactions. Therefore, I identified the two *S. sclerotiorum* NADPH oxidases-encoding genes (*nox1* and *nox2*) which are believed to be a primary source of ROS generation. RNAi revealed that both genes are essential for sclerotial development. However, only *Nox1*-silenced mutants (SsNOX1-RNAi mutants) showed reduced virulence in infection assays. Additionally, I observed significantly reduced OA production in the SsNOX1-RNAi mutant. The reduced virulence of the SsNOX1-RNAi mutant also correlated with an increased plant oxidative burst. In contrast, inhibition of *nox2* genes showed a negligible effect in pathogenesis. Based on findings in this study, I conclude that both Nox1 and Nox2 have distinct functions in pathogenic development of *S. sclerotiorum*, whereas only Nox1 plays an essential role in pathogenesis.

Several studies have shown the importance of NADPH oxidases in plant-fungus interactions. In the case of the endophyte *Epichloë festucae*, wild type *E. festucae* appears to utilize ROS from NoxA that plays a signaling role to maintain a mutualistic association with ryegrass (Tanaka et al. 2006). Nox1 in *S. sclerotiorum* also appears to have a crucial role in plant-*Sclerotinia* disease interaction. This study clearly shows that the *S. sclerotiorum nox1* gene is essential for full pathogenicity in *Sclerotinia* disease. This is in accordance with previous data showing that ROS correlated with plant PCD

(Kim et al. 2008b), suggesting that ROS from *S. sclerotiorum* Nox1 may activate host PCD pathways. This study also reveals a possible functional correlation between OA and ROS. In addition, it is interesting to note that OA can restore virulence to the *Nox1*-silenced mutant. Collectively, these results suggest that interplay between ROS and OA may modulate host PCD pathways. For further study, discovery and investigation of target proteins of ROS and OA in the host PCD pathways will be needed.

The A2 mutant phenotype is reminiscent of the HR (hypersensitive response), which is generally observed in plant-biotroph interactions. Therefore, I asked whether the HR-like response triggered by the A2 mutant is mechanistically similar to the HR. The HR-like response displayed callose deposition, lignin formation, ROS generation, and cell wall cross-linking, all of which correlated with the HR and plant defense. Several defense-related genes were also up-regulated in tomato plant leaves infected with A2. In contrast, these defense responses were suppressed in plant leaves infected with wild type *S. sclerotiorum*. Furthermore, an anti-apoptotic *ced-9* gene showed negligible effect on the HR-like response, compared to PCD induced by wild type *S. sclerotiorum*. Preliminary data, using Lysotracker dye, also suggest a possibility that autophagy is involved in the HR-like response, but not in PCD induced by wild type. These findings suggest that HR-like cell death may be different from wild type-induced PCD and may utilize a different signaling pathway. Coupled with previous work, this chapter show that oxalate appears to not only activate plant PCD pathways, but it also appears to moderate the plant defense response (Kim et al. 2008b). Further experiments

are required to determine how oxalate can suppress various defense responses and what pathways are involved in the HR-like responses.

These studies provide several novel findings to help in our understanding of the underlying mechanisms of *Sclerotinia* disease. Chapter II reveals the importance of *S. sclerotiorum* NADPH oxidases in pathogenic development. Importantly, Nox1 is correlated with OA production which can direct PCD in plant tissues. Although the exact mechanism is still elusive, this study offers a new idea that the NADPH oxidase may impact the production of a secondary metabolite important for virulence. Chapter III provides evidence that the phenotype of plant leaves infected with oxalate deficient A2 mutant is similar to the HR response. Collectively, these studies may shed light on understanding of control mechanisms and cooperation between ROS, OA and plant PCD in *Sclerotinia* disease. This and other similar approaches should provide new insight into our understanding of plant-necrotroph interactions.

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