MOLECULAR INTERPLAY AMONG THE REDOX-RESPONSIVE REGULATOR AaAP1, THE TWO-COMPONENT HISTIDINE KINASE AND THE MITOGEN-ACTIVATED PROTEIN (MAP) KINASES IN Alternaria alternata OF CITRUS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2010
To my wife, Ms. Hui-Yu Hsieh, and my sisters, Ms. Mei-Ling Lin and Mei-Jyun Lin for their thorough support and encouragement
ACKNOWLEDGMENTS

This research project would not have been possible without the support of many people. I would like to express my gratitude to my supervisor, Dr. Kuang-Ren Chung, whose expertise, understanding, and patience, added considerably to my graduate experience. I appreciate his invaluable vast knowledge and skills in many areas and his assistance in my writing. I would like to thank the other members of my committee, Dr. Fredy Altpeter, Dr. Jeffrey A. Rollins, and Dr. Jeffrey B. Jones for their helpful assistance and critical evaluation of this dissertation. Special thanks also to all my graduate friends: Andrew Funk, Franklin Behlau, Qiang Chen, Xiaoen Huang, and Chang-Hua Huang, Sarsha, and Carol. They each helped make my time in the PhD program more fun and interesting. I also want to thank our Lab members, Siwy Ling Yang, Nan-Yi, Wang and Mr. Lenny Venderpool for their assistance to some aspects of my research project. Most importantly, I thank my family for the support they provided me through my entire life and in particular, I must acknowledge my wife without her endless love and encouragement I would not have finished this thesis.
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May 2010

Chair: Kuang-Ren Chung
Major: Plant Pathology

Alternaria brown spot is caused by the tangerine pathotype of Alternaria alternata. The disease affects tangerine, grapefruit and their hybrids, resulting in severe agronomic and economic losses in Florida. This research determined the important roles of signaling pathways that are mediated by three MAP kinases (AaHOG1, AaSLT2 and AaFUS3), a redox-responsive transcription regulator (AaAP1), and a two-component histidine kinase (AaHSK1) in the life cycle of A. alternata.

The results revealed that AaAP1 is necessary for cellular response and adaption to oxidative stresses. Disruption of the AaAP1 gene in A. alternata abolished antioxidant activities and increased sensitivity to H₂O₂. The AaAP1 null mutant failed to induce any visible necrotic lesions on citrus leaves, primarily due to its inability to detoxify ROS produced by the host plant.

Molecular characterization of a conserved AaFUS3 gene, encoding a FUS3-type MAP kinase, from A. alternata revealed that AaFUS3 is required for vegetative growth, conidiation, pathogenicity, and production of several hydrolytic enzymes. Two genes encoding putative Major Facilitator Superfamily (MFS) transporters were identified from
a suppression subtractive hybridization library. Expression of these two genes was coordinately regulated by AaAP1 and AaFUS3, suggesting a synergistic regulation between AaFUS3 MAP kinase and redox-responsive regulator AaAP1.

Furthermore, the AaHSK1 gene encoding a group III “two-component” histidine kinase, the AaHOG1 gene encoding a HOG1-type MAP kinase, and the AaSLT2 gene encoding a SLT2 MAP kinase, were also cloned and characterized in A. alternata. AaHSK1 is a primary regulator for cellular resistance to sugar osmotic stress and for sensitivity to dicaboximide or phenylpyrrole fungicides. AaHOG1, which conferred cellular resistance to salts and oxidative stress, bypasses AaHSK1 even though deletion of AaHSK1 affected AaHOG1 phosphorylation. These functions are likely modulated by unknown mechanisms rather than directly by the AaHOG1–mediated pathway.

AaSLT2 is necessary for conidiation, maintenance of cell-wall integrity, and fungal virulence but is dispensable for toxin production. As with AaAP1 and AaFUS3, AaHOG1 and AaSLT2 are necessary for fungal pathogenicity; yet AaHSK1 is completely dispensable for pathogenicity. Fungal mutants impaired in AaHSK1, AaHOG1, AaAP1, AaSLT2 or AaFUS3 were all hypersensitive to 2-chloro-5-hydroxypyridine (CHP) or 2,3,5-triiodobenoic acid (TIBA). Overall, this study highlights the dramatic flexibility and uniqueness in the signaling pathways that are involved in pathogenicity and respond to diverse environmental stimuli in Alternaria alternata.
Introduction of *Alternaria alternata* (Fr.) Keissler

**Taxonomy**

*Alternaria* brown spot is caused by the necrotrophic fungus, *Alternaria alternata* (Fr.) Keissler that belongs to the kingdom Fungi, phylum Ascomycota, class Dothideomycetes, subclass Pleosporomycetidae, order Pleosporales, and family mitosporic Pleosporaceae. *Alternaria* brown spot was first reported on Emperor mandarin (*Citrus reticulate* Blanco) in Australia in 1903 (Cobb 1903). The causal agent was identified as a species of *Alternaria* in 1959 (Kiely 1964) and grouped as *Alternaria citri* Ellis & Perce (Pegg 1966). Later, the pathogen which affects tangerines and rough lemon was re-classified as *A. alternata* (Kohmoto et al. 1979).

*Alternaria* species usually form dark-colored mycelia and produce short conidiophores bearing single or branched chains of conidia. Conidia are dark-pigmented, long, or pear shaped and multi-cellular with both transverse and longitudinal cross walls with the size 25-40 × 10-15 μm (Timmer 1999).

**Host-Selective Toxins Produced by *A. alternata* to Citrus**

Many pathotypes of *A. alternata* produce phytotoxins and in total more than 70 phytotoxins are known to be produced by *A. alternata* (Nishimura and Kohmoto 1983; Walton 1996). On citrus, three diseases caused by *Alternaria* species have been identified (Akimitsu et al. 2003). *Alternaria* black spot, caused by *A. citri* Ellis & Pierce is a post-harvest problem affecting all commercial citrus worldwide (Akimitsu et al. 2003). *Alternaria* brown spot is caused by the tangerine pathotype of *A. alternata* (Fr.) Keissler, whereas *Alternaria* leaf spot is caused by the rough lemon pathotype of *A. alternata*. 
These two pathotypes are indistinguishable using phylogenetic and morphological analyses. Yet, each pathotype produces a host-selective toxin with a distinct mode of action (Peever et al. 2003). The tangerine pathotype produces the host-selective ACT toxin containing a core structure of 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid. ACT toxin causes rapid electrolyte leakage from susceptible citrus hosts, such as tangerine (*Citrus reticulata*), grapefruit (*C. paradisi* Macfad.), their hybrids, and hybrids from tangerines and sweet oranges (*C. sinensis* (L.) Osbeck) (Kohmoto et al. 1993). In contrast, the rough lemon pathotype, producing the host-selective ACRL toxin, primarily attacks rough lemon (*Citrus jambhiri* Lush) and Rangpur lime (*Citrus × limonia* Osbeck). ACRL toxin affects mitochondrial functions by causing metabolite leakage and malfunction of oxidative phosphorylation (Gardner et al. 1986). ACRL toxin is not toxic to tangerines, grapefruit, and their hybrids. Host-selective toxins produced by *A. alternata* have long been known to be essential for fungal pathogenesis (Gardner et al. 1986; Kohmoto et al. 1993) and important determinants of host ranges (Kohmoto et al. 1991; Otani et al. 1995). The genes involved in the biosynthesis of host-selective toxins in *Alternaria* species are often clustered on a conditionally dispensable chromosome (Hatta et al. 2002).

**Disease Symptoms of Alternaria Brown Spot**

The tangerine pathotype of *A. alternata* infects young fruit, leaves and twigs inducing brown spots within 24 hours of infection (Timmer et al. 2000). Lesions usually display brown spots surrounded by a yellow halo which is caused by the host-selective ACT-toxin (Kohmoto et al. 1993). Necrotic lesions can extend along the veins even beyond the area of tissue colonization as the toxin is translocated through the vascular
system (Fig. 1-1). On fruit, lesions can vary from small spots to large crater-like lesions (Akimitsu et al. 2003).

**Life Cycle**

*Alternaria alternata* has no known sexual stage and has a relatively simple life cycle in citrus (Fig. 1-2) (Timmer 1999). Conidia (fungal spores) with dark-pigmented cell walls can tolerate unfavorable environmental conditions. Conidia are produced from infected leaves and can survive for a long period of time in the field. Conidia can be dispersed by wind or rain splash. Under humid conditions, conidia quickly germinate to form penetration hyphae on susceptible hosts (Akimitsu et al. 2003). The minimum period for symptom appearance is around 4-8 hours under favorable conditions (Canihos et al. 1999). The optimum temperature for infection is 27°C (Canihos et al. 1999). Penetration can occur through stomata without the formation of appressoria or direct penetrate the host cuticle with the formation of appressoria (Solel and Kimchi 1998).

**Economic Significance and Disease Control**

*Alternaria* brown spot has been widespread in Florida since first appeared in 1974 (Whiteside 1976). The disease was later documented in Israel (Solel 1991), South Africa (Schutte et al. 1992), Turkey (Canihos et al. 1997), Spain (Vicent et al. 2000), Brazil and Argentina (Peres et al. 2003). *Alternaria* brown spot can be a major problem on many citrus cultivars because the disease weakens tree development and damages the fruit. Alternaria brown spot needs to be controlled, particularly if the fruit are intended for the fresh market. One of the effective strategies to control Alternaria brown spot is frequent application of fungicides. Many fungicides, such as phthalimides (captan, folpet), dithiocarbamates (maneb, metiram), dicarboximide fungicides
(iprodione, procymidine), procloraz manganese, flutriafol, and copper fungicides are effective against *A. alternata* (Solel et al. 1996). However, frequent application of fungicides also raises concerns of pathogen resistance and off-target environmental effects.

**Oxidative Burst and Strategy of Antioxidant Defense in Fungi**

**Roles of Reactive Oxygen Species in Plants**

Plants cope with many threats from the environment through efficient defense systems that protect them from biotic and abiotic stresses (Benhamou 1996). Upon exposure to pathogen attack, plants may induce various defense mechanisms to restrict or kill pathogens. Those defense reactions may include modification of preexisting cell wall structures, production of phytoalexins, phenolic compounds, and antimicrobial proteins, induction of hypersensitive response and programmed cell death (Kombrink 1995). One of earliest defense responses to pathogen attacks in plants is the oxidative burst, described as a rapid transient production of large amounts of reactive oxygen species (ROS) around the infection site (Greenberg 1997). ROS include superoxide radicals (\(\cdot\)O\(_2\)), hydrogen peroxide (H\(_2\)O\(_2\)) and hydroxyl radicals (\(\cdot\)OH). ROS can be generated at low levels in chloroplasts and mitochondria during normal metabolic processes, but are dramatically induced in response to pathogens (Wojtaszek 1997). In plants, the main source of ROS is primarily generated by a membrane-bound NADPH-oxidase which converts NADPH and O\(_2\) to form O\(_2\)\(^{-}\) and further to H\(_2\)O\(_2\) (Lamb and Dixon 1997). Since production of ROS is rapid and transient, the roles of ROS may vary, depending on the intimate interactions between plants and the challenging factors (Doke et al. 1996; Lindner et al. 1988; Davis et al. 1993).
ROS is virtually toxic to all macromolecules including proteins, nucleic acids, and lipids. In addition to antimicrobial effects, \( \text{H}_2\text{O}_2 \) is involved in linkage of cell wall polymers. \( \text{H}_2\text{O}_2 \) can also serve as a signal for induction of programmed cell death, a characteristic of hypersensitive reactions (Greenberg 1997; Neill et al. 2002; Veal et al. 2007). In plants, ROS act as a secondary messengers for abscisic acid (ABA) and ethylene-mediated signaling pathways during stress (Chen et al. 1993; Leon et al. 1995). Apart from stress responses, ROS also modulates plant growth and development. In *Arabidopsis*, \( \text{H}_2\text{O}_2 \)-induced MAPK cascade represses auxin-inducible gene expression (Walker and Estelle 1998).

**Detoxification of ROS and Fungal Pathogenesis**

Antioxidants can be produced via enzymatic or non-enzymatic mechanisms (Cessna et al. 2000; Mayer et al. 2001; Moye-Rowley 2003). Several antioxidant enzymes are known from the microbial world. These include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (Gpx). SOD is involved in conversion of \( \text{O}_2^- \) and \( \cdot\text{OH} \) to \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \). Hydrogen peroxide can be further converted to oxygen and water by catalases and peroxidases (Mayer 2001). Glutathione (GSH) and thioredoxin are small proteins that serve as non-enzymatic antioxidants (Carmel-Harel and Storz 2000). Glutathione and thioredoxin convert \( \cdot\text{OH} \) and \( \text{H}_2\text{O}_2 \) to \( \text{O}_2 \) and water by oxidation and therefore function directly as free radical scavengers (Carmel-Harel and Storz 2000). The oxidized glutathione (GSSG) and thioredoxin can be reverted by an NADPH-dependent reaction, thus resetting the cycles. In *S. cerevisiae*, mutation in either *gpx3*, *trx2* (encoding thioredoxin), *trr1*, *trr2* (encoding thioredoxin reductase) or *tpx* (encoding thioredoxin peroxidase) resulted in strains that are
hypersensitive to H₂O₂ and t-butyl-hydroperoxide (Kuge et al. 1994; Inoue et al. 1999; Machado et al. 1997; Pedrajas et al. 1999).

The roles of ROS-detoxification in relation to pathogenesis vary among phytopathogenic fungi. For example, disruption of an sod gene (encoding SOD) in Claviceps purpurea (Moore et al. 2002), or disruption of a cat1, cat2, or cat3 gene in Cochliobolus heterostrophus (Robbertse et al. 2003) resulted in fungal strains that are hypersensitive to oxidizing agents but remain pathogenic to their hosts. However, the Botrytis cinerea ∆bcsod1 mutant defective in superoxide dismutase was reduced considerably in lesion development (Rolke et al. 2004).

Transcriptional Regulation in Response to Oxidative Stress

The mechanisms regulating the fungal response to oxidative challenge can be broadly classified into two types. In S. cerevisiae, the mechanisms are primarily regulated by the Yap1p-mediated detoxification systems (Kuge et al. 2001). In contrast, Schizosaccharomyces pombe utilizes a Sty1 mitogen-activated protein kinase (MAPK) to regulate oxidative-stress tolerance (Toone and Jones 1999). YAP1 protein homologs have been identified in a number of fungal species, such as Pap1 in S. pombe, Cap1 in Candida albicans, Yap1 in Ustilago maydis, Chap1 in C. heterostrophus, and AfYap1 in Aspergillus fumigatus (Toone and Jone 1999; Molina and Kahmann 2007; Lev et al. 2005; Lessing et al. 2007). All AP1-like proteins contain a basic leucine zipper (bZIP) and two cysteine rich domains, called the amino terminal- (n-CRD) and carboxy terminal- (c-CRD) domains (reviewed in Toone and Jone 1999). Kuge and colleagues (1997) first demonstrated that subcellular localization of Yap1 occurs in response to oxidative stress. This change depends on another protein transporter called Crm1 that binds to the nuclear export sequence (NES) in Pap1 and functions as a nuclear exporter
(Toone et al. 1998). Crm1 actively transfers Yap1 proteins from the nucleus to the cytoplasm under normal conditions. Deletion of the *crm1* gene or a point mutation within the NES region of *Yap1* blocks Crm1 binding to NES, thus Yap1 regulators are dominantly localized in the nucleus (Toone et al. 1998).

The Sty1-mediated signaling pathway of *S. pombe* resembles the HOG1 (high osmolarity glycerol) MAP kinase pathway in *S. cerevisiae* and the mammalian JNK and p38 protein kinase cascades (Toone and Jones 1998). In response to environmental stress, Wak1 (a MAPKKK) is first phosphorylated within the TXY (threonine/X/tyrosine) motif. The phosphorylated Wak1 subsequently phosphorylates the downstream Wis1 (a MAPKK), which in turn phosphorylates Sty1 (Samejima et al. 1997; Shieh et al. 1998). In response to oxidative stress, the phosphorylated Sty1 activates a second bZIP-containing transcription activator, called Atf1, in addition to PaP1. Atf1 is phosphorylated directly by Sty1 and transferred into the nucleus upon exposure to oxidative stress (Shieh et al. 1998). Several HOG1-like MAP kinases have also been found to be required for resistance to ROS in phytopathogenic fungi, including those from *B. cinerea*, *C. heterostrophus*, and *Mycosphaerella graminicola* (Igbaria et al. 2008; Liu et al. 2008; Mehrabi et al. 2006). In *S. pombe*, the H$_2$O$_2$-dependent activation of the Sty1 pathway is mediated via a histidine-containing phosphotransferase (Mcs4) that is regulated by the ‘two-component’ histidine sensor kinases Mak2 and Mak3 (Buck et al. 2001).

**Signal Transduction Cascades That Regulate Fungal Development and Virulence**

**MAP Kinase Cascade**

In eukaryotic cells, MAP kinases are responsible for transducing a variety of extracellular signals for cell growth and differentiation (Gustin et al. 1998; Kultz 1998). In *S. cerevisiae*, five MAP kinase-mediated signaling pathways have been characterized
and demonstrated to control diverse functions. Filamentous fungi have three analogous MAP kinases: FUS3/KSS1, SLT2, and HOG1-type signal transduction pathways (Banuett 1998; Gustin et al. 1998; Herskowitz 1995; Xu 2000). The FUS3 and KSS1 pathways in S. cerevisiae are partially redundant in that both share a number of components through the MAPKKK-MAPKK signaling pathway (Fig. 1-3). However, FUS3 is responsible for regulating the mating process, whereas KSS1 is involved in filamentous growth (Madhani and Fink 1998). In the maize pathogenic fungus U. maydis, the mating process is absolutely required for pathogenesis (Banuett 1995). Deletion of a ubc3 gene, a FUS3 homolog, strongly attenuated the formation of dikaryotic hyphae, blocked pheromone secretion and response, and reduced virulence (reviewed in Xu 2000). Fus3-like MAP kinases have also been shown to be involved in fungal development, formation of conidia and/or appressoria, penetration and pathogenicity in many phytopathogenic fungi (Cho et al. 2007; Zheng et al. 2000; Lev et al. 1999; Takano et al. 2000; Jenczmionka et al. 2003; Pietro et al. 2001; Xu and Hamer 1996; Ruiz-Roldan et al. 2001; Solomon et al. 2005; Rauyaree et al. 2005).

In S. cerevisiae, the SLT2 MAP kinase-mediated signaling pathway is mainly responsible for cell wall integrity and cytoskeleton reorganization (Lee et al. 1993). The M. grisea MPS1, an SLT2 homolog, is required for sporulation, appressoria formation, cell wall integrity and penetration to its host (Xu et al. 1998). Similarly, deletion of an SLT2-like gene in C. purpurea or C. heterostrophus created fungal strains defective in conidiation and pathogenicity, and increased sensitivity to lytic enzymes (Mey et al. 2002; Igbaria et al. 2008).
The high osmolarity glycerol (HOG) pathway is not only responsible for cellular response to osmotic stress, but also is required for responses to heat shock, UV radiation, cold, and oxidative stresses in the budding yeast (Shieh et al. 1998). In filamentous fungi, HOG1 kinase homologs also play an important role in stress response. For example, a HOG1 homolog, SakA in *Aspergillus nidulans* or Fphog1 in *Fusarium proliferatum*, is required for osmotic, oxidative and heat shock stresses (Adam et al. 2008; Kawasaki et al. 2002). In some phytopathogenic fungi, maintenance of intracellular osmotic balance and generation of turgor pressure are crucial for vegetative growth and pathogenicity (Howard and Valent 1996; Money and Howard 1996). In the rice blast fungal pathogen *M. grisea*, turgor pressure in the appressorium is generated due to the accumulation of intracellular glycerol (de Jong et al. 1997). However, accumulation of glycerol or generation of turgor pressure in appressoria is not controlled by a HOG1 ortholog (*OSM1*) in *M. grisea* because the gene deletion strains were still pathogenic (Dixon et al. 1999). In contrast, the HOG1-type MAP kinase gene homologs are required for pathogenicity in *C. heterostrophus* and *Botrytis cinerea* (Igbaria et al. 2008; Segmuller et al. 2007).

**Two-Component Histidine Kinases**

Similar to MAP kinases, a two-component histidine kinase (HK) has been shown to regulate diverse cellular processes, including differentiation, metabolite production and virulence in fungi (Alex et al. 1996). In prokaryotes, two-component signaling systems contain a histidine kinase (HK) and a response regulator (RR); each is encoded by a separate gene (Parkinson and Kofoid 1992). In contrast to prokaryotic HKs, all fungal HKs harbor both the HK and RR domains within the same peptide (West and Stock 2001; Wolanin et al. 2002). In response to environmental changes, a series of
phosphate transfers between histidine (His) and aspartate (Asp) residues takes place in a pattern of His-Asp-His-Asp (Loomis et al. 1997; Thomason and Kay 2000). First, the HK is autophosphorylated at a conserved His residue. The phosphate is then transferred to a conserved Asp residue located within the RR protein, then to a protein containing a His phosphotransfer (HPT) domain, and subsequently to Asp of a second RR protein. This activated RR in turn regulates downstream signaling pathways, such as mitogen-activated protein (MAP) kinase cascades, and eventually produces a change in gene expression (Wurgler-Murphy and Saito 1997; Thomason and Kay 2000; Kruppa and Calderone 2006). In yeasts and fungi, osmosensing or fungicide resistance is modulated via two-component HK systems, often in conjunction with the HOG1 signaling pathway (Ota and Varshavsky 1993; Dongo et al. 2009; Kojima et al. 2004; Yoshimi et al. 2005). However, deletion of a HK- or a HOG1 gene may result in distinct phenotypes among yeasts and fungi. For example, deletion of a histidine kinase gene homolog, OS-1/NIK in *Neurospora crassa* or dic1 in *C. heterostrophus*, generated fungal mutants that were hypersensitive to salt and sugar stresses (Schumacher et al. 1997; Yoshimi et al. 2004). However, deletion of a histidine kinase homolog, *HIK1*, in *M. grisea* yielded fungi that were only hypersensitive to sugars, but not to salts (Motoyama et al. 2005). These discrepancies open a window of opportunity for elucidation of the evolutionary relationships in the context of osmotic adaptation mechanisms in different fungi.

**Mitogen-Activated Protein Kinase (MAPK) Network**

Cross talk between the MAP kinase- and cAMP-mediated signaling pathways for regulation of mating, appressoria formation, and filamentous growth have been documented in various fungi (Banuett 1998; Xu 2000). Furthermore, different MAP
kinase pathways may also interact antagonistically or synergistically (Gustin et al. 1998). For example, pheromone treatment simultaneously activates both FUS3 and SLT2-mediated signaling pathways in *S. cerevisiae* (Zarzov et al. 1996). FUS3 and KSS1 MAPK pathways share several upstream components (Schwartz and Madhani 2004). The *C. heterostrophus* *chk1* (*a* FUS3 homolog) mutants appear to have both the phenotypes observed in *M. grisea* *pmk1* (*a* FUS3 homolog) and *mps1* (*a* SLT2 homolog) mutants (Lev et al. 1999; Xu et al. 1996). However, in each of the pathways, divergent components have evolved to ensure pathway specificity. For example, trimeric G protein, Ste5 and FUS3 are exclusively used for the mating process but not in the filamentous (KSS1) pathway (Elion 2001). Moreover, HOG1 MAP kinase is specifically required for response to osmotic stress (O'Rourke and Herskowitz 1998) (Fig. 1-3). Furthermore, it has been widely proposed that scaffold proteins that can bind two or more signaling components of a pathway promotes signaling specificity and prevents cross-talk between pathways. Ste5 and Pbs2 scaffold proteins in FUS3- and HOG1-type MAP kinase pathways (Elion 2001; Harris et al. 2001; O'Rourke and Herskowitz 1998), respectively, appear to regulate and maintain the response specificity (Fig. 1-3). Finally, activation of one pathway can cause the inactivation of the other pathway. During mating response, the activated FUS3 kinase may inhibit the downstream Tec1 transcription factor specifically required for filamentous development (Gavrias et al. 1996; Zeitlinger et al. 2003; Shock et al. 2009). The HOG1 MAP kinase may activate expression of *Msg5*, encoding a phosphatase which in turn dephosphorylates FUS3 and KSS1 MAP kinases, thereby suppressing their functions (Bardwell et al. 1996; Andersson et al. 2004).
Research Overview

The major goal for this research was to determine the functions of the redox-responsive transcriptional factor AaAP1, three MAP kinase proteins (AaHOG1, AaSLT2 and AaFUS3), and a two-component histidine kinase protein (AaHSK1) in Alternaria alternata. Specific objectives were to determine the regulation of AaAP1, each of the MAP kinase and a two-component histidine kinase; and to identify if any cross-talk occurs between them. Through genetic and molecular analyses, I intend to investigate the fungal response to host-generated reactive oxygen species by characterizing the AaAP1 gene of A. alternata, which encodes a polypeptide resembling many YAP1-like transcriptional activators implicated in cellular responses to stress. I provide experimental evidence to support the idea that ROS detoxification is critical in the pathogenicity of A. alternata (Chapter 2). Downstream genes whose expression is regulated by AaAP1 were also identified using suppression subtractive hybridization (Chapter 2). Targeted disruption of a FUS3 MAP kinase gene homolog resulted in fungal strains that are nonpathogenic to citrus (Chapter 3). I also demonstrate that A. alternata utilizes specialized or synergistic regulatory interactions between the AP1 and MAPK signaling pathways for diverse physiological functions (Chapter 3). Functional characterization of a gene encoding a group III histidine kinase (AaHSK1) and a yeast HOG1 analog (AaHOG1) shows that the two gene products to operate, both uniquely and synergistically, in a number of physiological and pathological functions (Chapter 4). Disruption of AaHSK1 acquired resistance to dicarboximide and phenylpyrrole fungicides and displayed hypersensitivity only to sugar osmotic stress. By contrast, AaHOG1 plays minor role in fungicide sensitivity and is involved in cellular resistance to oxidants and salts, but not sugars (Chapter 4). Further studies revealed that fungal
mutants impaired in *AaHSK1, AaHOG1, AaAP1, AaSLT2* or *AaFUS3* are all hypersensitive to 2-chloro-5-hydroxypyridine (CHP) or 2,3,5-triiodobenzoic acid (TIBA) (Chapter 3, 4 and 5). These phenotypes are completely novel and have never been described in any fungus previously. Overall, the results derived from my studies highlight a dramatic flexibility and uniqueness in the signaling pathways that are involved in responding to diverse environmental stimuli in *Alternaria alternata*. 
Figure 1-1. Symptoms of Alternaria brown spot. (A) Alternaria brown lesions on the leaves of Minneola tangelo. (B) Alternaria brown spots on Dancy tangerine. (C) Fungal infection occurs early in the season leading to large lesions and may induce defoliation. (D) Lesions with corky protuberances on the fruit of Minneola tangelo (Courtesy Dr. L. W. Timmer).
Figure 1-2. Disease cycle of Alternaria brown spot, caused by the tangerine pathotype of *Alternaria alternata* (Redrawn based on the work of Timmer 1999).
Figure 1-3. The *S. cerevisiae* mating (FUS3), filamentation (KSS1), cell integrity (SLT2) and high osmolarity glycerol (HOG1) MAPK pathways. Mating pathway-specific components: open circles, only one of several possible transcription factor combinations depicted; Filamentation pathway-specific components: open squares; SLT2 pathway-specific components: filled ellipse; HOG1 pathway-specific components: open hexagons, one of several known transcription factors depicted. Shared components: filled diamonds (Redrawn based on the work of Schwartz and Madhani 2004; Jurgen et al. 1999).
CHAPTER 2
THE Alternaria alternata AaAP1 TRANSCRIPTION FACTOR INVOLVED IN DETOXIFICATION OF REACTIVE OXYGEN SPECIES IS A KEY PATHOGENICITY FACTOR ON CITRUS

Due to the universal toxic effects of reactive oxygen species (ROS) and their important roles in plant defense responses, plant pathogens must develop strategies to breakdown ROS. In this work, a YAP1 homolog, designated AaAP1, was characterized in the necrotrophic fungus, Alternaria alternata, and found to play an important role in ROS detoxification and pathogenesis to citrus. The A. alternata AaAP1 contains all conserved domains required for cellular localization of YAP1 and for YAP1-mediated resistance to oxidative damage. Upon exposure to H₂O₂, the AaAP1::sGFP (synthetic green fluorescent protein) fusion protein became localized in the nucleus. Expression of AaAP1 was responsive to oxidative stress. Disruption of the AaAP1 gene resulted in mutants that are highly sensitive to H₂O₂, menadione, and tert-butyl-hydroperoxide and displayed a marked reduction in several antioxidant enzymatic activities. The AaAP1-null mutants retained normal conidiation and ACT toxin production but failed to incite necrotic lesions on Minneola leaves. Application of NADPH oxidase inhibitors partially restored lesion formation in the AaAP1-disrupted mutants. Furthermore, several downstream genes potentially regulated by AaAP1 were identified by subtractive suppressive hybridization. Introduction of a full-length AaAP1 into the AaAP1 disruptant restored resistance to oxidative stresses as well as pathogenicity to wild type levels. Taken together, I present information below that AaAP1 plays an essential role for ROS detoxification and lesion development and thus, is an important pathogenicity factor in A. alternata.
Introduction

In response to pathogen invasion, plant cells often rapidly and transiently generate reactive oxygen species (ROS), including superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical (OH) (Greenberg 1997). This defense-associated process is called the oxidative burst.

ROS may have antimicrobial effects, as well as ability to trigger programmed cell death and hypersensitive response (HR) at the site of infection (Greenberg 1997). ROS may also serve as a signal for activation of other defense responses against pathogen attacks (Neill et al. 2002; Veal et al. 2007). To survive within the harsh oxidative environment of host plants, fungal pathogens must develop strategies to detoxify or repress ROS-mediated defense system via enzymatic or nonenzymatic mechanisms (Cessna et al. 2000; Mayer et al. 2001; Moye-Rowley 2003). Indeed, plant pathogens may produce a wide array of enzymes that are capable of breaking down ROS produced by host plants. Those enzymes include superoxide dismutases (SOD), catalases (CAT), peroxidases, glutathione peroxidases, glutathione reductases, as well as thioredoxin reductases and thioredoxin peroxidases (Staples and Mayer 1995).

In *Saccharomyces cerevisiae*, the YAP1 transcription factor has been intensively studied for controlling oxidative-stress response (Moye-Rowley 2003). YAP1 was identified as an ortholog of mammalian AP-1 transcriptional activator based on its ability to bind to an AP-1 response element (ARE: TGACTAA) in the promoter region (Harshman et al. 1988). YAP1, containing a basic leucine zipper (bZIP) domain, is responsible for cellular resistance to $H_2O_2$, drugs and heavy metals (Toone et al. 2001). Two cysteine-rich domains: carboxyl terminus (c-CRD) and amino terminus (n-CRD) are required for appropriate nuclear exportation and subcellular localization, and thus are
critical for YAP1-mediated resistance to oxidative stress (Coleman et al. 1999; Delaunay et al. 2000; Kuge et al. 2001). In addition, a characteristic nuclear export sequence (NES) carrying a short stretch of leucine amino acids is present in the c-CRD domain of YAP1. This domain is required for binding by nuclear export protein Crm1p under normal conditions (Toone et al. 1998). Upon exposure to oxidative stress, redox signals induce formation of intramolecular disulfide bonds within YAP1, resulting in conformational changes. Since the NES is invisible to Crm1p, YAP1 remains localized in the nucleus and activates oxidative stress-related genes (Toone and Jones 1999). Indeed, numerous genes under YAP1 regulation have been identified to be related to oxidative damage in *Saccharomyces cerevisiae* (Toone et al. 2001). Those include *GSH1* encoding a *r*-glutamylcysteine synthetase for glutathione synthesis (Stephen et al. 1995; Wu et al. 1994); *GSH2* encoding a protein for glutathione biosynthesis (Sugiyama et al. 2000); *GLR1* encoding a glutathione reductase (Grant et al. 1996); *GPX2* encoding a GSH peroxidase (Inoue et al. 1999); *TRR1* encoding a thioredoxin reductase (Lee et al. 1999); *TRX2* encoding a thioredoxin (Kuge and Jones 1994); *FLR* encoding a multidrug resistance transporter (Nguyen et al. 2001); *YCF1* encoding an ABC transporter essential for cadmium tolerance, and many others (Morgan et al. 1997).

The YAP1 transcription factor involved in ROS detoxification has been identified as an essential virulence factor in the biotrophic maize pathogen *Ustilago maydis* (Molina and Kahmann 2007) and the opportunistic human pathogen *Candida albicans* (Enjalbert et al. 2007). However, disruption of a YAP1 gene homolog, *chap1* in *Cochliobolus heterostrophus* (Lev et al. 2005) or *AfYap1* in *Aspergillus fumigatus* (Lessing et al. 2007) did not change virulence on the respective hosts. Thus, the role of ROS in host defense
against fungal pathogens remains elusive. The interactions may depend on the lifestyle of the pathogen (Glazobrook 2005) and the effectiveness of its own ROS detoxification machinery.

In this Chapter, I report on the cloning and characterization of a gene, designated AaAP1 (*Alternaria alternata* AP-1 like), encoding a yeast YAP1 homolog from the tangerine pathotype of *A. alternata*. My objectives are to determine whether AaAP1 is involved in the oxidative stress response, to identify possible downstream genes regulated by AaAP1, and to evaluate whether fungal antioxidant systems are important for *A. alternata* pathogenicity.

**Materials and Methods**

**Fungal Strains and Culture Conditions**

The wild type EV-MIL31 strain of *Alternaria alternata* (Fr.) Keissler used in this study was single-conidium cultured from diseased leaves of Minneola tangelo, a hybrid between Duncan grapefruit (*Citrus paradise* Macfad.) and Dancy tangerine (*Citrus reticulate* Blanco) in Florida. Fungal isolates were cultured on potato dextrose agar (PDA, Difco Laboratories) at 28°C. Conidia were harvested from fungal cultures grown on PDA under cool-white fluorescent light for 3 to 4 days. For DNA and RNA purification, fungal strains were grown on PDA overlaid with sterile cellophane for 2 days. For preparation of protoplasts, fungal isolates were grown in 50 ml of potato dextrose broth (PDB, Difco Laboratories) for 5 days, blended, mixed with fresh 200 ml PDB, and incubated for an additional 24 h. Fungal mycelia were collected after centrifugation at 6,500 × g at 4°C for 10 min and resuspended in a wash solution (1 M NaCl, 10 mM CaCl₂). Fungal protoplasts released after treating with cell-wall-degrading enzymes for 2
h were harvested by centrifugation at 3000 \times g for 10 min, and resuspended in sterile STC solution (1.5 M sorbitol, 10 mM CaCl₂, 10 mM Tris-Cl pH 7.5) as described (Chung et al. 2002). Transformation of *A. alternata* EV-MIL31 using a CaCl₂ and polyethylene glycol-mediated method was performed by mixing PCR fragments or plasmid construct with protoplasts (1 \times 10⁶/ml) as described previously (Chung et al. 2002). Fungal transformants were regenerated and selected on regeneration medium (RMM) amended with hygromycin or sulfonylurea (Chung et al. 2002).

**Lipid Peroxidation Assays**

Lipid peroxidation assays were performed based on the production of malondialdehyde (MDA) from lipid derivatives reacting with thiobarbituric acid (TBA) during the oxidation of polyunsaturated fatty acid (Zawoznik et al. 2007). Briefly, 0.2 g Minneola leaves inoculated with or without conidial suspension was grounded in 2 ml of 20% trichloroacetic acid (TCA) and centrifuged at 10,000 \times g for 10 min at room temperature. The supernatant was collected and mixed with equal volume of 0.5% TBA and 100 \mu l of butylhydroxitoluene (BHT; 40 mg/ml). MDA was formed after heating at 95 °C for 30 min and measured spectrophotometrically at 532 nm. Absorbance value measured at 532 nm was normalized by subtracting that of non-specific absorption at 600 nm. The MDA concentration was calculated using its extinction coefficient 155 mM⁻¹ cm⁻¹.

**Detection of H₂O₂ in Citrus Leaves**

H₂O₂ accumulated in the citrus leaves was identified by the formation of brown polymerization product of 3,3’-diaminobenzidine (DAB) as described (Orozco-Cardenas and Ryan 1999) with some modifications. As described above, 5 \mu l conidial
suspensions (1 × 10^4 / ml) of A. alternata were inoculated onto detached Minneola leaves for 12 to 24 h. Leaves with no visualized lesions were immersed in 5 mM DAB solution (pH 3.8) in darkness for 16 h at room temperature. Leaves were photographed after decolorization by soaking in 95% ethanol for 2 days.

Cloning of AaAP1

A 0.6-kb AaAP1 DNA fragment was amplified from genomic DNA of the A. alternata EV-MIL31 strain using a Go-Taq DNA polymerase (Promega) with two degenerate primers AP-1F and AP-1R (Table A-1). The primers are complementary to the conserved N-terminal cysteine-rich domain. The amplicon was cloned into a pGEM-T easy vector (Promega). Sequence analysis revealed that the amplified DNA fragment displayed amino acid similarity to many AP1- like proteins. The entire AaAP1 open reading frame (ORF) as well as its promoter region was obtained with two inverse primers yap-31 and yap-32 from XhoI-digested and self-ligated DNA templates of A. alternata. ORF and exon/intron locations were verified by comparisons of genomic and cDNA sequences. The promoter region was analyzed using regulatory sequence analysis tools (van Helden 2003). Functional domains were predicted according to the PROSITE database using ExPASy (Henikoff et al. 2000) or Motif/ProDom and Block programs. The A. alternata AaAP1 sequence has been deposited in the EMBL/GenBank Data Libraries with accession number FJ376607.

Creation and Identification of the AaAP1 Null Mutants

All oligonucleotide primers used in this study are shown in Table A-1. To disrupt AaAP1, a 1.7-kb DNA fragment containing the entire AaAP1 ORF was amplified with two primers yap1DF2 and yap1DR2 and cloned into pGEM-T easy vector to create T-
yapDFR2. A 1.6-kb hygromycin phosphotransferase (HYG) gene cassette under control of the *A. nidulans* trpC promoter was obtained from pUCATPH (Lu et al. 1994) after digestion with *Bam*HI. The fragment was end-filled and cloned into T-yapDFR2 at the blunted *Nru*I site to generate a disruption construct, T-yapHyg. A split-marker strategy was used for gene disruption (Choquer et al. 2005). Two truncated but overlapping HYG fragments fused with either 5’ or 3’ end of *AaAP1* were amplified. A 1.5-kb DNA fragment containing 5’*AaAP1* and 3’ HYG was amplified with primers yapDR2 and hyg3; a 2.1-kb DNA fragment encompassing 3’ *AaAP1* and 5’ HYG was obtained with two primer yapDR2 and hyg4 (Fig. 2-3A). PCR fragments were directly transformed into protoplasts prepared from the wild type EV-MIL 31 strain, using CaCl₂ and polyethylene glycol as previously described (Chung et al. 2002). Fungal transformants were selected on RMM medium with 200 μg/ml hygromycin (Roche Applied Science), tested for the sensitivity to H₂O₂, and further confirmed by Southern and Northern blot analyses.

**Genetic Complementation of an AaAP1-Disrupted Mutant**

To complement an *AaAP1* null mutant, a 3.8-kb DNA fragment containing functional *AaAP1* with its endogenous promoter was amplified from *A. alternata* genomic DNA with two primers hypo1 and yap-taa using a high-fidelity DNA polymerase (Roche Applied Science). The amplified DNA products were co-transformed with the pCB1532 plasmid carrying the *Magnaporthe grisea* acetolactate synthase gene (*SUR*) cassette that confers sulfonylurea resistance (Sweigard et al. 1997) into protoplasts of the *AaAP1* null mutant. Transformants were selected on a medium containing 5 μg/ml sulfonylurea (chlorimuron ethyl) (Chem Service, West Chester, PA, U.S.A.) and tested for H₂O₂ sensitivity.
AaAP1 Localization

A joining PCR method was performed to generate a fusion construct between the 
*AaAP1* and *GFP* genes (Fig. 2-6A). A 4.0-kb DNA fragment containing a functional 
*AaAP1* gene and its endogenous promoter was amplified with two primers hypo1 and 
AP1::sGFP by a PFU DNA polymerase (Stratagene). A synthetic green fluorescent 
protein (sGFP)-coding DNA fragment was amplified from the plasmid pTdsGFP::ToxA 
with the primers sGFP::AP1 and sGFP.nos. The resulting fragments were mixed and 
further amplified with the primers hypo-1 and sGFP.nos to form a 5.0-kb *AaAP1::sGFP* 
fusion construct. Primers AP1::sGFP and sGFP::AP1 share complementary sequences. 
The *AaAP1::sGFP* fusion construct was co-transformed with pCB1532 into protoplasts 
prepared from an *AaAP1* null mutant. Transformants were selected on RMM medium 
containing sulfonylurea at 5 μg/ml and tested for H₂O₂ sensitivity and for green 
fluorescence.

Sensitivity Test of *AaAP1* Null Mutants

Assays for sensitivity to H₂O₂ or other chemicals were conducted by transferring 
fungal mycelia as a toothpick point inoculation onto PDA agar containing oxidants or 
compounds and incubating under constant fluorescent light. Fungal radial growth was 
measured at 4-5 days.

Pathogenicity Assays

Pathogenicity assays were conducted on detached Minneola leaves (4-6 days after 
emergence and approximate 2 to 3 cm) inoculated with conidial suspension. Conidia 
were isolated as previously described (Peever et al. 2000). Briefly, fungal strains for 
inoculation were incubated at 27°C under cool-white fluorescent light for 5-6 days and
conidia were harvested in sterile water with low-speed centrifugation (5000 × g). The concentration of conidia was adjusted to 1 × 10^4 conidia/ml. On each spot, 5 μl conidial suspension was inoculated on the Minneola leaves and the inoculated leaves were incubated in a moist chamber for lesion development for 2-6 days.

**Purification of ACT Host-Selective Toxin**

Production of ACT toxin by *A. alternata* was carried out in a modified Richard’s medium (25 g of glucose, 10 g of KNO₃, 5 g of K₂PO₄, 2.5 g of MgSO₄, 0.02 g of FeCl₃, and 0.005 g of ZnSO₄ per liter) as described (Kohmoto et al. 1993). Fungal isolates were grown in 200 ml Richard’s medium at room temperature for 24 days. Fungal mycelia were harvested by filtration through three layers of filter paper. The culture fluid was adjusted to pH 5.5 with 10% sodium phosphate buffer and mixed with 30 g of Amberlite XAD-2 resin (Aldrich) in a constant stir for 2 h. Amberlite XAD-2 was packed in a column and ACT toxin was eluted with 400 ml of methanol. Methanol was evaporated and the remaining solution was partitioned five times with equal volume of ethyl acetate. The organic solvents were collected, combined, and evaporated at 50°C. The final residue was dissolved in methanol and analyzed spectrophotometrically or separated by thin-layer chromatography utilizing TLC plates coated with a 60F254 fluorescent silica gel (5 by 20 cm; Selecto Scientific). The solvent system contained benzene/ethyl acetate/acetic acid (50:50:1, v/v). ACT toxin was visualized as a band using a hand-held UV light (UVP, San Gabriel, CA). Bands were marked, scraped from the plate, eluted with methanol, and tested for toxicity. A leaf necrosis assay for the toxicity of ACT toxin was performed by placing 5-10 μl of solution on detached Minneola leaves as described (Kohmoto et al. 1993). The treated citrus leaves were incubated in...
a moist chamber at 25°C under light and examined daily for appearance of necrotic lesions.

**Microscopy**

Conidial viability was tested by treating conidia with or without 0.1% H₂O₂ for 30 min and staining with 0.1% Evan’s blue dye as described (Taylor and West 1980). The percentage of non-blue cells over total cells was used as the index of viability.

To visualize *A. alternata* strains within the Minneola leaves, 5 μl conidial suspensions (1 × 10⁴ conidia/ml) prepared from wild type and the AaAP1 disrupted mutant were point inoculated on the leaves. After 2 days postinoculation (dpi), leaf samples were fixed with 3% glutaraldehyde dissolved in 0.1 M potassium phosphate buffer (pH 7.2) and 2% osmium tetroxide. The plant tissues were embedded in Spurr’s plastic after dehydration with an acetone series. Samples were sectioned and examined by light microscopy (Leica Microsystems Inc., Exton, PA, U.S.A).

GFP fluorescence was detected using a Leitz Laborlux phase contrast microscope equipped with a 450 to 490-nm excitation filter and a 520-nm barrier filter (Leica Microsystems). Fungal nuclei were stained with 4′-6-diamidine-2-phenylindole (DAPI) fluorescence as previously described (Chung et al. 2002) and detected using a 340-380 nm excitation filter and a 425 nm barrier filter.

**Enzymatic Assays**

Fungal proteins extracted with ice cold 250 mM potassium phosphate buffer (pH 7.0) after grinding fungal mycelia in liquid nitrogen were collected by centrifugation at 10,000 × g for 15 min at 4°C. Concentration of crude proteins was determined by a protein assay kit (Bio-Rad, Hercules, CA, U.S.A.). Total cellular catalase activity was
determined by measuring the decomposition of $\text{H}_2\text{O}_2$ with a colorimetric reagent (200 ml of 34.2 mM purpald dissolved in 480 mM hydrochloric acid) (Johanson and Borg 1988). Briefly, 50 $\mu$g of fungal proteins were mixed with 95% methanol and 10 $\mu$l of 0.3% $\text{H}_2\text{O}_2$ and incubated at room temperature for 20 min. The reaction was stopped by adding 100 $\mu$l of 7.8 M potassium hydroxide, mixed with 34.2 mM purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole), spun for 10 min to remove precipitate (Purple color), and measured at $A_{550}$. One unit of catalase is defined as that required to decompose 1.0 mM $\text{H}_2\text{O}_2$ per min at pH 7.0 at 25°C.

Peroxidase activity was determined by the formation of purpurogallin (2,3,4,6-tetrahydroxy-5H-benzocycloheptene-5-one) from pyrogallol (ACROS) in the presence of $\text{H}_2\text{O}_2$ (Abrash et al. 1989). The enzymatic reaction containing 50 $\mu$g crude extract proteins, 0.5% $\text{H}_2\text{O}_2$, and 5% pyrogallol in 100 mM phosphate buffer (pH 6.0) was incubated at 25°C for 1 min and measured at $A_{420}$.

The overall activity of superoxide dismutase (SOD) was determined by the reduction of nitrotetrazolium blue (NBT) chloride to NBT-diformazan by superoxide radical that is generated by xanthine oxidase during conversion of xanthine to uric acid and $\text{H}_2\text{O}_2$ (Giannopolitis and Ries 1997). The reaction containing 50 $\mu$g/ml of fungal protein extracts, 0.75 mM NBT, 3 mM xanthine, and 4.4 $\mu$l of 10 nM of xanthine oxidase, was incubated for 30 s and absorbance measured at $A_{550}$. One unit of SOD inhibits 50% of NBT-diformazan formation under the conditions of assay. Standard curves were constructed using pure catalase, peroxidase, or SOD (Sigma).

Lignin-type peroxidase activity was determined by formation of yellow color after reaction with 1 mM 3,3'-diaminobenzidine (DAB) dissolved in 50 mM potassium
phosphate. Fungal proteins (30 μg/ml) were mixed with 100 μl DAB for 1 h and the reaction was measured at A482 (Archibald 1992).

The Mn-type peroxidase using phenol red as a colorimetric reagent was evaluated as described (Kuwahara et al. 1984). Fungal proteins (30 μg) were added into a sodium phosphate buffer (pH 4.5) containing 0.2 mM MnSO4, 0.1 mM H2O2, and 0.0025% phenol red. The reaction was performed at 25°C for 1 h and absorbance measure at A431.

Ascorbate peroxidase (APX) activity was assayed according to the method of Nakano and Asada (1981). The hydrogen peroxide-dependent oxidation of ascorbate was determined by a decrease in the absorbance at 290 nm. The reaction mixture (2 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 0.02% H2O2 and 30 μg protein sample. APX activity was expressed as mmol ascorbate oxidized min⁻¹ g⁻¹ fungal dry weight.

Glutathione peroxidase (Gpx) was measured at A340 for decreasing absorbance after adding NADPH and tert-butyl-hydroperoxide into 0.1 M phosphate buffer (pH 7.0) containing fungal proteins, reduced glutathione (GSH), and glutathione reductase as described (Wheeler et al. 1990). Glutathione reductase (GR) was measured at A340 for decreasing absorbance in the presence of oxidized glutathione (GSSG), EDTA, and NADPH. One unit of glutathione peroxidase or reductase forms 1.0 μmol NADP⁺ from NADPH per min at pH 7.0 at 25°C.

Glutathione-S-transferase (GST) activity was determined by the formation of p-nitroanilide from glutamic acid p-nitroanilide (Zablotowicz et al. 1995). Fungal proteins (30 μg) were mixed with 50 μl of 20 mM glutathione and 20 mM 1-chloro-2,4-
dinifrobenzene dissolved in 95% ethanol, incubated for 30 min, and measured at $A_{450}$.
The regression line was established using pure glutathione-S-transferase (Sigma).

Laccase activity was determined spectrophotometrically using ABTS
[2,2:azinobis(3-ethylbenzthiazoline-6-sulfonic acid)] as a substrate (Niku-Paavola et al. 1998). The laccase reaction contained 0.5 ml of extracellular culture fluid and 14 nM of
ABTS dissolved in 50 mM glycine-HCl (pH 3.0). The reaction was monitored by
measuring the change in $A_{436}$ for 5 min. The laccase activity was expressed as
nanokatals (nanomoles/second). The extinction coefficient of 29,300 M$^{-1}$ cm$^{-1}$ was used
to calculate the amounts of the oxidized ABTS.

Total sulfhydryl content was determined by formation of thio-bis-nitrobenzene (TNB)
after reacting with 5,5'-dithio-bis-nitrobenzoic acid (DTNB) (Holmgren 1977). Fungal
proteins (30 $\mu$g) were mixed with 780 $\mu$l of 0.2 M Tris-HCl (pH 8.0) and 20 $\mu$l of 5 mM
DTNB, incubated at 25$^\circ$C for 30 min, and measured at $A_{412}$.

All experiments were carried out two times with at least three replicates. The enzyme
activities of the wild type, the AaAP1 mutants and the complementation strains were
compared by Analysis of Variance (ANOVA) within the SPSS statistical analysis
software (SPSS Inc.). A $p$-value of $< 0.05$ was interpreted as a significant difference.

**Molecular Techniques**

Plasmids propagated in *Escherichia coli* DH5-$\alpha$ were isolated with a Wizard DNA
purification kit (Promega). Fungal DNA was purified with a DNeasy Plant Mini kit
(Qiagen, Valencia, CA, U.S.A.). RNA was extracted with a TRIZOL RNA isolation kit
(Invitrogen, Carlsbad, CA, U.S.A.). DNA probes for Southern and Northern blot
analyses were labeled with digoxigenin (DIG)-11-dUTP (Roche Applied Science) by
PCR with specific primers yap-31 and yap-atg. Procedures and conditions for pre-hybridization, hybridization, washing and immunological detection of the probe with a CSPD chemofluorescent substrate for alkaline phosphatase were performed following the manufacturer’s recommendations (Roche Applied Science).

Results

**Stress Responses of Citrus Leaves Inoculated with A. alternata**

Lipid peroxidation is one of the hallmarks of cellular injury in plants and often used as an indicator of oxidative stress in cells and tissues (Hodges et al. 1999). To determine if A. alternata would induce lipid peroxidation, Minneola leaves were inoculated with conidial suspension prepared from the wild type strain. As shown in Fig. 2-1A, citrus leaves inoculated with A. alternata accumulated malondialdehyde (MDA), which is one of the most abundant carbonyl products of lipid peroxidation (Fig. 2-1A). Accumulation of H$_2$O$_2$ in citrus leaves was determined using 3,3’-diaminobenzidine (DAB) as a substrate. Inoculation of A. alternata in Minneola leaves resulted in brown polymerization as being the indicative of H$_2$O$_2$ accumulation (Fig. 2-1B). The results implicate the accumulation of ROS in citrus responding to A. alternata.

**Characterization of an AP1 Homolog in A. alternata**

The AaAP1 gene cloned from the tangerine pathotype of A. alternata has a 2021-bp ORF interrupted by two introns (50 and 81 bp). Further analysis of 832-bp upstream sequence from the putative ATG translational initiation codon found a putative stress responsive element (STRE: AGGGG) that can be induced by various stresses including oxidative damage in yeasts (Marchler et al. 1993).

The AaAP1 gene encodes a polypeptide of 629 amino acids, showing 44-87% similarity and 33-81% identity to numerous of AP1-like proteins in yeasts and fungi.
AaAP1 protein is most similar to the AP1-like proteins of *Pyrenophora tritici-repentis* (XP_001931984) and *C. heterostrophus* (AAS64313). The predicted AaAP1 polypeptide contains several conserved domains of YAP1 orthologs (Fig. 2-2A and B): a basic leucine zipper (b-ZIP) DNA binding domain (amino acids 161-224), an N-terminal cysteine-rich domain (n-CRD; amino acids 387-429), and a carboxyl-terminal cysteine-rich domain (c-CRD; amino acids 572-605). Additionally, a putative hydrophobic nuclear export sequence (NES; amino acids 564-577) located in c-CRD was found. This site can be recognized and bound by the Crm1p-like exporter (Yan et al. 1998) and is critical for subcellular localization of AaAP1 during oxidative stress.

**Targeted Disruption of AaAP1**

Two split-marker fragments carrying a truncated hygromycin phosphotransferase gene (*HYG*) fused with either 5'- or 3'- *AaAP1* sequence were amplified from the disruption construct (T-yapHyg) and directly transformed into wild type for targeted gene disruption. In total, two of 35 transformants recovered from media containing hygromycin were hypersensitive to 0.1% H$_2$O$_2$ and were analyzed further. Southern blot hybridization of *Spe*I-digested genomic DNA from wild type detected an expected 2.4-kb hybridizing band. However, two putative *AaAP1* disrupted mutants showed a 4.0-kb band, resulting from insertion of an additional 1.6-kb *HYG* gene cassette at *AaAP1* locus (Fig. 2-3B). The putative *AaAP1*-disrupted mutants were further analyzed by Northern blotting (Fig. 2-3C), confirming that the *AaAP1* gene has been successfully disrupted in *A. alternata*. Analysis of *Spe*I-digested genomic DNA isolated from six transformants that were resistant to both hygromycin and H$_2$O$_2$ by Southern blot hybridization identified the 2.4-kb bands similar to that of wild type (Fig. 2-3D).
**AaAP1 Is Required for Resistance to Oxidative Stress**

The AaAP1 mutants showed 30% growth reduction compared to wild type on PDA. Growth of AaAP1 null mutants was inhibited by 0.1% H$_2$O$_2$, 2 mM menadione, 0.02% tert-butyl-hydroperoxide, or 1 mg/ml KO$_2$ to various degrees (Fig. 2-4A and some data not shown). However, the AaAP1 mutants were not sensitive to 0.1% SDS, 1 mg/ml MTT ([4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), 1 M sorbitol, mannitol, NaCl, or KCl (Fig. 2-4A). Introduction of a functional AaAP1 gene with its endogenous promoter into a null mutant restored all defective phenotypes, as exemplified in the CP1 and Cp2 strains (Fig. 2-4A).

The toxicity of H$_2$O$_2$ to A. alternata was further evaluated for the viability of conidia after staining with 1% Evan’s blue. Dead cells cannot exclude dye and stain blue, whereas live cells effectively export dye and remain clear. In the absence of H$_2$O$_2$, all strains were viable and stayed clear with no obvious difference throughout the assay period. Conidia of both wild type and the Cp1 strain remained clear and viable in the presence of hydrogen peroxide (Fig. 2-4B). After exposure to H$_2$O$_2$ for 30 min, greater than 90% of the conidia of the AaAP1 null mutant were stained blue, indicative of cell death (Fig. 2-4C).

**AaAP1 Null Mutants Have Defective H$_2$O$_2$ Metabolism**

To determine if A. alternata strains are able to detoxify H$_2$O$_2$, fungal cultures of the wild type, the AaAP1 mutants, and the complementation Cp1 strain were immersed with 0.1% H$_2$O$_2$ for 30 min and stained with DAB. H$_2$O$_2$ reacted with DAB, forming a brownish polymer around fungal colonies. The AaAP1 mutant colonies became dark brown 5 h after staining with DAB, indicating H$_2$O$_2$ accumulation around the fungal hyphae (Fig. 2-5A). However, the wild type and Cp1 colonies remained largely white. To
further evaluate if the AaAP1 null mutant was impaired in H₂O₂ metabolism, detoxification of hydrogen peroxide by Alternaria strains was assessed by measuring H₂O₂ reduction over time in solution. The wild type and Cp1 strains quickly consumed H₂O₂: more than 65% of H₂O₂ was consumed or detoxified within 30 min (Fig. 2-5B). In contrast, degradation of H₂O₂ by the AaAP1 null mutant was significantly slower.

Expression of AaAP1 Is Induced by Oxidative Stress

Northern blot hybridization was performed to evaluate if expression of the AaAP1 gene responds to oxidants in axenic culture. The wild type AaAP1 transcript was barely detectable when the fungal culture was grown on PDA, but accumulated to higher level after H₂O₂, menadione, or tert-butyl-hydroperoxide treatment, and to a lesser extent in response to KO₂ (Fig. 2-5C). Treatment with SDS, rose Bengal, or MTT did not induce AaAP1 expression.

Nuclear Localization of AaAP1::sGFP upon Exposure to H₂O₂

To investigate the mode of AaAP1 activation, the AaAP1 gene was fused translationally in frame with the gene encoding a synthetic green fluorescent protein (sGFP) (Fig. 2-6A). To ensure that the AaAP1::sGFP fusion protein was functioning correctly, the fusion construct was transformed into an AaAP1 null mutant and only strains with restored phenotype for H₂O₂ resistance were chosen for microscopic analysis. In the absence of H₂O₂, the AaAP1::sGFP fusion protein shows diffuse fluorescence in cytoplasm (Fig. 2-6B). After treatment with H₂O₂, the fusion protein became localized in the nucleus.

Regulation of ROS-Related Enzymatic Activities by AaAP1

Compared to the wild type and the complementation strains, the AaAP1 null mutants displayed a marked reduction in glutathione-S-transferase (GST), glutathione
peroxidase (Gpx), glutathione reductase (Grx), catalase, peroxidase, and SOD activities (Fig. 2-7). The AaAP1 null mutants also showed a significant reduction in lignin-type peroxidase activity. In contrast, AaAP1 null mutants did not alter Mn-type peroxidase, ascorbic peroxidase, laccase activities, total sulfhydryl (-SH) or glutathione (non-protein-SH) contents (data not shown).

Identification of the Genes Whose Expression is Regulated by AaAP1

More than 40 expression sequence tags (EST) using suppression subtractive hybridization (SSH) were recovered from the wild type cDNA library subtracted with that of the AaAP1 null mutant (Table 2-1). Northern blot analyses revealed that deletion of the AaAP1 gene downregulated expression of the clones #2 (encoding a conserved hypothetical protein), #8 (encoding a putative fatty acid synthase subunit α reductase), #10 (encoding a NmrA-like Hscarg dehydrogenase), #19 (encoding a MFS transporter), and #54 (encoding another MFS transporter) (Fig. 2-8). Expression of the clone #62 (encoding a non-ribosomal peptide synthase) was up-regulated in the AaAP1 mutant.

AaAP1 Is Required for the Virulence in A. alternata

To determine if the AaAP1 gene product plays an essential role during fungal pathogenesis, conidia prepared from the wild type, the AaAP1 mutants (D1 and D2), and the complementation strains (Cp1 and Cp2) were inoculated on detached Minneola leaves using point or spray inoculation techniques. The AaAP1 disruptants failed to incite visible lesions on unwounded leaves at 4 days postinoculation (dpi), whereas both the wild type and Cp1 strains developed typical necrotic lesions surrounded by yellow halos at 2-4 dpi (Fig. 2-9A). As much as 18% of total spots inoculated by AaAP1 null mutants induced small lesions at 4 dpi (Fig. 2-9B), which probably were caused by the host-selective ACT toxin produced by the AaAP1 mutants. Pathogenicity assessment
carried out by spray inoculation also verified that the AaAP1 null mutants cannot cause visible symptoms on Minneola leaves (Fig. 2-9C). Furthermore, wounding the leaves prior to inoculation did not facilitate colonization and lesion formation by the AaAP1 mutants D1 and D2 (Fig. 2-9D).

The AaAP1 Null Mutant Is Impaired in Penetration and Colonization Stages

Minneola leaves inoculated with the wild type, the AaAP1 disruptant (D1), and the complementation Cp1 strains were investigated using light microscopy. The wild type and the Cp1 strains successfully invaded plant cells and disrupted epidermal layers and cellular organelles (Fig. 2-10A and C). By contrast, the disrupted mutant did not cause degradation of cell organelles and conidia were arrested on the leaf surface (Fig. 2-10B).

Disruption of the AaAP1 Gene Did Not Affect Host-Selective Toxin Production

The tangerine pathotype of A. alternata produces the host-selective ACT toxin that has been demonstrated to be essential for fungal pathogenesis (Hatta et al. 2002). Northern blot hybridization of RNA prepared from the wild type, the AaAP1 null mutant, and the complementation strains to an AKT homolog (encoding a 9,10-epoxy-8-hydroxy-9-methyldecatatrienoic acid for ACT toxin biosynthesis) probe (Masunaka et al. 2000) identified a 3.6-kb transcript with similar intensities (Fig. 2-11A). A leaf necrosis assay was used to determine if ACT toxin was produced by A. alternata in axenic culture (Kohmoto et al. 1993). Culture filtrates of wild type, the AaAP1 mutant, and the complementation Cp1 strains all induced similar necrotic lesions on Minneola leaves (Fig. 2-11B). ACT toxin was purified using Amberlite XAD-2 resin and ethyl acetate from culture filtrates. Spectrophotometric scanning revealed that the ethyl acetate extracts prepared from all test strains displayed a strong absorbance at 210 nm (Fig. 2-11C). Thin-layer chromatography (TLC) analysis also revealed no significant differences
among the culture filtrates (Fig. 2-11D). One of the bands ($R_f$ 0.53, the ratio of the distance migrated by a substance compared with the solvent front) was scraped from the silica gel and showed to incite necrotic lesions on detached Minneola leaves (Fig. 2-11E). An $R_f$ 0.58 band did not cause any visible lesions (data not shown). Therefore, deletion of the $AaAP1$ gene in $A. alternata$ did not affect ACT toxin production.

**NADPH Oxidase Inhibitors Partially Restore Pathogenicity of the $AaAP1$ Null Mutant**

In plants, NADPH oxidases are involved in the production of $H_2O_2$ and superoxide in response to pathogens (Doke et al. 1996). A NADPH oxidase inhibitor, apocynin (hydroxyl-3 methoxyacetophenone) or diphenylene iodonium (DPI) was co-applied with conidia of the $AaAP1$ mutant on detached Minneola leaves to determine if the inhibitors would affect pathogenicity of the mutant. Co-inoculation of the $AaAP1$ disruption mutant with apocynin or DPI induced necrotic lesions at 5 dpi, and the lesions continued to expand at 8 dpi (Fig. 2-12). However, application of NAPHD oxidase inhibitors or the $AaAP1$ null mutant did not incite any visible lesions on Minneola leaves. The wild type strain of $A. alternata$ induced necrotic lesions on Minneola leaves at 2 dpi; thus, the NADPH oxidase inhibitors only partially restored virulence of the $AaAP1$ disruption mutant.

**Discussion**

Redox regulation is one of the important mechanisms for controlling cellular differentiation, cellular defense, and cell signaling in all eukaryotic cells (Aguirre et al. 2005; Apel and Hirt 2004; Mittler 2002; Neill et al. 2002). Of the key determinants that trigger a battery of defensive reactions during the hypersensitive cell death, one of the early responses is the transient production and accumulation of toxic ROS near the
infection courts (Greenberg and Yao 2004). In plants, the defense response mechanism by generating ROS is triggered by pathogen invasion. ROS can induce considerable damage to macromolecules, including fatty acids, proteins, enzymes, sugars and nucleic acids and may further result in programmed death to protect cells against biotic or abiotic stress (Glazebrook 2005; Spoel et al. 2007). In addition to ROS, lipid peroxidation derived from the oxygenated byproducts of lipid also plays a crucial role in plant early defensive responses (Deighton et al. 1999). As shown in the present study, citrus cv. Minneola attacked by *A. alternata* quickly provoked lipid peroxidation and accumulated H$_2$O$_2$ around the inoculation site, indicative of early defense responses.

In budding yeast, regulation of gene expression by oxidative stress has been demonstrated to be mediated by the bZIP-containing transcription regulator, YAP1 (Moye-Rowley 2003). Similar to YAP1 in *S. cerevisiae*, AaAP1 transcription factor contains a bZIP domain, two CRDs, and a NES that have been shown to be important for YAP1 cellular localization in the budding yeast. The genes, whose products are involved in the detoxification of ROS, such as catalase, peroxidase, SOD, glutathione reductase, glutathione synthase, thioredoxin reductase, and multidrug resistance transporter, have been demonstrated to be regulated by YAP1 (Godon et al. 1998). Similar mechanisms might be applicable in *A. alternata* as well. Indeed, deletion of the *AaAP1* gene resulted in a fungal strain that is hypersensitive to several oxidants and impaired in H$_2$O$_2$ metabolism. Several antioxidant enzymes involved in ROS detoxification were shown to be controlled by AaAP1. They include catalase, SOD, peroxidase, glutathione reductase, glutathione peroxidase, and glutathione-S-transferase. Thus, the *A. alternata* AaAP1 is a crucial regulator for ROS detoxification.
when cells are exposed to oxidative stress. Moreover, I demonstrated that two genes encoding putative fatty acid synthase subunit $\alpha$ reductase and NmrA-like hscarg dehydrogenase recovered from the SSH library were positively regulated by AaAP1. However, I did not recover any genes encoding putative catalase, SOD, or peroxidase from the library. Interestingly, $NPS$ encoding a putative non-ribosomal peptide synthase was negatively regulated by AaAP1. In $C.\ heterostrophus$, the nonribosomal peptide synthase ($NPS6$) has been shown to be required for virulence, siderophore-mediated iron metabolism, and resistance to oxidative stress (Lee et al. 2005; Oide et al. 2006). Nevertheless, the results indicated that AaAP1 is functioning in the regulation of the genes involving in the redox homeostasis and ROS detoxification.

The nuclear export sequence (NES) within the CRD domain of YAP1 has been known to promote nuclear exportation and subcellular localization of YAP1 in yeasts and fungi (Lessing et al. 2007; Molina and Kahmann 2007; Toone et al. 1998). In yeast, nuclear localization of YAP1 is a critical step for the function of YAP1 in transcriptional regulation (Coleman et al. 1999). A nuclear export protein, Crm1p is a negative regulator of YAP1. Under the condition of oxidative stress, Crm1p fails to bind to the NES because YAP1 is induced to experience a conformation change that simply masks the nuclear export sequence (Yan et al. 1998). Nuclear localization of the YAP1-like proteins responding to oxidative stress has been well established in yeasts (Toone et al. 2001) and $A.\ fumigatus$ (Lessing et al. 2007) as well as in phytopathogenic fungi (Lev et al. 2005; Molina and Kahmann 2008). It is likely that AaAP1 of $A.\ alternata$ also complies with this function based on the conserved domains and cysteine residues.
Indeed, nuclear localization of AaAP1 was observed in *A. alternata* when cells encountered H$_2$O$_2$.

ROS has been shown to play diverse roles in plant-microbe interactions and the production of ROS is strongly associated with HR (Lamb and Dixon 1997). If plants use ROS as a defensive response against microorganisms, successful pathogens may have evolved unique abilities to counteract toxic effects of ROS (Apel and Hirt 2004; Miller and Britigan 1997; Moye-Rowley 2003; Toone and Jones 1999). Unlike biotrophic fungi, most of the necrotrophic pathogens produce toxins or cell wall-degrading enzymes to kill the plant cells prior to invasion (Divon and Fluhr 2007). Many necrotrophic fungi obtain nutrients from the oxidative response-induced cell death to facilitate colonization (Cessna et al. 2000; Govrin and Levine 2000; Keon et al. 2007). To thrive in harsh environments, necrotrophic fungi have to evolve intricate strategies against the toxicity of ROS.

The production of ROS is not only essential for differentiation, development, and signaling (Aguirre et al. 2005; Apel and Hirt 2004), but is also critical for cellular defense against pathogens in both animals and plants. However, the roles of ROS-responsive mechanisms and YAP1-mediated antioxidant activity in relation to fungal pathogenicity or virulence are divergent among species and highly dependent on the types of plant-microbe interactions (Giesbert et al. 2008; Glazebrook 2005; Keon et al. 2007; Lev et al. 2005; Mayer et al. 2001; Molina and Kahmann 2007; Tanaka et al. 2006). For example, YAP1 homolog in the biotrophic fungal pathogen *U. maydis* was shown to play a role in fungal virulence (Molina and Kahmann 2007). However, deletion of a YAP1-related gene, *chap1*, in the necrotrophic plant pathogen *C. heterostrophus*, did not affect fungal
virulence (Lev et al. 2005). Similarly, the AfYap1-disrupted mutant of Aspergillus fumigatus remains normal virulence (Lessing et al. 2007). Disruption of Nox-like gene encoding a NADPH oxidase in Magnaporthe grisea, Claviceps purpurea, and Botrytis cinerea prevented in-planta growth (Egan et al. 2007; Giesbert et al. 2008; Segmuller et al. 2008). However, in the Epichloë festuca-ryegrass interaction, NoxA is essential for maintaining a mutualistic interaction with ryegrass. Mutation of the noxA gene in Epichloë festuca enhanced fungal virulence, causing severe stunting on ryegrass and thus, disrupting the symbiotic interactions between E. festucae and its host (Takemoto et al. 2006).

Many Alternaria species produce host-selective toxins (HSTs) with unique modes of toxicity. HSTs have been demonstrated to play a profound role during fungal invasion and lesion formation in various plant-Alternaria interactions (Ito et al. 2004). As assayed on detached Minneola leaves, AaAP1 null mutants were nonpathogenic to the host plants even when inoculated onto wounded leaves. It seems that AaAP1 is not required for production of host-specific ACT toxin, conidiation, and germination (data not shown). Recently, a YAP1 homolog RLAP1 was shown to be essential for fungal pathogenicity in the rough lemon pathotype of A. alternata (Yang et al. 2010). It seems likely that the inability of the AaAP1-disrupted mutant to incite necrotic lesions is related to defects in detoxifying ROS-mediated plant defense. Indeed, several antioxidant-related enzymes and oxidative-responsive genes have been identified to be regulated by AaAP1 in this study. Increasing evidence indicates that peroxide-signaling mechanisms via antioxidant enzymes are required for sensing and detoxifying hydrogen peroxide in living cells (Mayer et al. 2001). This hypothesis was further supported by the
fact that co-application of conidial suspension of the AaAP1 null mutant with a NADPH oxidase inhibitor, apocynin or diphenylene iodonium, partially restored its pathogenic ability. Thus, in a low-ROS setting, AaAP1-disrupted mutants were able to infect and exert pathogenicity.

It seems very likely that the YAP1-mediated antioxidant activity is not a common mechanism by which all fungal pathogens alleviate the toxicity of ROS-mediated plant defenses. The relative importance may be likely dependent on the type of plant-microbe interactions and affected by the balance between ROS-generating and ROS-detoxifying systems in hosts and pathogens. The results derived from my studies strongly not only support the host-selective toxin produced by A. alternata being important for fungal pathogenicity, but also that AP1-mediated detoxification of ROS is necessary for successful colonization in citrus. Thus, the results contribute to the understanding of how necrotrophic plant pathogens deal with toxic ROS, which they may confront during infection.
Table 2-1. Expression sequence tags (EST) that are possibly regulated by AaAP1 were recovered from the wild type cDNA library after subtracted with that of the AaAP1 null mutant.

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<th>Putative function</th>
<th>E-value</th>
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Figure 2-1. Detection of lipid peroxidation and \( \text{H}_2\text{O}_2 \) in Minneola leaves inoculated with \( A. \) \textit{alternata}. (A) Lipid peroxidation was determined by the content of malondialdehyde (MDA) generated from thiobarbituric acid (TBA). Time-course analyses of lipid peroxidation of citrus leaves after challenged with \( A. \) \textit{alternata} (WT) or water only. (B) Detection of hydrogen peroxide in Minneola leaves inoculated with conidial suspension for 24 h was determined by staining with 3,3-diaminobenzidine (DAB) before any necrotic lesions were visible.
Figure 2-2. Functional domains of AaAP1 in the tangerine pathotype of A. alternata. (A) Schematic illustration of the putative AaAP1 containing 629 amino acids showing a basic region leucin zipper (bZIP) domain, a transcription factor PAP1, an N-terminal cysteine rich domain, a C-terminal cysteine rich domain and the position of the nuclear export sequence (NES). (B) Alignment of bZIP domains, (C) n-CRD, or (D) c-CRD of AP-1 homolog proteins of C. heterostrophus, C. albicans, S. cerevisiae, and U. maydis. Upper letters and * indicate that all proteins are identical; whereas lowercase letters and colons (:) indicate that three or more proteins are similar.
Figure 2-3. Targeted disruption of AaAP1 in A. alternata. (A) Schematic illustration of the split-marker strategy for AaAP1 gene disruption. (B) Southern blot analysis of SpeI-digested genomic DNA of the wild type (WT) and two putative disruption mutants (D1 and D2) were hybridized with a specific AaAP1 probe as indicated in A. (C) Northern blot analysis identified a 2.0-kb transcript from the wild type but not from two AaAP1 deletion strains, D1 and D2. (D) Southern blot hybridization of SpeI-digested DNA isolated from the wild type, transformants (T3 to T8) displaying resistance to both hygromycin and H2O2, and an AaAP1-complemented strain (Cp1) to an AaAP1 probe.
Figure 2-4. The AaAP1 gene plays a crucial role in resistance to oxidants. (A) Sensitivity test of the wild type, the AaAP1 null mutants (D1 and D2), and the complementation strains (Cp1 and Cp2) of A. alternata was determine by radial growth on PDA supplemented with different oxidants or compounds as indicated. MTT: methylthiazolyldiphenyl-tetrazolium bromide; SDS. Sodium dodecyl sulfate. (B) The toxicity of H$_2$O$_2$ to A. alternata was determined by the inability of the fungus to export Evan’s blue. Live cells export dye and remain clear; whereas dead cells stain blue. (C) Quantitative determination of conidial viability of A. alternata treated with or without H$_2$O$_2$ for 30 min.
Figure 2-5. The Alternaria alternata AaAP1 is required for H$_2$O$_2$ detoxification and expression of AaAP1 in response to oxidative stress. (A) The wild type, the AaAP1 null mutant D1, and the complementation Cp1 strains were cultured on potato dextrose agar, flooded with H$_2$O$_2$, and stained with 3,3’-diaminobenzidine (DAB) to form brownish polymers. (B) Consumption of H$_2$O$_2$ by A. alternata strains was determined by monitoring a decrease of absorbance at 240 nm over time. The mock control contains no fungal hyphae. (C) Accumulation of the AaAP1 transcript in response to oxidative stress in A. alternata. MND: menadione; t-BHP: tert-butyl-hydroperoxide; MTT: methylthiazolyldiphenyl-tetrazolium bromide; RB: rose bengal; SDS: sodium dodecyl sulfate.
Figure 2-6. Oxidative stress-regulated nuclear localization of AaAP1::sGFP. (A) Schematic representation of AaAP1::sGFP with an endogenous promoter. The putative NES region is indicated. (B) Nuclear localization of AaAP1::sGFP upon exposure to 0.01% H₂O₂ for 0, 20, 40, and 60 min. Samples were analyzed by fluorescence microscopy. 4'-6-diamidine-2-phenylindole (DAPI) fluorescence indicates distribution of nuclei as indicated by arrows.
Figure 2-7. AaAP1 regulates the production of antioxidant activities in *A. alternata*. Total proteins were extracted with cold phosphate buffer. The AaAP1 deletion strains D1 or D2 displayed reduced activities in catalase (A), peroxidase (B) superoxide dismutase, SOD (C), glutathione S transferase, GST (D), glutathione peroxidase, Gpx (E), glutathione reductase, Grx (F), and lignin-type peroxidase (G), compared to the wild type (WT) and the complementation strains Cp1 or Cp2. *a, b, and c* were different groups (*p* < 0.05).
Figure 2-8. Identification of the genes that are regulated by AaAP1. Total RNA prepared from the wild type (WT) and the AaAP1-disrupted mutant (D1) was hybridized to a digoxigenin-labeled AaAP1 probe. Gel stained with ethidium bromide indicates relative loading of the RNA samples.
Figure 2-9. The *A. alternata* AaAP1 is required for pathogenicity on citrus cv. Minneola. (A) Pathogenicity was assayed on detached Minneola leaves inoculated with 5 μl of conidial suspension (10^4 conidia/ml) from the wild type (WT), the AaAP1-disrupted mutants D1 and D2, and the complementation strains Cp1 and Cp2. (B) Quantitative analysis of lesion formation on Minneola leaves inoculated with conidia suspension of *A. alternata* strains. (C) Fungal pathogenicity assayed on detached Minneola leaves uniformly sprayed with conidial suspension of *A. alternata* strains. (D) Development of necrotic lesions by the *A. alternata* WT and AaAP1 null mutants D1 and D2 on detached Minneola leaves with wounding prior to inoculation. The mock controls were treated with water only.
Figure 2-10. Light microscopy of Minneola leaves inoculated with (A) the wild type and (C) the complementation strains of A. *alternata* 2 dpi, revealing deformed plant tissues and fungal hyphae (Hp) within the plant tissues. (B) Inoculation of the AaAP1-disrupted mutant did not show destruction and fungal hyphae within the plant cells. Fungal hyphae (Hp) and conidia (Cn) are indicated by arrows.
Figure 2-11. The *A. alternata* AaAP1 gene is not required for the production of host-specific ACT toxin. (A) RNA purified from the wild type (WT), the AaAP1 null mutant (D1), and the complementation strain (Cp1) of *A. alternata* was hybridized with an ACT biosynthetic gene probe. (B) Culture filtrates of the WT, D1, and Cp1 strains grown in modified Richard’s medium (Kohmoto et al. 1993) were applied onto detached Minneola leaves. (C) Spectrophotometric scanning of the ethyl acetate crude extracted from WT and D1 strains. (D) Thin-layer chromatography (TLC) analysis of the ethyl acetate extract separated displaying two major bands with $R_f$ 0.58 and $R_f$ 0.53. (E) Detached Minneola leaves treated with $R_f$ 0.53 bands recovered form silica gel and eluted with methanol, developing similar necrotic lesions. The mock controls were treated with methanol (5 µl) only.
Figure 2-12. NADPH oxidase inhibitors partially restored pathogenicity of the AaAP1-null mutant. Conidial suspension (5 μl of 10^4 conidial/ml) of the AaAP1 null mutant was applied with or without NADPH oxidase inhibitor, apocynin (APC) or diphenylene iodonium (DPI) onto detached Minneola leaf. The inoculated leaves were incubated in a moist chamber for lesion development. The mock controls were treated with APC or DPI dissolved in dimethyl sulfoxide (DMSO).
CHAPTER 3
THE FUS3-TYPE MITOGEN-ACTIVATED PROTEIN KINASE AND THE REDOX-RESPONSIVE AP1 REGULATOR FUNCTION COOPERATIVELY IN Alternaria alternata

Mitogen-activated protein (MAP) kinases are involved in cellular signal transduction pathways and play diverse roles for differentiation, pathogenesis, and growth. In this study, a well-conserved Fus3-type MAP kinase gene homolog, AaFUS3, from A. alternata was characterized. Our studies revealed that AaFUS3 is required for vegetative growth, conidiation, fungicide resistance, melanin biosynthesis, and penetration ability on its citrus hosts. AaFUS3 deletion strains were highly resistant to salt stress and displayed altered activities in several hydrolytic enzymes. A mutant disrupted in both AaFUS3/AaAP1 genes increased sensitivity to 2,3,5-triiodobenzoic acid (TIBA), 2-chloro-5-hydroxypyridine (CHP), and diethyl maleate (DEM) compared to the strain mutated at AaFUS3 or AaAP1 alone. Expression of AaFUS3 and AaAP1 as well as phosphorylation of AaFUS3 were also induced by TIBA, CHP, and DEM. Phosphorylation of AaFUS3, however, was negatively regulated by AaAP1. Furthermore, two putative MFS coding genes were regulated by both AaFUS3 and AaAP1. Thus, our results indicate that a synergistic regulation occurs between the FUS3-type MAP kinase and the redox-responsive transcription regulator AaAP1 for diverse physiological functions.

Introduction

Like all living organisms, fungi are challenged by environmental changes. Thus, fungal pathogens may have evolved strategies to perceive chemical and physical signals from environments and effectively respond with intracellular physiological changes. In eukaryotic cells, the mitogen-activated protein (MAP) kinases have been
shown to be capable of responding to a variety of exterior stimuli. The MAP kinase-mediated signaling pathway is required for regulation of numerous cellular activities, such as mitosis, differentiation, and cell survival (Pelech and Sanghera 1992; Robinson and Cobb 1997). This signaling cascade consists of three serine/threonine protein kinases: MAP kinase kinase kinase (MAPKKK or MEKK), MAPK kinase kinase (MAPKK or MEK) and MAP kinase (MAPK). MAPKKKK phosphorylates MAPKK, which in turn phosphorylates and activates MAPK (Gustin et al. 1998; Kultz 1998). The MAP kinases-mediated signaling cascade is evolutionarily well-conserved from yeasts to mammals (Herskowitz 1995; Xu 2000). However, the biological functions of each component kinase are highly dependent on the lifestyles of the species and their environment (Bardwell 2006).

In *Saccharomyces cerevisiae*, several MAP kinase-mediated pathways involved in mating responses (Fus3-type MAPK), filamentous growth (Kss1-type MAPK), cell integrity (Slt2-type MAPK) and osmotic stress response (Hog1-type MAPK) have been identified (Banuett 1998; Gustin et al. 1998; Herskowitz 1995). One of the best-studied MAP kinases is the Fus3/Kss1-type MAP kinase which is responsible for the mating pheromone response, nitrogen starvation, and filamentous growth in yeasts. Both Fus3 and Kss1 pathways are regulated by Ste 20, MEKK (Ste11) and MEK (Ste7) (Madhani and Fink 1998) (Fig. 1-3).

The Fus3-type MAP kinase homologs have recently been shown to play an important role for pathogenicity in various fungal pathogens, including *Alternaria brassicicola* (Cho et al. 2007), *Botrytis cinerea* (Zheng et al. 2000), *Cochliobolus heterostrophus* (Lev et al. 1999), *Collectotrichum lagenarium* (Takano et al. 2000),
Fusarium graminearum (Jenczmionka et al. 2003), F. oxysporum (Pietro et al. 2001), Magnaporthe grisea (Xu and Hamer 1996), Pyrenophora teres (Ruiz-Roldan et al. 2001), Stagonospora nodorum (Solomon et al. 2005), Ustilago maydis (Mayorga and Gold 1999), and Verticillium dahlia (Rauyaree et al. 2005). In addition, Fus3-like MAP kinases are required for the formation of conidia and/or appressoria in the fungal pathogens.

The M. grisea PMK1, a Fus3 homolog, is essential for formation of appressoria and conidia (Xu and Hamer 1996). Deletion of a Fus3 homolog, CHK1 in C. heterostrophus and PTK1 in P. teres resulted in poorly developed aerial hyphae and affected the formation of both conidia and appressoria (Lev et al. 1999; Ruiz-Roldan et al. 2001). Disruption of BMP1 in gray mold fungus B. cinerea or FMK1 in F. oxysporum, however, did not affect conidiation, yet compromised fungal pathogenicity (Zheng et al. 2000; Pietro et al. 2001). Fus3 MAP kinases also play important roles for production of cell-wall-degrading enzymes (CWDE) and hydrolytic enzymes as evidenced in several phytopathogenic fungi (Cho et al. 2007; Pietro et al. 2001).

Previous studies have shown that YAP1-like transcription regulators are essential for A. alternata pathogenicity to citrus by detoxifying reactive oxygen species (ROS) (Lin et al. 2009; Yang et al. in press). In this Chapter, I characterized a Fus3 gene homolog, designated AaFUS3 (Alternaria alternata Fus3-type MAP kinase) and revealed a critical role in pathogenesis. I also provided a possible link or synergistic interaction between AaFUS3 and AaAP1.

Materials and Methods

Fungal Strains and Growth Conditions

The wild type EV-MIL31 strain of A. alternata (Fr.) Keissler used for transformation, mutagenesis, and conidia isolation has been previously described (Lin et al. 2009).
Cloning of *AaFUS3*

To obtain a *Fus3* MAP kinase homolog, a 0.5-kb DNA fragment was amplified with two primers MAPK-5F and MAPK-6R (Table A-1) from genomic DNA of *A. alternata* by using a Go-Taq DNA polymerase (Table A-1) (Promega). The resulting amplicon was cloned into a pGEM-T easy vector (Promega) for sequence analysis. The cloned gene was named *AaFUS3*. Subsequently, the entire *AaFUS3* ORF sequences as well as its promoter region were amplified with two inverse primers MAPK-98 and MAPK-293 from restriction enzyme-digested and self-ligated DNA templates. Sequence data from this chapter can be found in the EMBL/GenBank Data Libraries under accession number GQ414506 (*AaFUS3*).

Identification of *AaFUS3* Null Mutants

To disrupt *AaFUS3*, a 1.2-kb DNA fragment containing the entire *AaFUS3* ORF was amplified with two primers MAPK-atg and MAPK-taa and cloned into pGEM-T easy to create T-AfMK1. A 2.2-kb *HYG* gene cassette under the control of the *A. nidulans* *trpC* promoter was amplified from pUCATPH (Lu et al. 1994) with the primers M13F and M13R, end-filled, and cloned into the *NcoI* site of T-AfMK1 to generate T-AfMKhyg. Two truncated *HYG* fragments fused with either 5’ or 3’ end of *AaFUS3* were amplified, mixed, and transformed into the wild type protoplasts. A 2.4-kb fragment encompassing 5’ *AaFUS3* and 3’ *HYG* was amplified with the primers MAPK-atg and hyg4; a 1.5-kb fragment containing 3’ *AfMK1* and 5’ *HYG* was obtained with the primers MAPK-taa and hyg3 (Figure 3-1B).

Genetic Complementation of *AaFUS3*-Null Mutant

For genetic complementation, a 2.6-kb DNA fragment containing the entire *AaFUS3* and its endogenous promoter region was amplified from genomic DNA with the
primers MAPK-P1 and MAPK-taa using a high fidelity PCR system (Roche Applied Science). The amplified product was co-transformed into a null mutant with the pCB1532 plasmid (Sweigard et al. 1997).

**Create Double Mutations at AaFUS3 and AaAP1 Genes in A. alternata**

To disrupt AaAP1 gene in the AaFUS3 null mutant, a PCR fusion method was performed to create split-marker DNA fragments (Fig. 3-9A). A 1.8-kb "SU" and a 1.7-kb "UR" fragments overlapping within the acetolactate synthase gene cassette (SUR) were amplified from pCB1532 (Sweigard et al. 1997) with the primers SUR-1/DR3 and surR and the primers SUR-2/DF3 and surF, respectively. A 0.8-kb DNA fragment containing the 5’ AaAP1 was amplified with the primers yap1DF2 and SUR1-DR3. A 0.8-kb 3’ AaAP1 fragment was amplified with the primers yap1DR2 and SUR2-DF3. In second-round PCR, a 2.6-kb DNA fragment containing 5’ AaAP1 fused with "UR" was amplified with primers yap-DF2 and surR from the PCR products described above. A 2.5-kb DNA fragment having 3’ AaAP1 fused with "SU" was amplified with the primers surF and yap-DR2. PCR fragments were directly transformed into protoplasts prepared from an AaFUS3 null mutant strain of *Alternaria alternata*. Fungal transformants were selected on the RMM medium with 5 μg/ml of sulfonylurea, tested for the sensitivity to H2O2, and further confirmed by PCR analyses with two AaAP1-specific primers yap1-atg and yap1-taa.

**Miscellaneous Assays for Enzymatic Activities**

Assays of endo-polygalacturase activities were carried out on the modified complete medium (Chen et al. 2005) by substituting glucose with 1% polygalacturonic acid as the sole carbon source. The pH of media was adjusted to 5 with 0.6 M Tris buffer. Fungal mycelia were blended and spread onto agar plates. The inoculated plates
were incubated at 27℃ for 3 days, overlaid with 1% hexadecyltrimethyl ammonium bromide, and examined daily for the formation of clear halos around the fungal colonies (Hubbell et al. 1978).

Proteolytic activities were determined by measuring the formation of clear zone around the fungal colonies on 10% skim milk medium (Difco Laboratories) dissolved in 0.05 M phosphate buffer (Ogrydziak and Mortimer 1977).

The alkaline and acid phosphatase activities were determined by the quantity of p-nitrophenol liberated from 4-p-nitrophenylphosphate (NPP) (Sigma) at 30℃ and measured at A410 (Dorn and Rivera 1996). Fungal strains were grown in 2-ml liquid minimal medium without phosphate for 3-4 hours. The culture filtrates were mixed with NPP (1:4 vol/vol) dissolved in 0.6 M Tris buffer (pH 9.5) for analysis alkaline phosphatase, whereas the culture filtrates mixed with NPP (1:4 vol/vol) dissolved in 0.6 M acetate buffer (pH 4.8) for measuring acid phosphatase activity.

Assays for lipolytic activities using 1% Tween-20 as a substrate were performed based on the appearance of visible precipitation around the fungal colonies grown on a Tween-20 agar medium (10 g peptone, 5 g NaCl, 0.1 g CaCl₂·H₂O, 20 g agar, and 10 ml Tween-20 in 1000 ml) (Gopinath et al. 2005).

Extracellular cutinase activity was determined by the formation of a yellow color after reaction with 5 mM para-nitrophenyl butyrate (PNPB) dissolved in 50 mM potassium phosphate (pH 5.0) (Stahl and Schafer 1992). Fungal isolates were grown on CM containing 0.1% 16-hydroxyhexadecanoic acid (HHDA; dissolved in 1% sodium acetate) for 5 days for induction before enzymatic assays. The supernatant of each
culture was mixed with PNPB solution (1:1, vol/vol), incubated for 1 h and measured at 
$A_{405}$. One unit of cutinase releases $1 \mu$mol $\rho$-nitrophenol per minute.

Extracellular activities of CWDEs were determined by measuring the amounts of 
reducing sugar released from 1% polygalacturonic acid (PGA), 1% citrus pectin, 0.5%
carboxymethyl-cellulose (CMC), or 0.5% xylan (hemicellulose), and reacted with 
dinitrosalicylic acid (DNS) reagent under alkaline conditions as described (Bailey et al. 
1993). Enzyme activities were calculated using a standard curve established with 
glucose. One unit of enzyme is defined as that required to release 1 $\mu$mol of glucose 
from the substrate per minute. Briefly, fungal isolates were grown on modified CM 
containing a polysaccharide as the sole carbon source for 7 days. Four agar plugs 
bearing fungal mycelia (5 mm) were inoculated in 0.1 M sodium acetate buffer (pH 5.0) 
containing 0.5% CMC or xylan, or in Tris buffer containing 1% PGA or citrus pectin (pH 
4.5 and 7.6) and incubated at $50^\circ C$ for 1 h. Culture filtrate (800 $\mu l$) was mixed with 200
$\mu l$ DNS reagent, boiled at $95^\circ C$ for 5 min, cooled down to room temperature, and 
measured spectrophotomtrically at $A_{540}$.

Melanin was extracted from mycelia with 2% NaOH boiled at $100^\circ C$ for 2 h and 
acidified to pH 2.0 with 5 N HCl. The pigment was separated by centrifugation at 6000 $\times$
g for 15 min, dissolved in 1 ml 2% NaOH, and measured at $A_{405nm}$ (Babitskaya et al. 
2000). The enzyme activities of the wild type, the $AaFUS3$ mutants and the 
complementation strains were compared by Analysis of Variance (ANOVA) of the SPSS 
statistical analysis software. A $p$-value of < 0.05 was interpreted as a significant 
difference.
Pathogenicity Test

Determination of fungal pathogenicity was conducted on detached Minneola leaves inoculated with mycelial mass. Briefly, fungal mycelia on PDA agar were transferred using sterile toothpicks onto detached Minneola leaves slightly away from the midribs. The inoculated leaves were incubated in a mist chamber at 27°C for lesion formation.

Detection of Phosphorylated AaFUS3 MAPK

Fungal culture grown in complete medium for 3 days was treated with 2.5 mM 2,3,5-triiodobenzoic acid (TIBA), 2.5 mM 2-cholor-5-hydroxypyridine (CHP), 0.1% diethyl maleate (DEM), or 0.1% H₂O₂ for 2 hours. Fungal mycelia were collected by filtration through three layers of filter paper. Crude proteins were extracted by grinding fungal mycelia in liquid nitrogen, mixed with ice-cold extraction buffer (10 mM Tris-Cl pH 7.5, 150 mM NaCl, 5 mM EDTA, 10 mM NaN₃, 1% Triton X-100), and collected via centrifugation at 10,000 × g for 15 min at 4°C. The protein samples were denatured in 2X SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 0.02% β-mercaptoethanol, 20% glycerol, 50 mM DTT and 0.002% bromophenol blue] by heating at 100°C for 10 min. Proteins were fractionated on a denaturing 12% SDS-polyacrylamide gel and either stained with Comassie brilliant blue or electroblotted onto a nitrocellulose membrane (Bio-Rad). Protein concentration was determined by a protein assay kit (Bio-Rad, Hercules, CA, U.S.A.).

The transferred membranes were incubated in a blocking buffer [TBS (20 mM Tris-HCl pH 7.6, 137 mM NaCl), and 0.1% Tween-20 with 5% w/v nonfat dry milk] for 1 h at room temperature and washed three time with TBS/T (TBS, 0.1% Tween-20). A rabbit anti-phosphate-p44/42 MAPK kinase antibody (Cell signaling Technology, Boston,
MA) and Fus3 (y-40) rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1000 dilution was used as primary antibodies. The anti-rabbit–IgG antibody conjugated horseradish peroxidase (HRP) (Cell signaling Technology) at a 1:2000 dilution was used as a secondary antibody. Detection of the HRP was performed using LumiGLO (Cell signaling Technology) as a chemofluorescent substrate.

Results

Cloning and Characterization of A Fus3 MAP kinase Gene Homolog in A. alternata of Citrus

I have previously shown that the A. alternata AaAP1 gene, encoding a YAP1-like transcription factor is essential for resistance to reactive oxidative species (ROS) and fungal pathogenicity. In the current study, I cloned and characterized a Fus3 MAPK homolog from the tangerine pathotype of A. alternata, using two primers that are complementary to the Amk1 gene of A. brassicicola (accession number AY515257; Cho et al. 2007). My goals were to determine if oxidative stress tolerance is also regulated by a Fus3 MAP kinase signaling pathway and if such a regulation cross-talks with the YAP1 signaling pathway. Sequence analysis of a 0.5-kb DNA fragment revealed that the deduced amino acids have high similarity to many Fus3-type MAP kinase proteins. The cloned gene was designated AaFUS3 (Alternaria alternata Fus3 MAP kinase gene).

The A. alternata AaFUS3 gene contains a 1264-bp ORF interrupted with four introns of 50, 53, 53, and 49 bp. The conceptually predicted AaFUS3 polypeptide has 74% to 98% similarity and 57% to 96% identity to a number of Fus3 MAP kinase homologs in fungi and yeasts. Phylogenetic analysis revealed that AaFUS3 is most similar to A. brassicicola AMK1, Pyrenophora teres PTK1, Bipolaris oryzae BMPK1, and Cochliobolus heterostrophus ChK1, yet has less similarity to M. grisea PMK1,
Botryotinia fuckeliana BMP1, and Mycosphaerella graminicola (data not shown). Further analysis of AaFUS3 protein identified a conserve serine/threonine protein kinase domain (Fig. 3-1A) with an ATP-binding region (amino acids 26-50), a characteristic MAP kinase signature (amino acids 54-156), and a protein kinase active site (threonine/glutamic acid/tyrosine; TEY; amino acids 180-183).

**Targeted Disruption of AaFUS3 of A. alternata**

To disrupt the AaFUS3 gene, split-marker fragments carrying truncated hygromycin phosphotransferase B gene (HYG) flanked by 5' or 3' AaFUS3 sequences were amplified from the T-AfMKhyg disruption construct (Fig. 3-1B) and directly transformed into protoplasts prepared from the wild type strain of *A. alternata*. In total, 2 of 15 transformants recovered from media containing hygromycin exhibited reduced radial growth and were considered putative AaFUS3-disrupted mutants. Southern-blot hybridization of the wild type genomic DNA digested with *Xho*I and *Bgl*II to an AaFUS3 probe identified an expected 1.0-kb hybridizing band. The putative AaFUS3 mutants (M1 and M2) had 3.0-kb hybridizing bands, resulting from the insertion of the 2.2-kb HYG gene cassette (Fig. 3-1C). Northern-blot analysis also detected a 1.2-kb transcript in RNA prepared from the wild type but not from putative AaFUS3 mutants (Fig. 3-1D). Thus, I concluded that they are AaFUS3 null mutants.

**AaFUS3 Is Required for Vegetative Growth, Resistance to Copper Fungicide but Negatively Modulates Salt Tolerance**

Both M1 and M2 mutants deleted at the AaFUS3 locus showed an average of 48% radial growth retardation on PDA compared to that of wild type (Fig. 3-2A and B). By contrast, the genetically complemented strains MCp1 and MCp2, containing a functional copy of AaFUS3, fully restored radial growth to wild type level (Fig. 3-2B). AaFUS3 null
mutants were not sensitive to 0.1% $\text{H}_2\text{O}_2$, 0.002% tert-butyl-hydroperoxide, 14 mM KO$_2$, 10 $\mu$M hematoporphyrin, eosin Y, cercosporin phytotoxin, or diethylenetriamine/NO (Fig. B-1 and some data not shown). However, growth of the AaFUS3 deletion strains was partially restored by 1 M glucose, but not by mannitol, sucrose, or sorbitol (Fig. 3-2C and Fig. B-1). Moreover, the AaFUS3 null mutants were highly resistant to 1 M KCl or NaCl, exhibiting faster growth compared with the wild type or the complementation strains (Fig. 3-2D and E). The AaFUS3-disrupted mutants displayed hypersensitivity to copper fungicide (Fig. 3-2F and G).

**AaFUS3 Is Essential for Conidiation**

Investigation through light microscopy revealed that the AaFUS3-disrupted mutants were defective in conidiation. The wild type strain produced mature conidia with both cross and longitudinal septae (Fig. 3-3A). However, no fully developed conidia were observed from the deletion mutants whose granulated hyphae were aberrant with distinct septae, often expanded into spherical swellings occurring chains (Fig. 3-3B to F). Although growth of the AaFUS3 null mutants was restored by NaCl, KCl, or glucose, none of these compounds was capable of restoring conidiation (data not shown). Furthermore, applying exogenous cAMP at various concentrations also could not restore conidial formation to the null mutants (data not shown). By contrast, the complementation strains MCp1 and MCp2 produced conidia morphologically similar to those produced by wild type (Fig. 3-3G and H).

**The AaFUS3 Is Required for Fungal Virulence**

Because the AaFUS3 null mutants produced no mature conidia, pathogenicity tests were performed on detached Minneola leaves using mycelial mass. The wild type and the genetically complemented strain Cp1 induced conspicuous necrotic lesions on
Minneola leaves at 4 dpi (Fig. 3-4A). In contrast, the leaves inoculated with the
AaFUS3-disrupted mutants (M1 and M2) failed to develop visible lesions (Fig. 3-4A).
However, M1 and M2 strains incited necrotic lesions similar to those of WT and MCp1
when leaves were wounded prior to inoculation (Fig. 3-4B). The results implicated
AaFUS3 as being important for fungal penetration to citrus.

When fungal inoculum was placed near the midribs, AaFUS3 deletion strains (M1
and M2) caused necrotic lesions in some of the leaves (Fig. 3-5A) but with considerably
less mycelia mass (Fig. 3-5B).

Expression of the AaFUS3 Gene Is Highly Induced by Leaf Extracts

To evaluate what factors might affect AaFUS3 gene expression, Northern blot
analysis was performed. The AaFUS3 transcript accumulated to relatively higher levels
when the fungus was grown on potato dextrose agar or minimal medium (MM)
containing leaf extracts from Minneola or rough lemon (Fig. 3-6). However, expression
of the AaFUS3 gene was not affected by the types of nitrogen or by eliminating nitrogen
or carbon sources from the medium (Fig. 3-6).

AaFUS3 Regulates the Production of Hydrolytic Enzymes and Melanin

The extracellular activities of CWDEs, hydrolytic enzymes, and melanin produced
by the wild type, the AaFUS3-disrupted mutants (M1 and M2), and the complementation
(Cp1 and Cp2) strains were measured. Deletion of AaFUS3 resulted in a fungal mutant
that produced higher levels of alkaline phosphatase, lypolytic, and cutinase activities
compared to the wild type and the genetically reverted strains (Fig. 3-7A, B and C). By
contrast, M1 and M2 produced lower endo-PG activities and melanin than WT, Cp1,
and Cp2 (Fig. 3-7D and E). However, there were no significant differences in proteolytic,
acid phosphatase, xylanase, pectinase and cellulose activities in all *Alternaria* strains tested (data not shown).

**AaFUS3 and AaAP1 Share Common Phenotypes and Confer Pleiotropic Drug Resistance**

The *AaFUS3*-disrupted mutants were insensitive to H$_2$O$_2$, t-butyl-hydroperoxide, menadione, KO$_2$, hematoporphyrin, cercosporin, and eosin Y (Fig. B-1 and some data not shown). I further tested whether or not *AaFUS3* was required for thiol-oxidizing agent resistance. Unexpectedly, both *AaFUS3* and *AaAP1* null mutants were hypersensitive to 2-chloro-5-hydroxypyridine (CHP; Matrix Scientific, Columbia, SC). This phenotype was discovered accidentally (Fig. 3-8). Irrelevant to the authentic thiol-oxidizing compound, diamide (Sigma-Aldrich), 2-chloro-5-hydroxypyridine, is also named “diamide” by the carrier (Matrix Scientific). Sensitivity tests revealed that the *AaFUS3* null mutants were highly sensitive to 2,3,5-triiodobenzoic acid (TIBA), dithiobis-2-nitrobenzoic acid (DTNB), rose bengal (RB), pyridoxine, pyridoxal-5-phosphate, diethyl maleate (DEM), and 2,6-dichloroisonicotinic acid (INA), and diamide (Fig. 3-8). Interestingly, the *AaAP1* null mutants were also hypersensitive to CHP, TIBA, DTNB, diethyl maleate, INA, and diamide (Fig. 3-8). The *AaAP1* null mutants were slightly sensitive to RB and pyridoxal-5-phosphate. All genetically complemented strains expressing a functional copy of *AaFUS3* in the M1 or *AaAP1* in the *AaAP1* mutant restored chemical resistance to wild type levels.

**Double Mutation at AaFUS3 and AaAP1 Genes in A. alternata Caused Greater Sensitivity to TIBA or CHP**

To understand if a cooperative regulation exists between *AaFUS3*- and *AaAP1*-mediated signaling pathways, a fungal strain carrying disruption at both *AaFUS3* and *AaAP1* genes was created. Transformation of split-marker fragments containing
truncated acetolactate synthase gene (SUR) flanked by either 5’ or 3’ AaAP1 sequence (Fig. 3-9A) into an AaFUS3 null mutant resulted in sulfurylurea-resistant transformants. Those transformants were screened by PCR with two AaAP1-specific primers yap1-atg and yap1-taa. The primers produced an expected 2.0-kb DNA fragment from genomic DNA of the wild type or the AaFUS3-null mutant, whereas a 4.7-kb band was amplified in a transformant presumably disrupted in the AaAP1 gene (Fig. 3-9B). Sensitivity assays revealed that the double mutant exhibited an elevated sensitivity to CHP and TIBA compared to the strain mutated at AaFUS3 or AaAP1 alone (Fig. 3-9C and D). The double mutant, similar to the AaAP1 null mutant, was also highly sensitive to oxidants (Fig. 3-9C and D). The results implicated a synergistic association between AaFUS3 and AaAP1.

Expression of the AaFUS3 and AaAP1 Genes in Response to Chemical Stress in A. alternata

Expression of the AaAP1 gene was increased when fungal cultures were shifted to a medium containing TIBA, CHP, DEM, or H2O2 (Fig. 3-10). Similarly, accumulation of the AaFUS3 gene transcript was also elevated in response to these compounds (Fig. 3-10), even though the effects were not as great as those observed for the expression of the AaAP1 gene.

Activation of AaFUS3 MAP Kinase Phosphorylation

The phosphorylation levels of AaFUS3 were assessed by Western blot analyses using a phospho-p44/42 monoclonal antibody. The results revealed that a 40 kDa band was detected in the samples of wild type (WT) and the AaAP1 null mutant (Y1), yet no signal was detected in the AaFUS3 (M1) or the AaFUS/AaAP1 (YM) deletion strain (Fig. 3-11A). After treating with CHP, DEM, or low concentration of TIBA (0.1 mM or 1 mM),
AaFUS3 was phosphorylated to higher levels (Fig. 3-11B and C). By contrast, when fungal cultures were treated with H$_2$O$_2$ or 2.5 mM TIBA, phosphorylation of AaFUS3 was decreased slightly or unchanged (Fig. 3-11B and C). Interestingly, disruption of the AaAP1 gene promoted phosphorylation of AaFUS3 (Fig. 3-11B), indicating AaAP1 suppressed AaFUS3 phosphorylation in A. alternata.

**A Synergistic Regulation of Expression of Two MFS Transporters by AaFUS3 and AaAP1**

TIBA is an inhibitor for a transporter of the plant hormone indoleacetic acid (IAA) (Prusty et al. 2004). Sensitivity of the AaFUS3 or AaAP1 null mutant to TIBA was probably attributable to defective functions of some membrane transporters. Expression of three genes encoding putative membrane transporters was examined. The gene clones were recovered from the wild type cDNA library after subtraction with that of an AaAP1 null mutant using the suppression subtractive hybridization (SSH) (Chapter 2). Northern blot analyses revealed that expressions of the gene clones #19 and #54 encoding putative MFS were down-regulated in fungal mutants disrupted in the AaFUS3 or AaAP1 gene (Fig. 3-12). The gene clone #57 encoding a putative efflux pump was only down-regulated in the AaFUS3 null mutant (Fig. 3-12).

**Discussion**

MAP kinase-mediated signal transduction pathways have been demonstrated to play diverse roles in fungi and yeasts (Dickman and Yarden 1999; Herskowitz 1995; Xu 2000). Three distinct MAP kinase pathways were identified in filamentous fungi. The pheromone response signaling pathway controlled by the Fus3/KSS1-type MAPK is necessary for pathogenesis, mating, conidiation, and appressoria formation. The HOG1-type MAPK cascade controls resistance to high osmolarity. The SLT2 is primarily
involved in cell wall integrity, conidiation and pathogenicity. In this study, I characterized the function of *A. alternata* AaFUS3 gene which has high similarity to Fus3 of *S. cerevisiae*, PMK1 of *M. grisea*, and many Fus3 homologs of other phytopathogenic fungi (Madhani and Fink 1998; Xu and Hamer 1996; Ruiz-Roldan et al. 2001; Mayorga and Gold 1999; Zheng et al. 2000).

Deletion of the AaFUS3 gene rendered defects in fungal penetration, pathogenicity, and several physiological functions in *A. alternata*. In *A. nidulans* and *Colletotrichum lagenarium*, the HOG1-type MAP kinases are phosphorylated under high osmotic conditions or stresses induced by fludioxonil fungicides (Kojima et al. 2004; Furukawa et al. 2005). AaFUS3 was shown to be responsible for resistance to copper fungicide in *A. alternata*. Moreover, the AaFUS3 null mutants displayed an increased resistance to salt stress. Possible interactions between FUS3- and HOG1-type MAP kinases signaling pathways will be described in Chapter 5.

The AaFUS3 deletion strains failed to produce mature conidia. Applying exogenous cAMP did not restore conidiation. The FUS3-type kinase has been characterized to be essential for pathogenicity in many fungal pathogens (Zheng et al. 2000; Lev et al. 1999; Pietro et al. 2001; Xu and Hamer 1996; Mayorga and Gold 1999; Cho et al. 2007). It is not surprising that AaFUS3 also plays an important role in pathogenicity. It seems that the inability of the AaFUS3 null mutants to cause necrotic lesion was primarily due to the loss of penetration ability. As demonstrated in the present study, the AaFUS3 null mutants failed to cause any necrotic lesions unless the leaves were wounded prior to inoculation.
Cell-wall-degrading enzymes (CWDE) have been shown to promote fungal virulence although the relative importance of CWDE varies among fungi (Rogers et al. 2000; ten Have et al. 1998; Tonukari et al. 2000; Voigt et al. 2005). MAP kinases are also known to be required for expression of the CWDE-coding genes in *F. oxysporum*, *C. heterostrophus*, and *A. brassicicola* (Gomez-Gomez et al. 2001; Lev and Horwitz 2003; Cho et al. 2007). The *AaFUS3* deletion strains exerted lower endo-PGase activity, even though endo-PGase is not required for the disease development in *A. alternata* (Isshiki et al. 2001). Similar results were also reported in the *Gpmk1*-disrupted mutant of *F. graminearum* (Jenczmionka and Schafer 2005). The product of *AaFUS3* negatively regulated the production of lipolytic enzymes, alkaline phosphatases, and cutinase activities. In *A. brassicola*, expression of the cutinase and lipase coding genes by the *Amk1* null mutant was also slightly increased in axenic culture but decreased during infection (Cho et al. 2007). It appears that the FUS3-type MAP kinase may have distinct functions as a negative or positive regulator in the production of CWDE during saprophytic growth or plant infection (Cho et al. 2007).

Disruption of the *AaFUS3* gene yielded fungi that were highly sensitive to CHP, TIBA, DTNB, pyridoxine, pyridoxal-5-phosphate, and INA. These phenotypes were not previously found to be associated with the FUS3-type MAP kinase signaling pathway. Interestingly, the *AaAP1* deletion mutants defective in the oxidative stress response also displayed severe growth retardation in the presence of CHP, TIBA, and diethyl maleate (DEM). The *AaFUS3* or *AaAP1* gene transcript was up-regulated after chemical treatments. Furthermore, AaFUS3 was phosphorylated to higher levels in response to TIBA, CHP, or DEM in the wild type strain or in the *AaAP1* deletion strain. Finally, the
genetically modified mutant defective in both AaFUS3 and AaAP1 genes displayed an increased hypersensitivity to TIBA and CHP. These results implied that AaFUS3- and AaAP1-mediated signaling pathways may function in an additive manner in A. alternata. The toxicity of TIBA to the AaFUS3 or AaAP1 mutant strains might be likely due to the defect regulation for expression of the genes encoding membrane transporters or efflux systems (Gulshan and Moye-Rowley 2007). Indeed, I observed that two genes encoding putative MFS transporters were coordinately regulated by AaFUS3 and AaAP1.

Taken together, this study demonstrates that the AaFUS3-mediated signaling pathway regulates physiological functions, development, the CWDE production and pathogenicity of A. alternata. Most importantly, I provide several lines of evidence to support the notion that a synergistic regulation, by controlling membrane transporters, exists between the AaFUS3 MAP kinase-mediated signaling pathway and the redox-responsive transcription factor AaAP1 in A. alternata.
Figure 3-1. The *Alternaria alternata* AaFUS3 conserved domains and targeted disruption of the *AaFUS3* gene. (A) Schematic illustration of AaFUS3 conserved domains (B) Predicted physical maps of the *AaFUS3* locus before and after disruption by inserting a 2.2-kb hygromycin phosphotransferase gene (*HYG*). (C) Southern blot hybridization of *BglII/Xhol* digested genomic DNA of the wild type and two putative *AaFUS3* disruptants (M1 and M2) to a DNA probe as indicated in B. (D) Northern blot analysis identified a 1.2-kb hybridizing band from the wild type, but not from RNA of two putative mutants M1 and M2.
Figure 3-2. The AaFUS3 gene whose product is necessary for vegetative growth and involved in response to salt sensitivity and fungicide resistance. (A) Radial growth of the wild type and the AaFUS3 null mutant (M1 and M2) strains grown on potato dextrose agar for 7 days. (B to G) Growth rates of the A. alternata strains were determined by measuring the colony diameter over time. Each point is the mean ± the standard deviation of the colony diameter from two independent experiments with at least three replicates.
Figure 3-3. The AaFUS3-disrupted mutants are defective in conidiation. Conidia of the A. alternata wild type (A), AaFUS3 null mutants (B, C, D, E, and F), and complementation strains (G and H) were examined with light microscopy.
Figure 3-4. The *AaFUS3* gene is required for fungal penetration and lesion development. (A) Inoculation of mycelial mass of wild type, the *AaFUS3* mutants (M1 and M2), and the complementation (Cp1) strains on unwound detached Minneola leaves at 4 dpi. (B) Mycelial mass was inoculated onto pre-wounded Minneola leaves at 2 dpi.
Figure 3-5. The *Alternaria alternata* AaFUS3 is required for full virulence. (A) Mycelial mass of wild type (WT), the AaFUS3 null mutants (M1 and M2), and the complementation strains (Mcp1) was inoculated on the midribs of unwound Minneola at 4 dpi. (B) Diameter of fungal hyphae was determined by measuring the fungal growth on the leaves shown in A. a, b, and c were different groups (p< 0.05).
Figure 3-6. Expression of AaFUS3 was up-regulated by leaf extracts. Northern blot hybridization of total RNA prepared from the wild type strain of *A. alternata* grown on PDA, minimal medium (MM), modified MM containing exogenous nitrogen, leaf extracts of Minneola or rough lemon, or no nitrogen or carbon source to an AaFUS3 probe. The RNA samples loaded in the gel were stained with ethidium bromide.
Figure 3-7. *AaFUS3* is involved in the production of hydrolytic enzymes, cutinase activities and melanin. (A) Lipolytic activities were assayed by measuring the formation of visible precipitations around the fungal colonies on the Tween-20 agar plates. (B) Alkaline phosphatase was determined by measuring the p-nitrophenol liberated from *p*-nitrophenyl phosphate (NPP) at 30°C at A_{410} nm. (C) Cutinase activities were determined by formation of a yellow color after reaction with *para*-nitrophenyl butyrate and measured at A_{405}. (D) Endo-PG activities were determined by measuring the amounts of reducing sugar release from 1% polygalacturonic acid (PGA) and reacted with 1% hexadecyltrimethyl ammonium bromide. (E) Production of melanin pigment was measured at A_{459}. *a*, *b*, *c*, and *d* were different groups (*p* < 0.05).
Figure 3-8. Sensitivity tests of the wild type, *AaFUS3* (M1 and M2) and *AaAP1* (Y1 and Y2) null mutants, and their complementation strains (YCp1, 2 and MCp1, 2) to different chemicals. Sensitivity of all *A. alternata* strains was determined by radial growth on PDA containing a chemical as indicated and was quantified by calculating the percentage of growth reduction of *AaFUS3*- or *AaAP1*-disrupted mutants compared to the wild type strain.
Figure 3-9. Schematic illustration of a strategy used for creation of an $AaFUS3/AaAP1$ double mutation and phenotypic assays. (A) Physical map of the split-marker fragments fused with an overlapping $SUR$ (acetolactate synthase gene) for targeted disruption of the $AaAP1$ gene in an $AaFUS3$ null mutant. (B) Two primers yap1-atg and yap1-taa was used to amplify the genomic DNA from WT, the $AaFUS3$ null mutant, and a putative $AaFUS3/AaAP1$ double mutant. (C) Radial growth of fungal strains on potato dextrose agar (PDA) in the presence of TIBA, CHP, $H_2O_2$, $t$-BHP, menadione was measured. (D) Percentage of growth reduction was calculated as a cumulative percentage of growth of WT and null mutants (M1: $\Delta AaFUS3$; Y1: $\Delta AaAP1$; YM: $\Delta AaFUS3/AaAP1$) grown on the same plate.
Figure 3-10. Induction of the AaAP1 or AaFUS3 gene transcript in A. alternata. Northern blot hybridization of total RNA with a digoxigenin-labeled AaFUS3 or AaAP1 probe. The wild type isolate was grown on PDA with a layer of cellophane for 3 days and shifted to media containing TIBA, CHP, DEM, or H₂O₂. The mock treatment (WT) contains RNA from fungal culture shifted to the nonamended PDA. A gel stained with ethidium bromide is shown to indicate the relative amounts of the RNA samples.
Figure 3-11. Immunological detection of AaFUS3 phosphorylation. (A) Western blots of total proteins of the WT, the AaFUS3 null mutant (M), the AaAP1-disrupted mutant (Y), and the AaFUS3/AaAP1 double mutant (YM) were probed with anti-dually phosphorylated P44/42 and anti-FUS3 antibodies. (B) Overall proteins of the AaAP1 null mutant or WT grown on CM in the presence of TIBA, CHP, DEM, and H$_2$O$_2$ were probed with anti-phospho P44/42 or anti-FUS3 antibodies. (C) Western blotting of the wild type grown on CM containing 0.1, 1, or 2.5 mM TIBA were detected by using anti-phospho P44/42 or anti-FUS3 antibodies.
Figure 3-12. A synergistic regulation of two MFS membrane transporters coding genes by AaFUS3 and AaAP1. Total RNA prepared from the wild type, the AaFUS3 null mutant, and the AaAP1-disrupted mutant was hybridized to digoxigenin-labeled probes as indicated.
CHAPTER 4
DISTINCT AND SHARED ROLES OF THE TWO-COMPONENT HISTIDINE KINASE (AaHSK1)- AND THE MITOGEN-ACTIVATED KINASE (AaHOG1)-MEDIATED SIGNALING PATHWAYS IN RESPONSE TO OSMOTIC STRESS AND FUNGICIDES IN Alternaria alternata

The AaHSK1 gene, encoding a group III histidine kinase and the AaHOG1 gene encoding a mitogen-activated protein kinase (MAPK) of Alternaria alternata were cloned and characterized. Mutational inactivation in AaHSK1 or AaHOG1 resulted in fungal strains displaying distinct phenotypic alterations, yet sharing several common deficiencies as well. The AaHSK1 null mutant acquired resistance to the dicarboximide and phenylpyrrole fungicides, and exerted hypersensitivity to sugar but not salt osmotic stress. In contrast, AaHOG1 played a moderate role in fungicide sensitivity. AaHOG1 was required for resistance to oxidants and salts but not sugars. The AaHOG1 null mutants were impaired in virulence, while the AaHSK1 mutants remained pathogenic to citrus. Fungal mutants disrupted at AaHSK1 or AaHOG1 were hypersensitive to 2-chloro-5-hydroxypyridine (CHP) or 2,3,5-triiodobenzoic acid (TIBA). Unlike Neurospora crassa or Aspergillus nidulans, the A. alternata two-component AaHSK1-mediated signaling pathway had little connection with the AaHOG1 MAPK pathway for osmotic, oxidative stress, and fungicide sensitivity. Yet, both AaHSK1 and AaHOG1 shared common functions for resistance to CHP or TIBA. The results implicate a complex regulatory network in response to environmental stimuli in A. alternata.

Introduction

The phosphorelay transduction pathway involving two-component histidine kinases (HK) is essential for perception and adaptation to the environments in bacteria, fungi and plants (Alex and Simon 1994; Chang et al. 1993). Saccharomyces cerevisiae has a single HK, yet Schizosaccharomyces pombe contains three HKs. The whole genome
analyses in filamentous fungi have revealed that *Neurospora crassa*, *Cochliobolus heterostrophus*, *Fusarium verticillioides* and *Botrytis cinerea* contain multiple HK proteins that can be divided into 11 classes (Catlett et al. 2003). Among them, six groups including III, V, VI, VIII, IX, and X HKs are commonly found in many filamentous fungal species (Catlett et al. 2003). Except for group III HK, the function of other histidine kinases remains largely unknown.

Group III histidine kinase such as the OS1 (also known as NIK1) in *Neurospora crassa* has been characterized in relation to osmotic resistance and fungicide sensitivity. This group HK contains unique HAMP (HKs, adenylate cyclases, methyl-accepting chemotaxis proteins, and phosphatases) domain repeats in the N-terminal region (Catlett et al. 2003). Disruption of group III histidine kinase homologs in *Alternaria brassicicola*, *Alternaria longipes*, *C. heterostrophus*, *B. cinerea*, *Magnaporthe grisea*, and *N. crassa*, created fungal mutants that were resistant to fludioxonil fungicide but sensitive to osmotic stress (Avenot et al. 2005; Dongo et al. 2009; Luo et al. 2008; Motoyama et al. 2005; Ochiai et al. 2001; Oshima et al. 2001; Yoshimi et al. 2005).

Furthermore, a point mutation within the HAMP domain of BcOS-1 in *B. cinerea* resulted in fungi highly resistant to decarboxyimide fungicide and sensitive to osmotic stress (Oshima et al. 2001). Similar phenotypes were also found in *N. crassa* and *C. heterostrophus* mutated by changing a single amino acid in the HAMP repeats (Ochiai et al. 2001; Yoshimi et al. 2004).

In addition to HK, cellular signaling pathways mediated by MAPKKK-MAPKK-MAPK cascades have also been well characterized in many fungi (Dickman and Yarden 1999; Lengeler et al. 2000; Xu 2000). However, the activators acting in the upstream
cascade and the regulatory mechanisms of phosphorylation of MAPKs in filamentous fungi remain unclear. In budding yeast *Saccharomyces cerevisiae*, the HOG1-type MAP kinase signal pathway is involved in adaptation to high-osmolarity response. This is regulated by two osmosensors: the sole two-component histidine kinase Sln1p and the membrane protein Sho1 (Fig. 1-3). Sln1p is required for osmotic adaption through the Sln1p-Ypd1p (a histidine phosphotransfer)-Ssk1p or Skn7p (response regulator) pathway (Posas et al. 1996; Li et al. 1998). Under normal osmolarity, Sln1p is autophosphorylated and subsequently activates Ypd1p and Ssk1p by a phosphorelay mechanism (Maeda et al. 1995; Posas and Saito 1998). The phosphorylated Ssk1p is inactive. In contrast, high osmolarity results in Sln1p inactivation and prevents Ssk1p from being phosphorylated. The unphosphorylated Ssk1p can activate Ssk2/22p (MAPKKK) that subsequently activates Pbs2p and Hog1 (Maeda et al. 1995; Posas and Saito 1998). The osmosensing receptor Sho1 triggers Hog1 activity through the stimulation of Ste11p (MAPKKK) and Pbs2p (MAPKK) in response to high osmotic stress (Maeda et al. 1995). In *S. pombe*, the H$_2$O$_2$-dependent activation of the Hog1 ortholog (Sty1) pathway is also activated by two-component sensor kinases, Mak2 and Mak3, in response to the oxidative stress (Samejima et al. 1997; Shieh et al. 1998).

Several studies in several filamentous fungi have shown that group III HK often regulates HOG1 during osmosensing (Dongo et al. 2009; Yoshimi et al. 2005). The HK-HOG1 signaling pathway is also responsible for fungicide sensitivity (Kojima et al. 2004). However, this regulation may vary among yeasts and fungal species. For example, disruption of the *hik1* gene (a group III HK homolog) in the rice blast fungus, *M. grisea*, resulted in fungi with an increased sensitivity to sugar but not salt stress (Motoyama et
al. 2005). The *N. crassa* os-1 mutants or the *C. heterostrophus* dic1 deletion strains, likely defective in the HK-Hog1 pathway, were sensitive to both salts and sugars (Schumacher et al. 1997; Yoshimi et al. 2004). However, the HOG1-like MAP kinase Sak1 of *Botrytis cinerea* was negatively regulated by the histidine kinase Bos1 and was not involved in fungicide sensitivity (Liu et al. 2008).

For some fungi such as *M. grisea*, building intracellular osmotic pressure in appressoria is critical for penetration into host plant (de Jong et al. 1977; Howard and Valent 1996; Money and Howard, 1996). Generation of turgor pressure in appressoria has been documented to be mediated via the HK-HOG1 regulatory mechanism in *M. grisea*. However, deletion of the *osm1* gene, a Hog1 homolog, in *M. grisea* did not alter fungal pathogenicity (Dixon et al. 1999). Unlike *M. grisea*, the *B. cinerea* BOS1 gene encoding a HK-like protein is required for full virulence (Viaud et al. 2006). Thus, the pathological function of group III histidine kinase in conjunction with HOG1 MAP kinase may rely on different lifestyle of each species and their hosts.

Previous studies have shown that the *A. alternata* AaFUS3 MAP kinase was not involved in the oxidative response (Lin et al. 2010). However, AaFUS3 cooperating with the redox-responsive AaAP1 transcription regulator confers resistance to diverse chemicals. Little is known about the relationships between HOG1 MAPK and histidine kinase in *A. alternata*. In the present study, I cloned and characterized two genes: *AaHSK1* and *AaHOG1*, encoding a Group III two-component HK and a HOG1 MAP kinase, respectively, in the tangerine pathotype of *A. alternata*. I provided experimental evidence to define their roles for pathogenicity, cellular responses to osmotic and
oxidative stresses, sensitivity to fungicides, and resistance to multiple drugs in this important citrus pathogen.

**Materials and Methods**

**Cloning of AaHSK1 and AaHOG1**

The AaHSK1 gene was amplified from genomic DNA of the A. alternata with two primers HSK-2 and HSK-8 that are complementary to a group III histidine kinase gene of A. brassicicola (Table A-1). The amplified fragment was cloned into pGEM-T easy vector (Promega) for sequence analysis. The 5'- AaHSK1 as well as its promoter region were amplified with two inverse primers HSK-69 and HSK-136 from restriction enzyme-digested and self-ligated DNA templates. The 3'-end of AaHSK1 gene was obtained by PCR with two inverse primers HSK-548 and HSK-564 (Table A-1).

The primers Hog-1Fand Hog-1R were used for PCR amplification of an AaHOG1 gene fragment. The full-length AaHOG1 and its 5’ and 3’-flanking sequences were obtained with two sets of inverse primers: Hog-256R paired with Hog-316F and Hog1001R paired with Hog-1068F (Table A-1).

**Construction and Identification of the AaHSK1- and AaHOG1-Null Mutants**

For AaHSK1 gene disruption, a 1.3-kb DNA fragment was amplified with two primers Hsk-2 and Hsk-2374 and cloned into pGEM-T easy vector to generate T-HskDM. The AaHSK1 disruption construct, T-HskDMhyg, was created by inserting the hygromycin phosphotransferase gene (HYG) cassette at a BglII site of T-HskDM. A 2.2-kb DNA fragment containing 5' AaHsk1::5' HYG fusion DNA was amplified with the primers HSK-2 and hyg3. A 2.1-kb fragment encompassing 3' AaHsk1 and 3' HYG was amplified with two primers HSK-2374 and hyg4. The amplified fragments were mixed, and transformed into the wild type protoplasts (Fig. 4-2A).
To disrupt *AaHOG1*, a PCR fusion method was carried out to create *AaHOG1/HYG* split-marker fragments as illustrated (Fig. 4-3A). The "HY/g" and "h/YG" of *HYG* were amplified using the primers M13R/hyg3 and M13F/hyg4 primers, respectively, from pUCATPH. The 5' *AaHOG1* was amplified with the primers Hog-316F and Hog-F2. The 3' *AaHOG1* was amplified with Hog-tr and Hog-F3 (Fig. 4-3). In second round PCR, a 2.2-kb DNA fragment (5' *AaHOG1*: h/YG) was amplified with primers Hog-316F and hyg4, whereas a 1.8-kb HY/g::3' *AaHOG1* was amplified with primers Hog-tr and hyg3. The putative *AaHSK1* and *AaHOG1* disruptants were screened for sensitivity to 1 M glucose and 1 M NaCl, respectively, and further confirmed by Southern and Northern blot analyses.

**Genetic Complementation of an *AaHSK1*-Null Mutant**

For genetic complementation, a 5.7-kb DNA fragment containing the entire *AaHSK1* gene and its endogenous promoter was amplified with the primers HSK-P1 and HSK-tga using a high fidelity DNA polymerase (Roche Applied Science). The amplified product was co-transformed with a pCB1532 plasmid (Sweigard et al. 1997) into protoplasts prepared from an *AaHsk1* null mutant.

**Molecular Techniques**

An *AaHSK1* cDNA fragment was amplified with the primers Hsk-up and Hsk-tga. A cDNA fragment of *AaHOG1* was amplified with the primers Hog1-atg and Hog-tr. Both *AaHOG1* and *AaHSK1* DNA probes were labeled with digoxigenin (DIG)-11-dUTP (Roche Applied Science) by PCR with gene-specific primers Hog-316F / Hog-F2 or Hsk-2/Hsk-2374, respectively.
Detection of Phospho-AaHOG1 MAPK

Fungal strains of *A. alternata* were grown in complete medium (CM) for 3 days at room temperature, treated with 0.6 M NaCl, 0.05% H₂O₂, or 1 μg/ml iprodione, and incubated for additional 2 hours. A rabbit anti-phosphate-p38 MAPK kinase antibody (Cell Signaling Technology, Boston, MA) and anti-Hog1 rabbit polyclonal antibody (Santa Cruz Biotechnology) at a 1:1000 dilution were used as primary antibodies. The anti-rabbit–IgG antibody conjugated horseradish peroxidase (HRP) (Cell Signalling Technology, Boston, MA) at a 1:2000 dilution was used as a secondary antibody.

Nucleotide Sequence

Sequence data from this chapter can be found in the EMBL/GenBank Data Libraries under Accession no. GQ414508 (*AaHSK1*) and GQ414509 (*AaHOG1*).

Results

Cloning of the *AaHSK1* and *AaHOG1* Genes of *A. alternata*

A 3.7-kb DNA fragment was amplified from *A. alternata* genomic DNA. Sequence analysis revealed that the deduced amino acid sequence shares high similarity to many group III histidine kinases and the cloned gene was named *AaHSK1*. By comparing with the cDNA sequences, the overall *AaHSK1* open reading frame (ORF) contains 4275-bp nucleotides interrupted with six introns of 50, 49, 55, 49, 51, and 58 bp. The deduced 1329 amino acids showed 96% to 99% similarity to two-component histidine kinases of *A. longipes*, *A. brassicicola*, or *C. heterostropus*, and 76% similarity to Os1 (NIK1) of *N. crassa*. The AaHSK1 polypeptide has a HAMP repeat domain (histidine kinase domain, adenylyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases), a response regulator domain, a histidine kinase-like ATPase domain, and a signal receiver domain (sensor domain) (Fig. 4-1A).
The primers Hog-1F and Hog-1R amplified a 0.9-kb DNA fragment from the tangerine pathotype of *A. alternata*. The predicted amino acid sequence displayed high similarity to many HOG1-type MAP kinases of yeasts and fungi and thus was designated *AaHOG1* (*Alternaria alternata* HOG1-like gene). *AaHOG1* contains a 1409 bp ORF interrupted by seven introns of 50, 50, 49, 48, 50, 47, and 47 bp. *AaHOG1* contains several conserved domains: a protein kinases ATP-binding region (amino acids 26-50), a MAP kinase (amino acids 55-153) and a serine/threonine protein kinase signature (amino acids 137-149) (Fig. 4-1B). *AaHOG1* is most similar to the HOG1-like MAP kinases of *Pyrenophora tritici-repentis* (XP_001935555) and *Phaeosphaeria nodorum* (QOU4L8), showing 98 to 100% identities.

**Targeted Disruption of *AaHSK1* and *AaHOG1* in *A. alternata***

Transformation of split *AaHSK1/HYG* fragments into the wild type strain of *A. alternata* recovered 19 transformants from medium containing hygromycin. Among them, four were highly sensitive to 1 M glucose and were considered as putative *AaHSK1* disruption mutants. Southern-blot analysis revealed that hybridization of *Xho*I-digested genomic DNA of four putative disruptants to an *AaHSK1* probe detected a 4.7-kb hybridizing signal, owing to the integration of *HYG* cassette. In contrast, a 2.5-kb hybridizing band was detected in the wild type DNA (Fig. 4-2B). Furthermore, Northern-blot analysis further confirmed that the four putative disputants did not accumulate any detectable transcript of the *AaHSK1* gene (Fig. 4-3C), indicating that they are *AaHSK1* null mutants.

Transformation of two fragments containing truncated *HYG* flanked by either 5’ or 3’ *AaHOG1* sequence (Fig. 4-3A) into wild type protoplasts identified three putative mutants out of four transformants recovered. These three mutants were highly sensitive
to 1 M NaCl and KCl and were considered as the putative AaHOG1-disrupted mutants. Southern-blot hybridization of NruI and EcoRV-digested genomic DNA isolated from three AaHOG1 mutants and wild type to an AaHOG1-specific probe revealed very different hybridizing patterns (Fig. 4-3B), indicating successful integration of HYG within AaHOG1. Northern-blot analysis further confirmed that the putative disruptants did not produce AaHOG1 transcript (Fig. 4-3C).

Phenotypic Characterization of the AaHSK1 and AaHOG1 Null Mutants

Sensitivity tests revealed that the AaHSK1-disrupted mutants were highly sensitive to glucose, sucrose, sorbitol, and mannitol, but not to tert-butyl-hydroxyperoxide, H2O2, menadione, NaCl, or KCl (Fig. 4-4A). The complemented strains by expressing a functional AaHSK1 fully restored the defective functions to wild type levels. In contrast, the AaHOG1-impaired mutants displayed hypersensitivity to NaCl, KCl, H2O2, menadione, or tert-butyl-hydroxyperoxide but not to sugar stress (Fig. 4-4B). The AaHOG1 null mutants did not produce protoplasts (see details in Chapter 5) and was not complemented. Interestingly, both AaHSK1 and AaHOG1 deletion strains were highly sensitive to TIBA and CHP (See details in Chapter 5).

The AaHSK1 and AaHOG1 null mutants were tested for sensitivity to dicarboximide (iprodione and vinclozolin) and phenylpyrrole (fludioxonil) fungicides. The wild type A. alternata was extremely sensitive to iprodione, vinclozolin, or fludioxonil. However, deletion of the AaHSK1 gene resulted in fungi highly resistant to these fungicides (Fig. 4-5). The AaHOG1 null mutant was only slightly resistant to those fungicides (Fig. 4-5), suggesting a limited link between AaHSK1 and HOG1-type MAP kinase signaling pathways in the context of fungicide sensitivity.
**AaHOG1 but not AaHSK1 Is Required for Fungal Pathogenicity**

Pathogenicity assays performed on detached Minneola leaves using point inoculation revealed that wild type, the AaHSK1 null mutants and the Cp1 strain all incited necrotic lesions at 3 days postinoculation (dpi) on wounded or unwounded leaves (Fig. 4-6A). By contrast, inoculation of the AaHOG1-disrupted mutants did not induce necrotic lesions (Fig. 4-6B). Similar results were observed using a spray inoculation technique, indicating that AaHOG1, but not AaHSK1, promotes fungal pathogenicity (Fig. 4-6C).

**AaHOG1 Phosphorylation Is Regulated by AaHSK1**

An anti-phosphate-p38 MAPK kinase antibody was used to detect phosphorylation of AaHOG1 MAP kinase. Compared with the untreated control, AaHOG1 was highly phosphorylated in wild type after being treated with 0.6 M NaCl, 0.05% H₂O₂, or 1 μg/ml Ipodine fungicide (Fig. 4-7). Interestingly, disruption of the AaHSK1 gene apparently reduced AaHOG1 phosphorylation (Fig. 4-7).

**Discussion**

eukaryotic cells can sense and respond to osmotic stress. This response plays important roles in growth and pathogenicity in filamentous fungi (de Jong et al. 1977; Howard and Valent 1996; Viaud et al. 2006). Cellular adaptation to changes in osmolarity has been investigated primarily in yeasts or *N. crassa* (Maeda et al. 1995; Posas and Saito 1998; Samejima et al. 1997; Schumacher et al. 1997; Shieh et al. 1998; Zhang et al. 2002), and osmotic response was poorly understood in plant pathogenic fungi. In the current study, I identified the AaHSK1 gene encoding a group III two-component histidine kinase and the AaHOG1 gene encoding a HOG1-type MAP kinase. Genetic analysis defined their functions in the signal transduction pathways related to
osmotic stress in *A. alternata*. Similar to the *N. crassa* OS-1 histidine kinase, the *A. alternata* AaHSK1 contains a HAMP repeat, a sensor and a response regulator conserved domains that are likely associated with fungicide sensitivity and osmotic resistance. The *A. alternata* AaHOG1 MAP kinase belonging to the protein kinase C superfamily was shown to be involved in response to oxidative, osmotic, and fungicide stresses.

In *N. crassa*, the OS-1-mediated signal transduction pathway comprised of *os-1* (*NIK*), *os-2* (*HOG1-like*), *os-4* (*MAPKKK*), and *os-5* (*MAPKK*), has been shown to be involved in response to the dicarboximide fungicides and osmotic adaptation (Noguchi et al. 2007). In contrast, the *AaHSK1* (OS-1 homolog) and the *AaHOG1* (OS-2 homolog)-mediated signal pathways seems to have very different regulatory functions in response to environmental stimuli in *A. alternata*. The *N. crassa os-1* or *os-2* mutants are hypersensitive to both salts and sugars but are resistant to fludioxonil fungicide (Noguchi et al. 2007; Schumacher et al. 1997; Zhang et al. 2002). However, disruption of the *AaHSK1* gene in *A. alternata* resulted in fungi that were highly sensitive to glucose, sucrose, sorbitol, and mannitol, but not to NaCl and KCl. Disruption of the *AaHOG1* gene, however, created fungi hypersensitive to NaCl, KCl, and oxidants, but insensitive to non-ionic osmoticants. The results indicated that *A. alternata* may have the ability to distinguish sugar from salt stimuli to cope with osmolarity conditions, using *AaHOG1* or *AaHSK1*-mediated signaling pathway. The *AaHSK1* null mutants showed an elevated resistance to the dicarboximide and the phenylpyrrole fungicides. However, the *AaHOG1*-disrupted strains were slightly resistant to these antifungal agents compared to wild type. Unlike other fungal systems (Furukawa et al. 2005; Noguchi et al.
group III histidine kinase-mediated fungicide sensitivity is not fully associated with the AaHOG1 MAP kinase signaling pathway in A. alternata. Thus, there may be one or more unknown signal routes under control of AaHSK1 for fungicide sensitivity. However, as demonstrated later (Chapter 5), both the AaHSK1 and AaHOG1 disruption mutants were hypersensitive to TIBA and CHP, suggesting the existence of shared functions between AaHSK1- and AaHOG1-signaling pathways. Indeed, a MFS-transporter coding gene (clone # 19) was found to be commonly regulated by AaHSK1 and AaHOG1 (Chapter 5).

Western blot analyses indicated that the AaHSK1 histidine kinase interferes with AaHOG1 phosphorylation in A. alternata. Unlike AaHOG1, AaHSK1 plays little or no role in resistance to H$_2$O$_2$, KCl and NaCl. In Aspergillus nidulans and N. crassa, the Hog1 MAP kinase-mediated signaling pathway is primarily activated by the group III two-component histidine kinase in response to osmotic stress or fungicides (Furukawa et al. 2005; Noguchi et al. 2007).

Accumulation of glycerol in appressoria by the rice blast fungus is vital for generation of mechanical force that is absolutely required for penetration to the leaf surfaces (de Jong et al. 1997; Money and Howard 1996). However, turgor generation and glycerol accumulation in appressoria were not controlled by OSM1 (a HOG1 ortholog) in M. grisea; the OSM1 gene deletion strain was pathogenic (Dixon et al. 1999). Inactivation of a histidine kinase gene (HIK1) in M. grisea had no effects on pathogenicity either (Motoyama et al. 2005), even though both OSM1 and HIK1 were required for resistance to high osmolarity. However, the histidine kinase gene homolog, BOS1, of the phytopathogenic fungus Botrytis cinerea is essential for colonization to its
host (Viaud et al. 2006). HOG1 homologs have been studied in many phytopathogenic fungi, including *M. grisea, Bipolaria oryzae, B. cinerea* and *Cryphonectria parasitica* for their roles in pathogenicity. The Hog1 homologs in *B. cinerea* and in *Cryphonectria parasitica* are essential for fungal pathogenicity (Park et al. 2004; Segmüller et al. 2007). However, disruption of a *HOG1* homolog in *M. grisea* or *B. oryzae* did not alter fungal virulence (Dixon et al. 1999; Moriwaki et al. 2006). In *A. alternata*, *AaHSK1* played no role in virulence. In contrast, *AaHOG1*-disruptants failed to incite any visible necrotic lesions even though citrus leaves were pre-wounded. *AaHOG1* is likely involved in oxidative detoxification of ROS, similar to the previously characterized redox-responsive *AaAP1* regulator.

The conserved HK-HOG1 signaling transduction pathway often functions together in the same cascade in many fungi (Furukawa et al. 2005; Noguchi et al. 2007). In *A. alternata*, the *AaHSK1*-mediated sugar resistance has little connection with the HOG1 pathway. Similarly, resistance to salt stress primarily modulated by *AaHOG1* is also not effected by mutation of the *AaHSK1* histidine kinase. The dicarboximide and the phenylpyrrole fungicides mainly target *AaHSK1* rather than *AaHOG1*. Thus, it becomes apparent that *A. alternata* has evolved unique mechanisms to adapt to environmental stresses.
Figure 4-1. Functional domains of AaHSK1 and AaHOG1. (A) Schematic illustration of AaHSK1 showing several conserved domains similar to group III histidine kinases of fungi. They include a repeat HAMP domain, a response regulator, and a receiver domain. (B) Physical map of AaHOG1 belonging to a protein kinase C superfamily contains a protein kinase ATP-binding region (I: aa 26 to 50), a MAP kinase signature (II: aa 55-153) and a serine/threonine protein kinase active site (III: aa 137-149).
Figure 4-2. Gene replacement of AaHSK1 in A. alternata. (A) Schematic illustration of generation of the truncated AaHSK1 gene fused with an overlapping hygromycin phosphotransferase gene (HYG) under control by the Aspergillus nidulans trpC promoter for AaHSK1 gene replacement using a split-marker approach. (B) Southern blot hybridization of Xhol digested genomic DNA with the AaHSK1-specific probe as indicated in (A). (C) Northern blotting of RNA prepared from the wild type (WT) and 4 putative AaHSK1 mutants with an AaHSK1 probe. A gel image stained with ethidium bromide indicates the relative amounts of RNA samples.
Figure 4-3. Targeted disruption of the AaHOG1 gene in A. alternata. (A) Predicted physical maps of the AaHOG1 locus before and after targeted disruption by hygromycin phosphotransferase gene (HYG). (B) DNA blot of NruI and EcoRV double digested genomic DNA of the wild type (WT), an ectopic strain (N), and three putative AaHOG1 disruptants, was hybridized with an AaHOG1 probe as indicated. (C) Northern hybridization of fungal RNA isolated from the WT, N and three putative disruptants with an AaHOG1 probe.
Figure 4-4. Phenotypic characterization of the wild type (WT), two AaHSK1-disrupted strains (Hk1 and Hk2), two AaHSK1 complementation strains (Cp1 and Cp2), and two AaHOG1 null mutants (Hg1 and Hg2). (A) The AaHSK1 null mutants displayed hypersensitive to glucose, sucrose, sorbitol, and mannitol. (B) Deletion of the AaHOG1 gene in A. alternata resulted in an elevated sensitivity to H₂O₂, menadione, t-BHP (tert-butyl-hydroperoxide), NaCl, or KCl. One representative replicate is shown.
Figure 4-5. Sensitivity of the wild type (WT), two AaHSK1 mutants (Hk1 and Hk2), and two AaHOG1 deletion strains (Hg1 and Hg2) to different fungicides. Fungal strains were grown on potato dextrose agar amended with 10 μg/ml of iprodine, vinclozolin (dicarboximide) or 0.1 μg/ml fludioxonil (phenylpyrrole) and incubated at 28°C for 3-4 days.
Figure 4-6. The *A. alternata* AaHOG1, but not AaHSK1, is required for pathogenicity. (A) Pathogenicity assays were performed on detached Minneola leaves inoculated with 5 μl of conidial suspension (10⁴ conidia/ml) prepared from the wild type (WT), the AaHSK1-disrupted mutants Hk1 and Hk2, and the complementation strain Cp1. (B) Minneola leaves inoculated with WT and two AaHOG1 null mutants Hg1 and Hg2 were incubated in a moist chamber. (C) Fungal pathogenicity was assayed on detached Minneola leaves uniformly sprayed with conidial suspension of *A. alternata* strains. Images were taken 3–4 days postinoculation (dpi). The mock controls were treated with water only.
Figure 4-7. Immunological detection of AaHOG1 phosphorylation. (A) Total proteins were prepared from the wild type (WT), the AaHSK1 null mutant and the AaHOG1-disrupted mutant grown in complete medium (CM) or in CM supplemented with 0.05% H$_2$O$_2$, 1 μg/ml iprodione or 0.6 M NaCl. The AaHOG1 was detected by anti-phosphorylated P38 and anti-Hog1 antibodies.
CHAPTER 5

The Alternaria alternata AaSLT2 gene, encoding an ortholog of the SLT2 mitogen-activated protein (MAP) kinase of Saccharomyces cerevisiae, was cloned and characterized. AaSLT2 was necessary for conidiation, maintenance of cell-wall integrity, melanin accumulation and fungal virulence but dispensable for toxin production. I compared the phenotypes of the mutants disrupted in each of three MAPK genes (AaFUS3, AaHOG1 and AaSLT2), the AaHSK1 gene, or the AaAP1 gene. This study revealed possible interactions among these pathways at transcriptional and post-translational levels, leading to proper regulation of a wide diversity of biological functions. Compared to the AaSLT2 null mutants, AaHSK1 and AaHOG1 null mutants were less sensitive to cell-wall-degrading enzymes. Accumulation of the AaHOG1 gene transcript was highly elevated in the AaSLT2 null mutant and was slightly increased in the AaFUS3 disruptant. AaSLT2 promoted AaFUS3 expression and vice versa. AaSLT2 elevated AaAP1 expression, whereas AaAP1 inhibited AaSLT2 expression. Furthermore, phosphorylation of AaHOG1 or AaFUS3 was affected when other genes were inactivated, indicating a functional antagonism or synergism among these signal transduction pathways. Interestingly, signaling transduction pathways-mediated by AaAP1, AaHSK1, AaHOG1, AaSLT2, and AaFUS3 play a critical and non-redundant roles in resistance to 2-chloro-5-hydroxyphridine (CHP) and 2,3,5-triiodobenzoic acid (TIBA) in A. alternata.
Introduction

Mitogen-activated protein (MAP) kinases-mediated signal transduction pathways are involved in diverse biological functions (Gustin et al. 1998; Kultz 1998; Xu 2000). The Saccharomyces cerevisiae SLT2 (MPK1) kinase pathway is involved in the formation of cytoskeleton components, cell wall integrity, polarization of cell growth, and responses to nutrient availability (Lee et al. 1993; Torres et al. 1991). SLT2 kinase is activated by the MAPKKK Bck1, which is activated by two redundant MAPKKs Mkk1 and Mkk2 (Irie et al. 1993; Kamada et al. 1995).

In the rice blast fungus, Magnaporthe grisea, an SLT2 homolog (Mpks) is essential for pathogen penetration and rearrangement of actin cytoskeleton (Xu et al. 1998). Similarly, an SLT2-like MAP kinase homolog in Claviceps purpurea, Cochliobolus heterostrophus or Fusarium graminearum is involved in developmental processes associated with sexual reproduction, plant infection, and cell wall integrity (Hou et al. 2002; Mey et al. 2002; Igbaria et al. 2008).

Although each of the MAP kinase pathways has been extensively studied in a number of fungi, interconnections between these signal pathways are not yet clear. In S. cerevisiae, the mating pathway mediated by FUS3 and the filamentous pathway by KSS1 are commonly regulated by multiple components including Ste20, Ste11, and Ste7 (Fig. 1-3) (Schwartz and Madhani 2004). Different MAP kinase pathways may also interact in a cooperative or antagonistic manner (Xu 2000), further diversifying their specificities. In S. cerevisiae, the specificity of MAP kinase signaling pathways is mainly determined by insulating the components in distinct subcellular compartments or by mutual inhibition (Whitmarsh and Davis 1998; Schwartz and Madhani 2004). For example, the KSS1 pathway has been shown to suppress the FUS3 or HOG1 pathway.
by degrading a Tec1 transcription factor or by preventing DNA binding from Tec1
(Gavrias et al. 1996; Zeitlinger et al. 2003; Shock et al. 2009). Tec1 is a regulator for
hyphal development and exclusively involved in the KSS1 signal pathway (Madhani and
Fink 1997). Tec1 often cooperates with Ste12 during filamentous growth. In contrast,
the Hog1 MAP kinase activates an Msg5 gene, encoding a phosphatase that facilitates
dephosphorylation in FUS3 and KSS1, thereby blocking their functions (Bardwell et al.
1996; Andersson et al. 2004).

Three MAP kinase genes and their functions have been characterized in the
pathogenic fungus \textit{M. grisea}. A \textit{FUS3/KSS1} homolog (Pmk1) is responsible for
appressoria formation and pathogenicity. A \textit{HOG1} homolog is involved in
osmoregulation but dispensable for pathogenesis. A \textit{SLT2} homolog is essential for
pathogenicity and cell wall integrity (Xu 2000). Moreover, the \textit{C. heterostrophus} mutant
disrupted in a \textit{FUS3} homolog displayed defective phenotypes similar to the mutants
disrupted in a \textit{SLT2} homolog. Those common phenotypes include autolytic appearance,
reduction in virulence and conidiation, suggesting that several downstream genes were
co-regulated by \textit{FUS3} and \textit{SLT2} MAPKs (Igbaria et al. 2008). The \textit{C. heterostrophus}
\textit{HOG1} is responsible for resistance and adaption to hyperosmotic and oxidative
stresses (Igbaria et al. 2008).

In this study, I first characterized an \textit{SLT2} MAP kinase gene homolog (designated
\textit{AaSLT2}). I further compared the signaling pathways that are mediated via three MAP
kinases (\textit{AaHOG1}, \textit{AaSLT2} and \textit{AaFUS3}), a redox-responsive transcription regulator
(\textit{AaAP1}), or a two-component histidine kinase (\textit{AaHSK1}) in \textit{A. alternata}. The studies
revealed possible interactions among these pathways at transcriptional and post-
translational levels, leading to proper regulation for a wide diversity of biological functions.

Materials and Methods

Fungal Strains

The wild type EV-MIL31 strain of *Alternaria alternata* (Fr.) Keissler has been described in Chapter 2. The genetically altered strains, defective in *AaAP1*, *AaHSK1*, *AaFUS3* or *AaHOG1*, were generated from previous studies.

Cloning of *AaSLT2*

A 0.9-kb DNA fragment was amplified by a Go-Taq DNA polymerase (Promega) from genomic DNA of *A. alternata* EV-MIL31 with two primers Slt2-1F and Slt2-1R (Appendix Table 1). The amplicon was cloned into pGEM-T easy vector (Promega) for sequence analysis. The cloned gene was named *AaSLT2*. The 5’- *AaSLT2* sequence as well as its promoter region were amplified with two inverse primers SLT-21R and SLT-52F, whereas the 3’-end of *AaSLT2* was amplified with two inverse primers SLT-946R and SLT-1024F from restriction endonucleases and self-ligated DNA templates. Sequence of *AaSLT2* has been deposited with EMBL/GenBank Data Libraries under accession no. GQ414510.

Creation and Identification of *AaSLT2* mutants

To disrupt *AaSLT2*, a PCR fusion method was used to create split-marker fragments (Fig. 5-1B). The fragments "HY/g" and "h/YG" overlapping within the hygromycin phosphotransferase cassette (*HYG*) were amplified from pUCATPH (Lu et al. 1994) with two sets of primers M13R/hyg3 and M13F/hyg4. A 1.0-kb DNA fragment of 5’ *AaSLT2* was amplified with the primers SLT-pro and SLT2-F2 and a 0.9-kb of 3’ *AaSLT2* amplified with the primers SLT2-taa and SLT2-F3. Primers SLT2-F2 and F3
contain sequences complementary to M13F and M13R primers, respectively. Subsequently, a 2.9-kb DNA fragment fused with 5' AaSLT2 and "h/YG" was amplified with the primers SLT-pro and hyg4. A 1.9-kb DNA fragment fused with "HY/g" and 3' AaSLT2 was amplified with the primers SLT-taa and hyg3.

**Genetic Complementation of AaSLT2-Null Mutant**

To complement an AaSLT2 null mutant, a 2.4-kb DNA fragment was amplified with the primers SLT-pro and SLT2-taa using a high fidelity PCR system (Roche Applied Science). The amplified PCR product was co-transformed with pCB1532 plasmid into protoplasts prepared from an AaSLT2 null mutant. Transformants were selected on a medium containing 5 μg/ml sulfonyleurea and tested for phenotypic restoration.

**Pathogenicity Test**

Fungal pathogenicity assays were conducted on detached Minneola leaves inoculated with conidial suspension as described in Chapter 2. Conidia were isolated as previously described (Peever et al. 2000).

**Statistical Analysis**

A two-tailed *t*-test was performed to indicate if changes in disease incidence were statistically significant. A *p*-value of < 0.05 in the two–tail *t*-test was interpreted as a significant difference, while *p*-values ≥ 0.05 were insignificant.

**Sensitivity of Cell-Wall-Degrading Enzymes (CWDEs) and Generation of Fungal Protoplasts**

Assays for sensitivity to 0.4 mg/ml β-glucanase or a CWDE mixture containing driselase, β-D-glucanase, β-glucuronidase, and lyticase were determined by the number of protoplasts released from fungal hyphae over time and examined by light microscopy (Leica Microsystems Inc., Exton, PA, U.S.A). Enzymes were dissolved in an
osmotic buffer for fungal protoplasts preparation as described previously (Chung et al. 2002).

**RNA Quantitative analyses**

Quantitative analyses with TotalLAB TL100 software (Nonlinear Dynamics) were performed to assess the intensity of each hybridizing band. 1D gel analysis is performed an automatic analysis using the selected modes following the manuscript instruction.

**Western-Blot Analysis**

Fungal isolates were grown in a complete medium for 3 days at room temperature and total proteins were extracted as previously described (Chapter 3). The procedures for protein separation, blotting to nitrocellulose membranes, hybridization, and washing have also been described in Chapter 3. Phosphorylation of AaFUS3 and AaHOG1 was detected by anti-phosphorylated P38 and P44/42 antibody, respectively.

**Molecular Techniques**

Procedures used for manipulation of nucleic acids were described in Chapter 2. The AaSLT2 cDNA fragment was amplified with the primers SLT-atg and SLT-taa using a high fidelity PCR Taq polymerase (Roche Applied Science) and cloned into pGEM-T easy vector for sequence analysis. DNA probes for Southern or Northern blot analysis were labeled with digoxigenin (DIG)-11-dUTP (Roche Applied Science) by PCR using gene-specific primers: yap-atg / yap-alpha3 (AaAP1), MAPK-98/ MAPK-taa (AaFUS3), Hog-atg / Hog-tr (AaHOG1), Hsk-2 /Hsk-2374 (AaHSK1), and SLT-52F/SLT-946R (AaSLT2). (Appendix Table A-1).
Results

Cloning of the AaSLT2 Gene in A. alternata

The A. alternata AaSLT2 gene has a 1677-bp ORF interrupted with five introns of 52, 52, 117, 50, and 155 bp. The translated AaSLT2 coding sequence contains 416 amino acids with a conserved serine/threonine domain (Fig. 5-1A). The AaSLT2 MAP kinase protein is most similar to SLT2-like proteins of A. brassicicola (AAU11317), C. heterostrophus (ABM54149) and Ajellomyce capsulatus (XP_001538584) showing 85 to 99% identity (data not shown).

Targeted Disruption of AaSLT2

As described in previous chapters, a split HYG marker strategy was performed to disrupt AaSLT2 gene in A. alternata, (Fig.5-1B). Of six transformants screened, five exhibited reduced growth on PDA and were analyzed further. Southern blot hybridization of SalI and StuI digested genomic DNA to an AaSLT2 probe detected an expected 1.2-kb band in the wild type. In contrast, a 3.4-kb hybridizing band was detected in DNA of five putative transformants owing to integration of the HYG gene cassette (Fig. 5-1C). Three putative AaSLT2 disruptants were analyzed further by Northern blotting (Fig. 5-1D), confirming that the AaSLT2 gene was successfully disrupted in A. alternata.

AaSLT2 Is Required for Virulence

Pathogenicity assessed on Minneola leaves sprayed uniformly with conidial suspensions revealed an apparent reduction of necrotic lesions induced by the AaSLT2 null mutant compared to those induced by wild type (Fig. 5-2). Statistical analysis using t-test indicated that the mean lesion number per leaf (Mean=40, n=10) induced by wild
type was significantly different from those induced by an AaSLT2 null mutant (Mean=12.3, n=10, \( p \leq 0.05 \)) (Table C-1).

**Production of Conidia and Protoplasts by A. alternata**

Disruption of the AaSLT2 gene in A. alternata resulted in a drastic reduction of conidia production \((1.0 \pm 0.25 \times 10^5)\) compared to the wild type strain \((3.75 \pm 0.55 \times 10^6)\).

Deletion of the AaFUS3 gene completely blocked conidial formation, consistent with previous findings (Fig. 5-3 and Chapter 4).

After treatment with cell-wall-degrading enzymes, the AaSLT2-impaired mutant released more protoplasts than wild type (Fig. 5-4A and B), whereas the AaHSK1 null mutant released fewer protoplasts. No protoplasts were produced by the AaHOG1 null mutant even after prolonged incubation with CWDEs.

**Phenotypic Assays in A. alternata**

In contrast to wild type, the AaAP1 and AaHOG1 null mutants were highly sensitive to oxidants (Table 5-1 or Fig. C-1). The AaHSK1-disrupted mutants exhibited hypersensitivity to sugars, whereas the AaHOG1 null mutants were hypersensitive to salts. The AaFUS3 and AaSLT2 deletion strains grew slowly on PDA. Inclusion of KCl or NaCl in PDA markedly enhanced radial growth of the AaFUS3 and AaSLT2 null mutants. The AaHSK1 null mutants, but not the AaAP1, AaFUS3 or AaSLT2-disrupted strains, became highly resistant to fludioxonil fungicide. The AaHOG1 null mutant displayed a slightly increased resistance to fludioxonil. Interestingly, all disrupted mutants were highly sensitive to TIBA or CHP.
AaAP1, AaFUS3, AaSLT2, AaHOG1 and AaHSK1 Cooperatively Regulate the Expression of a MFS Transporter Coding Gene

TIBA is an inhibitor of the plant hormone indoleacetic acid (IAA) transporter (Prusty et al. 2004). Previous studies revealed that two gene clones (#19 and #54), encoding membrane-bound major facilitator superfamily (MFS) transporters, were regulated by both AaAP1 and AaFUS3 (Chapter 3). Further analysis indicated that expression of the gene clone #19 was down-regulated considerably in fungal mutants disrupted in AaAP1, AaFUS3, AaSLT2, AaHOG1 or AaHSK1 (Fig. 5-5). Expression of the gene clone #54 was also down-regulated in the AaAP1, AaFUS3, AaSLT2 and AaHSK1 deletion strains, but slightly up-regulated in the AaHOG1 null mutant (Fig. 5-5).

Transcriptional Feedback Regulation

Accumulation of the AaAP1 gene transcript was elevated in the AaHSK1 null mutant, but decreased in the AaSLT2-disrupted mutant (Fig. 5-6). Expression of the AaHSK1 gene was up-regulated in the null mutants defective in either of the AaAP1, AaFUS3, AaSLT2 or AaHOG1 gene (Fig. 5-6). Expression of the AaFUS3 gene was slightly up-regulated in the AaHSK1 and AaHOG1 null mutant. Disruption of the AaAP1, AaHSK1 or AaHOG1 gene increased accumulation of the AaSLT2 gene transcript. Expression of the AaSLT2 gene was down-regulated in the AaFUS3 null mutant and vice versa. Expression of the AaHOG1 gene was greatly up-regulated in the AaSLT2 null mutant and only slightly elevated in the AaAP1- or AaFUS3-disrupted mutant (Fig. 5-6).

Cross-Talk between Signaling Pathways

Western blot analyses revealed that phosphorylation of AaFUS3 was reduced considerably in the AaHSK1 null mutant and was slightly increased in the AaAP1,
AaSLT2 or AaHOG1 null mutant (Fig. 5-7A). Phosphorylation of AaHOG1 was repressed in the AaHSK1 and AaSLT2 null mutants. The AaFUS3 null mutant had an apparent increased in AaHOG1 phosphorylation (Fig. 5-7B).

Discussion

In S. cerevisiae, each of the MAP kinase cascades responds to different environmental signals. Mounting evidence indicates that intricate interactions between these MAP kinase pathways occur in S. cerevisiae. For example, treatment of pheromone not only activates the FUS3-mediated signaling pathway, but also increases the cell wall integrity pathway and the tyrosine phosphorylation mediated by SLT2 MAP kinase (Zarzov et al. 1996). On the other hand, the Ste11 (MAPKKK) is involved in the regulation of three (FUS3, KSS1 and HOG1) MAP kinase pathways (Fig 1-3). Thus, different MAP kinase pathways may interact with each other to regulate cellular responses to different environmental stimuli including perhaps infection cycles in fungal pathogens as well (Xu 2000). In this study, three types of MAP kinase homologs, AaFUS3, AaHOG1 and AaSLT2, were independently disrupted. Deletion of the AaFUS3 and the AaHOG1 gene created mutants with distinct phenotypes in terms of the susceptibility to osmotic stresses. This observation indicated that FUS3- and HOG1-type MAP kinase signaling pathways function antagonistically to regulate osmotic adaption imposed by salts. The antagonistic interactions between AaFUS3 and AaHOG1 occurred at both transcriptional and post-translational levels, as judged by Northern blot and Western blot analyses. Inactivation of the AaHOG1 gene resulted in an increased accumulation of the AaFUS3 transcripts and phosphorylation of AaFUS3. Similarly, disruption of the AaFUS3 gene promoted expression of the AaHOG1 gene and phosphorylation of AaHOG1. However, the mechanism of how AaFUS3 and
AaHOG1 negatively regulate each other in *A. alternata* remains unclear. In *S. cerevisiae*, the HOG1 MAP kinase activates expression of a phosphatase-coding gene (*Msg5*) whose product specifically dephosphorylates FUS3 and KSS1 MAP kinases proteins and thus, inhibits their functions (Bardwell et al. 1996; Andersson et al. 2004).

Fungal strains defective in either *AaSLT2* or *AaFUS3* displayed growth retardation compared to wild type. However, addition of NaCl or KCl restored radial growth to the *AaSLT2* and *AaFUS3* null mutants. Thus, *AaSLT2* and *AaFUS3* negatively regulated salt tolerance. At the transcriptional level, *AaSLT2* and *AaFUS3* positively regulate each other. *AaSLT2* functions as a negative regulator for expression of the *AaHOG1* gene and vise versa. However, disruption of the *AaSLT2* gene promoted phosphorylation of *AaFUS3* but decreased phosphorylation of *AaHOG1*. In *C. heterostrophus*, phosphorylation of a SLT2-like MAP kinase was increased if the *HOG1* gene was inactivated (Igbaria et al. 2008). Although the *AaHSK1*-mediated signaling pathway had little connection with *AaHOG1* (Chapter 4), accumulation of the *AaHSK1* gene transcripts apparently was increased in the mutant strains disrupted in *AaAP1*, *AaFUS3*, *AaSLT2* or *AaHOG1*. Inactivation of *AaHSK1* reduced phosphorylation of both *AaHOG1* and *AaFUS3*. These results indicated additive or antagonistic interactions at both transcriptional and translational regulatory levels in *A. alternata*.

Both *AaHOG1* - and *AaAP1*-disrupted mutants were highly sensitive to oxidants. However, expression of the *AaAP1* gene was slightly up-regulated by the *AaHOG1* null mutant, but was positively regulated by *AaSLT2*. In *Schizosaccharomyces pombe*, the Sty1 kinase, a HOG1 MAP kinase homolog, directly regulates a bZIP transcription factor, Atf1, rather than the *AP1*-like gene during oxidative stress response (Toone and Jones
The mechanism by which proteins modulate AP1-like gene is still unknown. Interestingly, recent studies in this lab revealed that deletion of a NADPH oxidase-coding gene (NOXA) resulted in fungi hypersensitive to oxidants and impaired expression of the AaAP1 gene (Siwy Ling Yang, personal communication). NOXA may function in the production of hydrogen peroxide. It becomes apparent that intracellular hydrogen peroxide is important for AaAP1 expression and cellular responses to oxidative stress require both AaAP1 and NOXA.

The SLT2-like MAP kinases have been characterized to be involved in cell wall integrity and conidial production in yeasts and filamentous fungi (Hou et al. 2002; Igbaria et al. 2008; Mey et al. 2002; Xu et al. 1998; Zhang and Gurr 2001; Zarzov et al. 1996). The A. alternata AaSLT2 was also required for maintenance of cell wall integrity and conidiation. In contrast, AaHOG1 and AaHSK1 had a negative role in cell wall integrity since deletion of either AaHOG1 or AaHSK1 gene generated fungi that were highly resistant to CWDEs.

Both AaFUS3 and AaSLT2 were shown to be required for conidial production. In filamentous fungi, conidiation is often controlled by the membrane-bound heterotrimeric G proteins containing three subunits Gα, Gβ and Gγ (Li et al. 2007; Liu and Dean 1997; Wendland 2001). The Gα subunit is activated once it is released from Gβγ subunits. The active Gα in turn regulates downstream effectors, such as adenylate cyclase, phospholipase, and MAPK for numerous biological functions (Neves et al. 2002). The A. alternata AaGα1, encoding a fungal Class I Gα subunit of GTP-binding protein, was recently cloned and disrupted. The AaGα1 disruption mutant produced fewer conidia.
compared to the wild type (Wang et al. 2010), suggesting a possible link between
AaGα1, AaFUS3 and AaSLT2 in the context of conidiation.

Both AaFUS3 and AaHOG1 were demonstrated to be required for pathogenicity. The A. alternata AaSLT2 gene was also required for full virulence. In C. heterostrophus, all three MAP kinases were essential for virulence to its host (Igbaria et al. 2008). However, in M. grisea, only FUS3 and SLT2 homologs were necessary for fungal pathogenicity (Xu 2000). The HOG1 homolog (OSM1) of M. grisea had no role in the pathogenicity (Xu 2000). Thus, the biological processed downstream of each MAP kinase could be highly divergent among species (Bardwell 2006).

One of the most important findings of this study is the discovery of the common phenotypes that fungal strains disrupted in AaAP1, AaFUS3, AaSLT2, AaHSK1 or AaHOG1 gene were hypersensitive to TIBA and CHP. It is tempting to propose that the phenotypes were likely mediated via regulation of common membrane transporters. As demonstrated in the present study, an MFS transporter coding gene (clone #19) was synergistically regulated by AaAP1, AaFUS3, AaSLT2, AaHSK1 and AaHOG1. Expression of another MFS membrane transporter-coding gene (clone #54) was also regulated by AaAP1, AaFUS3, AaSLT2 and AaHSK1. Overall, my studies have established functional links and possible interactions involving different signaling pathways by phenotypic comparisons and molecular analyses at both transcriptional and translational levels (Fig. 5-8 and Fig. C-2). Thus, a regulatory interaction exists between AP1-, HK- and MAPK-mediated signaling pathways in A. alternata. In conclusion, the pioneering studies in S. cerevisiae and M. grisea may provide useful guidelines, but may not be directly applicable to understating the functions and
regulatory mechanisms of these signalling pathways in all fungal pathogens. Understanding each of the components in MAP kinase-mediated pathways and potent interactions with different signaling pathways in *A. alternata* will provide valuable information regarding molecular mechanisms underlying the infection processes as well as the evolution of fungal pathogenicity.
Table 5-1. Phenotypic characterization of wild type (WT) and mutant strains of *Alternaria alternata* grown on potato dextrose agar amended with oxidants, sugars, salts, fungicides, or chemicals.

<table>
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<tr>
<th>Phenotype</th>
<th>WT</th>
<th>ΔAaAP1</th>
<th>ΔAaFUS3</th>
<th>ΔAaHOG1</th>
<th>ΔAaSLT2</th>
<th>ΔAaHSK1</th>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>0.1% H₂O₂</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>100 mM Cyclo-</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
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<tr>
<td><strong>Osmotic stress</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 M Glucose</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>1 M Sucrose</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>1 M Sorbitol</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>1 M Mannitol</td>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
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<td><strong>Salt stress</strong></td>
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</tr>
<tr>
<td>1 M KCl</td>
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<td>R</td>
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<td>S</td>
<td>Rr</td>
<td>R</td>
</tr>
<tr>
<td>1 M NaCl</td>
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<td>R</td>
<td>Rr</td>
<td>S</td>
<td>Rr</td>
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<tr>
<td><strong>Fungicide</strong></td>
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<tr>
<td>0.1 μg/ml Fludioxonil</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>r</td>
<td>S</td>
<td>R</td>
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<tr>
<td><strong>Chemical Stress</strong></td>
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<tr>
<td>10 mM TIBA</td>
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<td>S</td>
<td>S</td>
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<tr>
<td>5 mM CHP</td>
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R: resistant; S: susceptible; Rr; highly resistant; r: minor resistant
Figure 5-1. Conserved domains of AaSLT2 and targeted disruption of the AaSLT2 gene. (A) Physical map of AaSLT2 (416 amino acids) showing a serine/threonine protein kinase domain. (B) Schematic illustration of a split-marker strategy for disruption of AaSLT2 by inserting a hygromycin phosphotransferase gene (HYG) under control of the Aspergillus nidulans trpC promoter. (C) Southern blot hybridization of SalI/StuI-digested genomic DNA of the wild type and five putative AaSLT2 disruptants (D1 to D5) to a specific probe as indicated in B. (D) Northern blot analysis identified a 1.6-kb hybridized band from the wild type, but not three putative mutants D1, D2 and D3.
Figure 5-2. *AaSLT2* is required for full virulence of *Alternaria alternata* as assayed on citrus cv. Minneola uniformly sprayed with conidial suspension. Lesions were recorded at (A) 2 dpi and (B) 4 dpi.
Figure 5-3. Quantitative analysis of conidia produced by the wild type (WT) and mutant strains of *Alternaria alternata* grown on PDA. Each column represents the mean number of conidia ± the standard error from two independent experiments, with at least three replicated.
Figure 5-4. Protoplasts released from the *Alternaria alternata* strains. Production of protoplasts was determined over time after the fungal strains were exposed to cell-wall-degrading enzymes containing lyticase, driselase, β-glucanase and glucuronidase cocktail (A) or β-glucanase alone (B). Release of protoplasts was determined with a hemocytometer by microscopy. Each point represents the mean number of protoplasts released ± standard error from two independent experiments with at least three replicates.
Figure 5-5. Expression of two MFS coding genes in *A. alternata*. Total RNA prepared from the wild type (WT) and mutant strains was hybridized to digoxigenin-labeled probes (#19 or #54). A gel stained with ethidium bromide is shown to indicate the relative amounts of the RNA samples.
Figure 5-6. Transcriptional regulation in *Alternaria alternata*. (A) Northern blot hybridization of total RNA purified from the wild type (WT) and mutant strains to digoxigenin-labeled probes as indicated. Ribosomal RNA stained with ethidium bromide indicates relative loading of the samples. (B) The relative intensities of hybridizing bands after normalizing from those of *actin* gene transcript using TotalLAB TL100 software.
Figure 5-7. Phosphorylation of AaFUS3 or AaHOG1 protein in *Alternaria alternata*. Western blots of total proteins of the wild type (WT) and mutant strains were probed with anti-dually phosphorylated P44/42 and anti-FUS3 antibodies (A) or anti-phosphorylated P38 and anti-HOG1 antibodies (B).
Figure 5-8. Summary of signal transduction modulated by the redox-responsive transcription regulator (AaAP1), the mitogen-activated protein (MAP) kinases (AaFUS3, AaSLT2, and AaHOG1), and the two-component histidine kinase (AaHSK1)-mediated pathways, in a specific and synergistic manner in Alternaria alternata. An arrow indicates positive regulation, whereas a T-bar indicates negative regulation based on the Western blot analyses (Fig. 5-7).
## Table A-1. Sequence of primers.

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<td>AaAP1</td>
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<td>AP-1R</td>
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<td>5'-gaattctacgcgcagcagat-3'</td>
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<td>5'-gctccgtaatgctgagctgagctg-3'</td>
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<td>5'-cgccggagatcgaggggaaggcaag-3'</td>
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Table A-1. Continued.
Figure B-1. The ΔAaFUS3 null mutants of *Alternaria alternata* are resistant to high osmolarity of KCl and NaCl. Sensitivity of *A. alternata* wild type (WT), AaFUS3 deletion strains M1 and M2, and two complementation strains Cp1 and Cp2 was determined by radial growth on potato dextrose agar (PDA). Only one representative replicate is shown.
### Table C-1. Statistical analysis of disease incidence caused by the wild type and ΔAaSLT2 on citrus leaves.

<table>
<thead>
<tr>
<th>Disease incidence</th>
<th>$t$-test: paired two samples for means</th>
<th>Wild type</th>
<th>ΔAaSLT2</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>44 10</td>
<td>Mean 40</td>
<td>12.3</td>
</tr>
<tr>
<td>ΔAaSLT2</td>
<td>27 10</td>
<td>Observations 10 10</td>
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<tr>
<td></td>
<td>103 5</td>
<td>Hypothesized Mean Difference 0</td>
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<tr>
<td></td>
<td>75 10</td>
<td>Degree of freedom 9</td>
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<tr>
<td></td>
<td>17 10</td>
<td>$t$ Statistic 2.83763221</td>
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<tr>
<td></td>
<td>12 8</td>
<td>$P(T \leq t)$ one-tail 0.00973913</td>
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<td></td>
<td>28 18</td>
<td>$t$ Critical one-tail 1.83311292</td>
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<tr>
<td></td>
<td>25 10</td>
<td>$P(T \leq t)$ two-tail 0.01947827</td>
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<td>$t$ Critical two-tail 2.26215715</td>
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<td>25 22</td>
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### SUMMARY

<table>
<thead>
<tr>
<th>Groups</th>
<th>Average</th>
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<tr>
<td>Wild type</td>
<td>40 $a$</td>
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<tr>
<td>ΔAaSLT2</td>
<td>12.3 $b$</td>
</tr>
</tbody>
</table>

$a$ and $b$ show statistically different groups ($t$-test)
Figure C-1. Sensitivity tests of the wild type (WT), the AaAP1-\textsuperscript{-}, the AaHSK1-\textsuperscript{-}, the AaFUS3-\textsuperscript{-}, the AaSLT2-\textsuperscript{-} and the AaHOG1-disrupted mutant strains. Radial growth of fungal strains was measured 4-7 days after incubation on potato dextrose agar (PDA) in different stresses or PDA containing oxidants, sugars, salts, fungicides, or chemicals. Only one representative photo is shown.
Figure C-2. Schematic illustration of transcriptional regulations between the AaAP1, the AaHSK1, the AaFUS3, the AaSLT2 and the AaHOG1 genes in Alternaria alternata. The transcriptional feedback regulation was determined based on the results described in Fig. 5-6B. Further details are discussed in the text. Positive regulation is indicated by an arrow. Negative regulation is indicated by a T-bar.
LIST OF REFERENCES


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Kuge, S., and Jones, N. 1994. YAP1 dependent activation of TRX2 is essential for the response of Saccharomyces cerevisiae to oxidative stress by hydroperoxides. EMBO J. 13:655-664.


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BIOGRAPHICAL SKETCH

Ching-Hsuan Lin was born in 1975 in Taiwan. He received a Bachelor of Science of Medical Technology in Chung Shan Medical University in 1997. After he finished his compulsory military service, he became a medical technologist in the Biochemistry Laboratory Department in Jiann Ren Hospital in 1999. He later joined in the Institute of Molecular Biology in the National Chung-Hsing University and received his Master of Science degree in May of 2004 and continued working in the same laboratory from 2004 to 2006. During that period, he was involved in several research projects, but mainly focused on the function and regulatory mechanism of a small heat shock protein in *Xanthomonas campestris pv. campestris*. Ching-Hsuan was awarded a Grinter Fellowship from the University of Florida and a Hunt Brothers Research Scholarship from the Citrus Research and Education Center, Department of Plant Pathology, University of Florida before he joined Dr. Kuang-Ren Chung’s Lab. Ching-Hsuan’s doctoral studies focus on determining the importance of redox-responsive AaAP1 transcriptional factor involved in detoxification of reactive oxygen species of citrus and the interaction and regulation between the AaAP1, mitogen-activated protein kinases and the histidine kinase in *Alternaria alternata*, a necrotrophic fungal pathogen of citrus. He was awarded a Doctor of Philosophy degree in May 2010.