

Review Article

Stress Response and Pathogenicity of the Necrotrophic Fungal Pathogen *Alternaria alternata*

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The production of host-selective toxins by the necrotrophic fungus *Alternaria alternata* is essential for the pathogenesis. *A. alternata* infection in citrus leaves induces rapid lipid peroxidation, accumulation of hydrogen peroxide (H₂O₂), and cell death. The mechanisms by which *A. alternata* avoids killing by reactive oxygen species (ROS) after invasion have begun to be elucidated. The ability to coordinate of signaling pathways is essential for the detoxification of cellular stresses induced by ROS and for pathogenicity in *A. alternata*. A low level of H₂O₂, produced by the NADPH oxidase (NOX) complex, modulates ROS resistance and triggers conidiation partially via regulating the redox-responsive regulators (YAP1 and SKN7) and the mitogen-activated protein (MAP) kinase (HOG1) mediated pathways, which subsequently regulate the genes required for the biosynthesis of siderophore, an iron-chelating compound. Siderophore-mediated iron acquisition plays a key role in ROS detoxification because of the requirement of iron for the activities of antioxidants (e.g., catalase and SOD). Fungal strains impaired for the ROS-detoxifying system severely reduce the virulence on susceptible citrus cultivars. This paper summarizes the current state of knowledge of signaling pathways associated with cellular responses to multidrugs, oxidative and osmotic stress, and fungicides, as well as the pathogenicity/virulence in the tangerine pathotype of *A. alternata*.

1. Introduction

Alternaria species have different lifestyles, ranging from saprophytes to endophytes to pathogens [1]. *Alternaria* species are a highly successful group of fungal pathogens that cause diseases in a wide variety of economically important crops, including apple, broccoli, cauliflower, carrot, citrus, pear, rice, strawberry, tomato, potato, and tobacco, as well as many ornamental and weed species. Due to their wide host range and worldwide distribution, *Alternaria* species cause severe economic problems. *Alternaria* species have been reported to cause diseases in nearly 400 plant species; *A. alternata* alone can infect more than 100 plant species [2–4]. One reason for the success of these pathogens may be attributed to their production of diverse phytotoxins [5, 6]. The host-selective toxins (HSTs) produced by many members of the genus *Alternaria* have unique modes of action and

toxicity to their respective host plants. Production of HST is critical for successful pathogenesis because HST-deficient mutants are incapable of attacking their host plants [7–11]. In addition to HSTs, many *Alternaria* species produce nonhost selective phytotoxins, such as brefeldin A, altertoxin, and tentoxin [1]. Others can produce mycotoxins that are harmful to humans and other animals [12]. Several *Alternaria* species can also cause upper respiratory tract infections and asthma in humans [13].

Alternaria alternata (Fr.) Keissler has several pathogenic variants, each producing a unique HST and causing disease in different host plants [5, 9, 10, 14, 15]. HSTs produced by *Alternaria* pathotypes are chemically diverse, ranging from low-molecular-weight compounds to cyclic peptides. The genes encoding polypeptides for biosynthesis of *Alternaria* HSTs have been shown to reside on a dispensable chromosome [9]. In citrus, *A. alternata* has two major

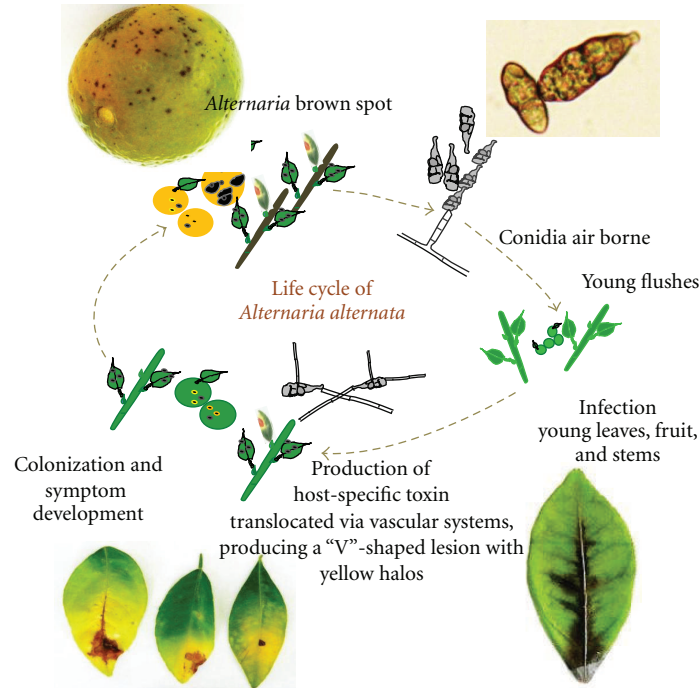


FIGURE 1: Life cycle of *Alternaria alternata*, the causal agent of citrus brown spot. ACT toxin produced by the tangerine pathotype of *A. alternata* is transported via the vascular system and formation of necrotic lesions on a detached calamondin leaf (bottom right).

pathotypes—the tangerine pathotype and the rough lemon type [16]. The citrus pathotypes are morphologically similar and can be differentiated only by pathological and genetic analyses [17]. The rough lemon pathotype, producing the host-selective ACRL toxin, is pathogenic exclusively to lemon (*Citrus jambhiri* Lush) and Rangpur lime (*Citrus x limonia* Osbeck). ACRL toxin affects mitochondrial function, disrupting posttranscriptional RNA splicing and causing metabolite leakage and malfunction of oxidative phosphorylation in susceptible host cells [18, 19]. In contrast, the tangerine pathotype of *A. alternata* produces the host-selective ACT toxin with a core 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid structure [20] and causes brown spots on citrus leaves and fruit. ACT toxin is highly toxic to tangerines (*C. reticulata* Blanco) and grapefruit (*C. paradisi* Macfad.), as well as hybrids from grapefruit and tangerine, or tangerine and sweet orange (*C. sinensis* Osbeck). ACT toxin does not affect rough lemon or Rangpur lime [20]. The toxin is quickly translocated outward through the vascular system, causing rapid electrolyte leakage and necrotic lesions along the veins (Figure 1). *A. alternata* infection in citrus leaves induces rapid lipid peroxidation and accumulation of hydrogen peroxide (H_2O_2) [21]. Studies show that *A. alternata* has evolved a dramatic flexibility and uniqueness in the signaling pathways in order to respond to diverse environmental stimuli and to thrive within host plants. This paper discusses signaling pathways related to oxidative and osmotic stress resistance, fungicide sensitivity, conidia formation, and pathogenesis of *A. alternata*.

2. Roles of Reactive Oxygen Species in Plant-Fungal Interactions

All organisms with an aerobic lifestyle inevitably generate toxic reactive oxygen species (ROS), primarily superoxide (O_2^-), and hydrogen peroxide (H_2O_2) during physiological metabolisms [22–26]. During the course of host colonization, fungal pathogens of plants need to overcome a wide range of potentially harmful environmental challenges, particularly an oxidative burst, which could result in the production and accumulation of highly toxic ROS. In addition to the direct toxicity of ROS to cells, when produced in abundance, ROS can also serve as secondary messengers in the pathogen-response signal transduction pathways [23, 27]. Among ROS, H_2O_2 is relatively stable and able to pass freely through membranes, serving as a signaling cue for defense responses in surrounding cells and as a substrate for oxidative cross-linking in the plant cell wall [27–32]. Hydrogen peroxide can react with O_2^- via the Haber-Weiss reaction or with metal ions via the Fenton pathway [33–35] to generate the extremely toxic hydroxyl radical. It has been well known that plants produce toxic ROS as a defense against pathogens [36–41]. In response to the microbe invasion, plant cells often produce excessive amounts of H_2O_2 by a specific plasma membrane NADPH oxidase, termed as the hypersensitive reaction (HR), which leads to programmed cell death and cellular defense against pathogen attack [42–46]. The HR plays a vital role in plant defenses against saprophytes and noncompatible or biotrophic pathogens; however, HR has been shown less

effective against necrotrophic phytopathogens [47–51]. ROS have been shown to be involved in nonhost resistance in plants as well [52, 53].

The burst of the HR may ironically provide an advantage for necrotrophic phytopathogens, as they acquire nutrients exclusively from dead cells [54]. ROS have been thought to enhance plant colonization by necrotrophic pathogens such as *Botrytis cinerea* and *Sclerotinia sclerotiorum* [50, 51, 55]. Because many necrotrophic pathogens are able to produce a wide array of HSTs or cell-wall-degrading enzymes that kill host cells before colonization, leading to the accumulation of ROS, the pathogens must have evolved effective mechanisms to cope with the toxicity of ROS [47, 49].

3. YAP1-Mediated ROS Detoxification in *A. alternata*

ROS damage a wide range of biological molecules, including fatty acids, proteins/enzymes, sugars, and nucleic acids; thus, exposure to ROS may result in cell death [56–58]. The relative sensitivity of the fungal pathogen to ROS is likely determined by the effectiveness of its own ROS detoxification ability. In order to survive under aerobic conditions, fungi must have detoxification systems that can effectively scavenge ROS, maintain reduced redox states within subcellular microenvironments, and repair ROS-triggered damage [22, 59, 60]. Molecular and genetic studies aimed at understanding the mechanisms by which cells cope with the oxidative stresses and are protected from the deleterious effects of ROS have been intensively studied in both prokaryotes and eukaryotes. In the budding yeast *Saccharomyces cerevisiae*, the YAP1 transcription regulator plays a central role in the cellular pathways associated with the oxidative stress response [61, 62]. YAP1 is responsible for transcriptional activation of genes involved in multidrug resistance as well. YAP1, resembling mammalian AP-1, has a basic leucine zipper (bZIP) domain and has been shown to be activated by H₂O₂ and various ROS-generating oxidants, as well as heavy metals [63–66]. In the absence of oxidative challenges, YAP1 can be found in the cytoplasm at low levels. Upon perceiving oxidative or chemical stimuli, YAP1 quickly forms disulfide bonds, changes conformation, and is translocated into the nucleus where YAP1 regulates the expression of genes responsible for stress alleviation [67–71]. Conserved cysteine residues in both the amino and carboxyl terminal domains are essential for the formation of the disulfide bonds, nuclear relocalization and transcriptional regulation of YAP1 [72].

Although all microorganisms employ complex mechanisms, both enzymatic and nonenzymatic to avoid ROS toxicity [47, 61, 73], the pathological roles of oxidative stress mitigation remain uncertain in pathogenic fungal species. The role of ROS in host resistance and pathogen invasion is likely dictated by the physiological conditions of the host, the lifestyle of the pathogen, and the combination of different stimuli [38, 41, 74–76]. Hence, ROS produced by plants may have different effects against different pathogens [49, 75]. YAP1-mediated detoxification of ROS is an essential virulence determinant in the opportunistic human pathogen

Candida albicans and the biotrophic maize pathogen *Ustilago maydis* [77, 78]. However, YAP1 is not required for virulence in the plant pathogens *Cochliobolus heterostrophus* and *B. cinerea* and in the animal pathogen *Aspergillus fumigatus*, even though the disrupted mutants exhibit increased sensitivity to H₂O₂ [79, 80]. The necrotrophic fungus *Sc. sclerotiorum* produces oxalic acid that suppresses host-generated ROS, and thus facilitates fungal evasion [73]. The *Magnaporthe oryzae* *MoHYR1* gene encoding a glutathione peroxidase (GSHPx) is required for detoxifying plant-generated ROS and full virulence [81]. In *S. cerevisiae*, HYR1 forms a disulfide bond with YAP1, inducing a conformational alteration and nuclear localization of YAP1 upon exposure to ROS [82].

To thrive within host plants, *A. alternata* must be able to detoxify or obviate the ROS-mediated plant defense barriers. Our studies have demonstrated that cellular detoxification of ROS regulated by the redox-responsive YAP1 transcription regulator is important for pathogenesis of *A. alternata* to citrus [83, 84]. Inactivation of the *A. alternata* *API* gene (designated *AaAPI*), encoding a YAP1-like transcription factor, resulted in fungal mutants that are hypersensitive to H₂O₂, menadione, and potassium superoxide (KO₂). The promoter of *AaAPI* contains a putative stress responsive element (STRE: AGAGGGG). Upon activation by H₂O₂, the *AaAPI::sGFP* fusion protein became localized in the nucleus. Fungal mutants lacking *AaAPI* ($\Delta yap1$) are weakly virulent on susceptible citrus cultivars even though they synthesize HST toxins normally. However, $\Delta yap1$ mutant is not sensitive to osmotic and salt stress-related compounds (e.g., sorbitol, mannitol, NaCl, and KCl) (Figure 2). $\Delta yap1$ mutant produces wild-type level of conidia that germinate at a rate and magnitude similar to the wild-type strain. The nonpathogenic phenotype of $\Delta yap1$ mutant was accompanied with reduced activities of fungal antioxidants, including catalase, peroxidase, superoxide dismutase (SOD), and glutathione reductase. The inability of $\Delta yap1$ mutants to incite necrotic lesions is likely due to the mutants' inability to detoxify ROS because coapplication of $\Delta yap1$ mutants with the NADPH oxidase inhibitor, apocynin, or diphenylene iodonium, partially restored lesion-forming capability to the mutants. $\Delta yap1$ mutant is impaired in the penetration and colonization stages because the impaired mutant did not cause any visible necrotic lesions on wounded or unwounded leaves of the citrus cultivar Minneola. All mutant phenotypes were completely restored to the wild type in fungal strains expressing a functional copy of *AaAPI*. $\Delta yap1$ mutant displayed severe defects in antioxidant activities and was unable to detoxify H₂O₂ effectively. Our studies concluded that effective detoxification of ROS via the *AaAPI*-mediated pathway is absolutely required for successful colonization of citrus by *A. alternata* [83, 84]. The tobacco pathotype of *A. alternata* impaired for the biosynthesis of mannitol, an antioxidant and quencher of the hydroxyl radical, also greatly reduces virulence [85, 86], consistent with the importance of ROS detoxification in the pathogenesis of *A. alternata*.

Furthermore, *AaAPI* was found to be required for full resistance to 2,3,5-triiodobenzoic acid (TIBA), 2-chloro-5-hydroxypyridine (CHP), diethyl maleate (DEM), and many pyridine-containing compounds [87]. Diethyl maleate is

a glutathione-depleting agent that has been shown to generate a nonreversible modification of cysteine residues in the *Schizosaccharomyces pombe* Pap1 protein. As a result, Pap1 is constitutively localized in nucleus and activates the genes required for ROS tolerance [88]. TIBA is often used as herbicides or as an inhibitor of indole-3-acetic acid (IAA) transportation [89, 90]. Pyridine is a heteroaromatic compound composed of five carbons and one nitrogen atom. Pyridine could accelerate the production of superoxide and hydroxyl radicals when Cu^{2+} and H_2O_2 are present [91, 92]. Pyridine and its derivatives serve as constituents of RNA and DNA, as electron carriers such as NADP/NADPH and flavin nucleotides (FAD/FADH) and as energy storage molecules such as ATP and GTP.

4. The “Two-Component” Histidine Kinase (HSK) Signaling Pathway

All living cells have a complicated yet well-regulated network often comprising different signaling transduction pathways to perceive changes in their environments and to adjust physiological and developmental processes [93–99]. “Two-component” histidine kinase (HSK) signaling transfer systems are commonly present in bacteria, slime molds, fungi, and plants; however, these systems have not yet been identified in animals [100, 101]. In bacteria, HSK signaling systems contain a histidine kinase (HSK) and a response regulator (RR); each is encoded by a separate gene [102, 103]. In contrast, all fungal HSKs have both the HSK and RR domains [101, 104, 105]. In response to environmental changes, a series of phosphate transfers between histidine (His) and aspartate (Asp) residues occurs in a pattern of His-Asp-His-Asp to regulate downstream signaling pathways such as mitogen-activated protein kinase (MAPK) cascades and eventually leads to a change in gene expression [100, 106, 107].

The *S. cerevisiae* histidine kinase, designated SLN1p, is required for osmotic adaption via the SLN1p-YPD1p (a protein containing a His phosphotransfer domain)-SSK1p or SKN7p cascade [108, 109]. SSK1p is the major regulator for osmolarity response; SKN7p plays only a minor role in osmosensing. Under normal osmolarity, the SLN1p kinase is phosphorylated and able to activate YPD1p and SSK1p with a phosphorelay mechanism (Figure 3). The phosphorylated SSK1p is inactive and incapable of activating the High Osmolarity-Glycerol 1 (HOG1) MAP kinase pathway (see below for details). In contrast, SLN1p is not phosphorylated under conditions of high osmolarity; therefore SSK1p is able to activate the HOG1-signaling cascade. The activated HOG1 pathway is responsible for glycerol accumulation, allowing the yeast to cope with the high osmolarity. *S. cerevisiae* also utilizes a non-HSK-related protein SHO1p to cope with osmotic stress [108, 110]. However, deletion of an *SHO1* homolog in *A. alternata* did not impact cellular tolerance to oxidative and osmotic stress, fungicide sensitivity or fungal virulence (L.-H. Chen, unpublished).

The budding yeast *S. cerevisiae* has only one HSK; all other fungi have multiple HSK signaling genes [111]. Fungal HSKs

are divided into 11 groups based on phylogenetic relationships inferred from the conserved HSK and RR domains. Among them, Group III HSK is one of the best characterized HSKs in the filamentous fungi. Collectively, Group III HSK has been implicated in osmotic and oxidative responses, toxin biosynthesis, hyphal development, conidia formation, and virulence, as well as sensitivity to dicarboximide and phenylpyrrole fungicides in different fungal species [112–121].

Signals sensed by HSK are often transduced down to the HOG1 MAP kinase pathway. Fungi lacking Group III HSK or HOG1 often became resistant to dicarboximide and phenylpyrrole fungicides and exhibited an elevated sensitivity to osmotic stress [117, 122, 123]. Although the HSK-HOG1 signaling pathway is conserved, it may be recruited for divergent functions in different fungal species. As discussed above, the *S. cerevisiae* SLN1p negatively regulates HOG1 phosphorylation under conditions of high osmolarity [100, 106, 107]. The filamentous fungus *Co. heterostrophus* Group III HSK (Dic-1) positively regulates phosphorylation of the HOG1 MAP kinase, which subsequently activates expression of genes responsible for osmotic resistance and fungicide sensitivity [117]. In *B. cinerea*, the HOG1-like MAP kinase is not required for fungicide sensitivity even though it is negatively regulated by the “two-component” HSK. Furthermore, the salt-tolerant yeast species, *Hortaea werneckii*, copes with osmotic stress using a Group VII HSK-HOG1 pathway [124]. Those studies indicate that the HSK-HOG1 signaling pathways can be operated in very different regulatory mechanisms in various species.

The *AaHSK1* gene, encoding a putative histidine kinase, was cloned from the tangerine pathotype of *A. alternata* [87]. *AaHSK1*, containing no transmembrane regions, is required for adaption to osmotic stress induced by sugars but not by salts (Figure 2). $\Delta hsk1$ displayed increased sensitivity to glucose, sucrose, sorbitol, or mannitol, but not to H_2O_2 , KCl, or NaCl [125]. Similarly, the *M. grisea* HSK is required for resistance to sugar, but not salt, osmotic stress [116]. The *F. oxysporum* histidine kinase Fhk1 is responsible for resistance to osmotic stress, menadione, but not H_2O_2 [126]. Similar to $\Delta yap1$ mutant, *AaHSK1* disruption mutants displayed an elevated sensitivity to TIBA and CHP, suggesting a possible link between YAP1 and HSK. $\Delta hsk1$ mutants displayed an elevated resistance to dicarboximide (iprodione and vinclozolin) and phenylpyrrole (fludioxonil) fungicides, suggesting that *AaHSK1* is one of the primary targets of these fungicides. Similarly, resistance to dicarboximide and phenylpyrrole fungicides has been demonstrated to be associated with a mutation within the gene encoding a Group III HSK and/or an HOG1 MAP kinase in a number of filamentous fungi [113–118, 121, 122, 126, 127]. However, HSK is not involved in dicarboximide susceptibility in *A. longipes*.

The *A. alternata* HSK1 is not required for response to oxidative stress. The *AaHSK1* gene product is not involved in pathogenicity or virulence because the *AaHSK1*-impaired mutants ($\Delta hsk1$) induced necrotic lesions at rates and magnitudes similar to the wild-type strain or the genetically reverted strain on wounded or unwounded leaves of citrus. In contrast, Group III HSK is a virulence determinant in

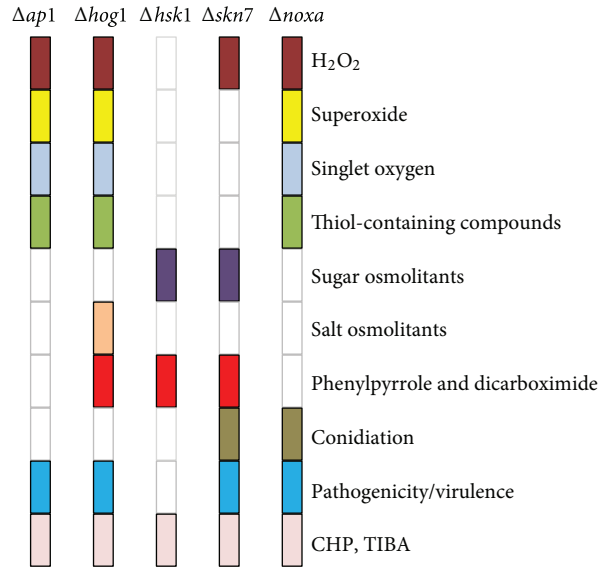


FIGURE 2: Phenotypic changes in *A. alternata* mutants lacking the transcription regulator (*YAP1*), the MAP kinase (*HOG1*), the “two-component” histidine kinase (*HSK1*), the response regulator (*SKN7*), or the NADPH oxidase (*NOXA*). Open rectangles denote wild-type phenotypes.

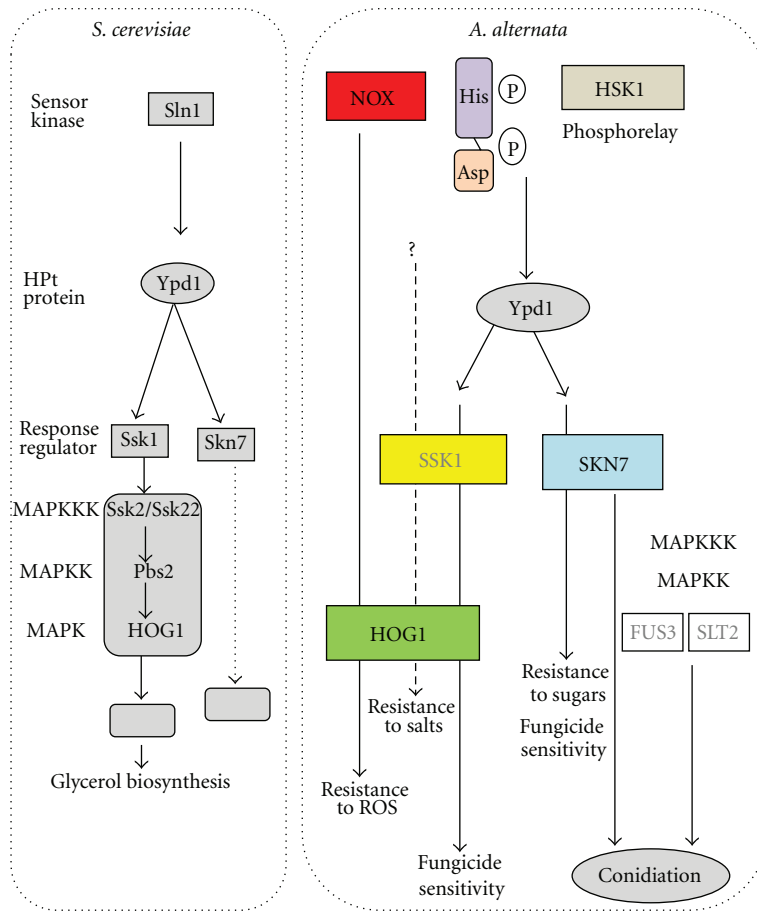


FIGURE 3: Schematic illustration and comparison of signaling pathways leading to osmotic stress resistance in the budding yeast, *S. cerevisiae*, and the pathways leading to ROS resistance, osmotic stress response, fungicide sensitivity, and conidia formation in the tangerine pathotype of *A. alternata*.

the phytopathogenic fungi *B. cinerea*, *Claviceps purpurea*, and *Fusarium oxysporum* and in the human pathogen *Cryptococcus neoformans* [118, 126, 128, 129].

5. The HOG1 Mitogen-Activated Protein Kinase (MAPK-) Mediated Signaling Pathway

The HOG1 MAPK-mediated signaling cascades in eukaryotic cells are vital for sensing environmental stimuli and for transmitting these signals to the nucleus to modulate gene expression [130, 131]. MAPK-mediated cascade pathways are composed of three serine/threonine protein kinases—MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and MAP kinase (MAPK). This signal transduction pathway, in conjunction with HSK, is well conserved in all eukaryotes and functions in perceiving environmental stimuli via phosphorylation and gene activation [132, 133]. The phosphorylated MAPK activates a set of genes via regulating appropriate transcription factors.

The *A. alternata* ortholog (AaHOG1) contains a distinct phosphorylation motif (TGY) involved in the osmotic stress response [134]. Inactivation of the HOG1 ortholog by targeted gene disruption in the tangerine pathotype of *A. alternata* resulted in mutants that are highly sensitive to the oxidants *tert*-butyl-hydroxyperoxide, H₂O₂, and menadione, salts (Figure 2), as well as TIBA and CHP [87]. Because fungal strain lacking the FUS3 MAP kinase ($\Delta fus3$) grew faster than wild type in the presence of KCl or NaCl [125]. Thus, AaHOG1 and FUS3 play an opposite role in KCl and NaCl tolerance. HOG1 has been shown to suppress the FUS3/KSS1 signaling cascade during hyperosmotic stress in *S. cerevisiae* [135, 136]. *A. alternata* strains impaired at *AaHOG1* ($\Delta hog1$) displayed wild-type levels of sensitivity to high concentrations of glucose, sucrose, sorbitol, or mannitol (Figure 2) even though sugar osmoticants increased AaHOG1 phosphorylation and subsequently nuclear localization in the $\Delta hsk1$ mutant background. In the wild-type background, sugar osmoticant had less effect on AaHOG1 phosphorylation and did not facilitate nuclear localization of AaHOG1.

The wild-type isolate of *A. alternata* is extremely sensitive to dicarboximide and phenylpyrrole fungicides, whereas fungal strain lacking *AaHSK1* is highly resistant to them. Compared to the resistance seen in mutants defective at *AaHSK1*, $\Delta hog1$ mutant displayed only slightly increased resistance to these fungicides. In the wild-type strain of *A. alternata*, the AaHOG1 protein was phosphorylated at low levels under normal conditions. Exposure to iprodione or fludioxonil fungicide, NaCl, or H₂O₂ elevated AaHOG1 phosphorylation to varying degrees. Although impairment of *AaHSK1* reduces AaHOG1 phosphorylation, *A. alternata* apparently recruits AaHSK1 and AaHOG1 to exert a unique function in resistance to sugar osmoticants and salt stress, respectively (Figure 3).

Under unchallenged conditions, expression of AaHOG1::sGFP fusion protein under control of the endogenous *AaHOG1* promoter in the wild-type strain resulted in green fluorescence uniformly diffused along the hyphal cytoplasm. However, the green fluorescence became dense

patches after exposure to H₂O₂, iprodione and fludioxonil fungicides, or NaCl. Thus, nuclear localization is important for proper functions of HOG1. Compared to $\Delta hsk1$ or $\Delta yap1$ mutant, $\Delta hog1$ mutant was highly resistant to cell wall-degrading enzymes (lyticase, driselase, β -D-glucanase, and β -glucuronidase), thereby failing to generate any protoplasts. As judged from distinct phenotypes in fungal mutants impaired in the *AaHSK1* or *AaHOG1* gene in *A. alternata*, it appears that AaHSK1 functions in osmotic tolerance and fungicide sensitivity via AaHOG1 and other gene (e.g., SKN7) activation branches.

AaHSK1 plays no role in pathogenesis of *A. alternata*. On the other hand, pathogenicity assays revealed that the *AaHOG1*-impaired mutants are nonpathogenic, producing no necrotic lesions on *Minneola* leaves that were unwounded or prewounded before inoculation. Similar to *AaAPI* disruption, the *AaHOG1*-impaired mutant is defective at the penetration and colonization steps. Inactivation of the *AaHOG1* gene did not impact the production of host-selective ACT toxin by *A. alternata*. HOG1 is required for virulence/pathogenicity in various fungal pathogens. These include *Co. heterostrophus*, *Cryphonectria parasitica*, *B. cinerea*, *Mycosphaerella graminicola*, *Ca. albicans*, and *C. neoformans* [137–142]. However, HOG1 is not a virulence determinant in *M. grisea*, *Colletotrichum lagenarium*, *Bipolaris oryzae* and *As. fumigatus* [122, 143–145]. Again, a conserved protein may have very different functions in fungi.

6. SKN7-Mediated ROS Detoxification

“Two-component” HSK-mediated signal transduction is vital for sensing and adapting to environmental changes in microorganisms. In *S. cerevisiae*, SLN1 histidine kinase transmits signals via a phosphotransfer process down to two response regulators, SSK1p and SKN7p, in response to osmotic stress. However, SKN7p is not regulated by the SLN1p-mediated phosphorylation in the oxidative stress response [146], indicating that there are two different activation mechanisms in response to osmotic and oxidative stress. Under oxidative stress, SKN7p is phosphorylated at serine or threonine residue, forming a heterodimer with YAP1; together they transcriptionally activate the genes involved in the oxidative stress response [146–152]. YAP1 regulates cadmium resistance independent of SKN7p [153]. SKN7p can also form a heterodimer with the heat-shock transcription factor, the cell cycle transcription regulator, the calcium responsive activator, or the Rho1 GTPase [154–157].

In filamentous fungi, SKN7 is required for oxidative stress adaptation, hypoosmotic stress response, cell cycle, sexual mating, sporulation, cell wall biosynthesis, and fungicide sensitivity [120, 146, 155, 158–163]. The *A. alternata* SKN7 homolog (*AaSKN7*) was cloned and characterized in the tangerine pathotype [164]. The promoter of *AaSKN7* contains a putative stress responsive element (STRE: AGAGGGG) that is often present in genes induced by various stresses such as oxidative damage in yeasts. AaSKN7 has a heat-shock transcription factor- (HSF-) type helix-turn-helix DNA-binding domain signature and a response regulatory (RR) domain.

Genetic mutation analysis revealed that *AaSKN7* is required for resistance to osmotic and oxidative stress and fungicide sensitivity, as well as conidiation and conidia morphology. *AaSKN7* is primarily localized in the nucleus, whereas *YAP1* and *HOG1* are quickly transported into the nucleus upon sensing oxidative stress. *AaSKN7* may interact directly with *AaAP1* in nucleus in response to oxidative stress as demonstrated in the budding yeast [151]. Both *A. alternata* *AP1* and *HOG1* are required for resistance to different types of ROS including hydrogen peroxide, superoxide, and singlet oxygen. On the other hand, *AaSKN7* is required for resistance to H_2O_2 , *tert*-butyl hydroperoxide, and cumyl peroxide, but not to superoxide-generating compounds—diamide, menadione and potassium superoxide (Figure 2). It appears that *AaSKN7* and *AaHOG1* contribute independently to oxidative stress in *A. alternata*. Because *AaHSK1* is not required for ROS resistance [87]; activation of *AaSKN7* and *AaHOG1* in response to ROS is likely mediated by unknown regulatory sensors other than *AaHSK1*. Furthermore, *A. alternata* *HSK1* apparently can recruit *SKN7* and *HOG1* to deal with sugar and salt osmoticants, respectively. Although *AaHSK1* and *AaSKN7* play no roles in resistance to salt-induced stress, *AaSKN7* is involved in resistance to sugar osmoticants likely via the *AaHSK1*—mediated signaling pathway (Figure 3). $\Delta skn7/\Delta hog1$ double mutants were hypersensitive to both salts and sugars, indicating that *A. alternata* is capable of sensing different environmental stimuli using distinct or shared signaling pathways. Furthermore, *AaSKN7*, independent of *AaHSK1*, is involved in conidia formation. Our studies also revealed that formation of conidia by *A. alternata* is closely regulated by the *FUS3* and *SLT2* MAP kinases-mediated signaling pathways, as well as by the G-protein and the NOX complex [125, 165–167]. However, recent studies revealed that cAMP-dependent protein kinase A (PKA) suppresses conidia formation by the tangerine pathotype of *Alternaria alternata* [168]. It remains uncertain if these signaling pathways actually interact during conidia formation.

A. alternata strains deleted for *HSK1* or *HOG1* showed an elevated resistance to dicarboximide and phenylpyrrole fungicides. $\Delta skn7$ mutant displayed an elevated resistance to those fungicides at levels between $\Delta hsk1$ and $\Delta hog1$ mutant strains, indicating that the involvement of *SKN7* in fungicide sensitivity is likely mediated by the *HSK1* signaling pathway. Fungal strain carrying *skn7/hog1* double mutations exhibited fungicide resistance, similar to the strain carrying a single *AaHSK1* gene mutation. The results indicated that the signals associated with fungicide sensitivity are transduced from *AaHSK1* simultaneously down to both *AaSKN7*- and *AaHOG*-mediated pathways. The *HSK*-*HOG* signaling pathways are associated with fungicide susceptibility in *A. brassicicola* and *N. crassa* [121]; yet *SKN7* is not involved in fungicide sensitivity in *N. crassa*. *HSK* governs *Ssk1p* (an upstream regulator of *HOG1*) and *Skp7p* for osmolarity adaption and fungicide sensitivity in the phytopathogenic fungus *Co. heterostrophus* and the human pathogen *C. neoformans* [128, 169, 170].

The roles of *SKN7* in pathogenicity/virulence vary among fungal pathogens. *A. alternata* *SKN7* is required for fungal colonization and lesion development in susceptible cultivars

of citrus. Similar to $\Delta yap1$ and $\Delta hog1$, mutational inactivation of *AaSKN7* in *A. alternata* resulted in reduced activities of catalase, SOD, and peroxidase, confirming further that the ability to detoxify host-generating H_2O_2 by *A. alternata* is crucial for successful pathogenesis in citrus. The *SKN7* homologs are required for virulence in the human pathogens *C. neoformans*, *Ca. albicans*, and *Ca. glabrata* [150, 171, 172]. Again, in contrast, *SKN7* is not a virulence determinant in the plant pathogens *Co. heterostrophus* and *M. oryzae* and in the human pathogen *As. fumigatus* [116, 162, 173].

7. The NADPH Oxidase (NOX): Mediated Signaling Pathway

The NADPH-dependent oxidase transfers electrons from NADPH to the oxygen molecule, leading to the production of a superoxide that is further metabolized to H_2O_2 by SOD [174, 175]. NOX complex is commonly found in animals, plants, and many multicellular microorganisms, but completely absent in prokaryotes [176]. Functionally, NOX complex plays a crucial role in cellular differentiation and defence response. In humans, the phagocytic NOX complex, involved in the production of superoxide and immunity, contains two major catalytic components $gp22^{phox}$ and $gp91^{phox}$ and multiple regulatory subunits Rac (a small GTPase), $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ [177]. NOX complex is also required for the regulation of hormone responses, cell proliferation, and apoptosis in animals ([178–180]. Activation of $gp91^{phox}$ is primarily regulated by $p67^{phox}$ and Rac2 [181]. Plants also have oxidases analogous to $gp91^{phox}$, designated respiratory burst homologs (Rboh), which are required for physiological metabolisms and for ROS generation in response to pathogen invasion [175, 182, 183].

Many fungi have NADPH oxidase orthologs, NOXA, NOXB and NOXC that have been documented by genetic analysis to be required for developmental, physiological and pathological functions [25, 76, 184, 185]. Both NOXA, and NOXB are analogous with mammalian $gp91^{phox}$. Expression of the NOXA/NOXB coding genes is closely regulated by the regulatory subunit, NOXR ($p67^{phox}$ homolog), and the small GTPase (Rac homolog) [185]. Fungal NOXC contains a calcium-binding EF-hand motif and is analogous to the mammalian NOX5 and the plant Rboh enzymes.

The functions of NOX complex in the regulation of multicellular development and pathogenicity vary markedly among fungal species that possess it [186]. Both NOXA and NOXB are involved in the regulation of sclerotia formation in both *B. cinerea* and *Sc. sclerotiorum* [141, 187]. In *B. cinerea*, only NOXB is required for the formation of the penetration structure, even though both NOX isoforms have a role in pathogenicity. In *M. grisea*, NOXA, and NOXB play a role in pathogenesis because both isoforms are required for the formation of penetration peg under the appressorium [188]. While NOXA is required for the development of sexual fruiting body in fungi, only NOXB is required for ascospore germination in *N. crassa* and *Podospora anserina* [189–191]. NOXA, but not NOXB, is required for establishing

the mutualistic association between the fungal endophyte *Epichloë festucae* and perennial ryegrass [76]. When inoculated into its grass host, *E. festucae* strain lacking NOXA or NOXR becomes pathogenic, showing increased branching and causing severe stunting and premature senescence of the host [192, 193]. NOXA is coordinately regulated by the small GTPase Rac and NOXR as evidenced by yeast two-hybrid and pull-down analyses [194]. Furthermore, the yeast polarity protein orthologs, Bem1 and Cdc24, have recently been proven to be parts of fungal NADPH oxidase complex [195].

The tangerine pathotype of *A. alternata* has NOXA, NOXB, and NOXR homologs. *A. alternata* NOXA (AaNOXA) contains a NADPH-binding domain and six transmembrane domains and a ferredoxin synthase-type FAD-binding domain, commonly found in the NOXA-like family. Genetic analysis revealed that AaNOXA is responsible for producing superoxide and H₂O₂. $\Delta noxA$ mutants accumulated less ROS within hyphae than the wild type, as judged by nitroblue tetrazolium (NBT), 3,3'-diaminobenzidine (DAB), and dichlorodihydrofluorescein diacetate (H₂DCFDA) staining for the presence of superoxide and H₂O₂. Moreover, deletion of AaNOXA in *A. alternata* resulted in an elevated sensitivity to H₂O₂, superoxide-generating compounds (menadione and KO₂), diamide, SDS, CHP, TIBA, and potent singlet oxygen-generating compounds (hematoporphyrin and rose Bengal) (Figure 2). These deficiencies are similar to the phenotypes previously seen for $\Delta yap1$ or $\Delta hog1$ mutant. Expression of the AaAPI and AaHOG1 genes is likely regulated by AaNOXA, as deletion of AaNOXA decreased the accumulation of the AaAPI and AaHOG1 gene transcripts. Reintroducing and expressing a wild-type AaNOXA in a $\Delta noxA$ mutant restored ROS resistance and expression of both AaAPI and AaHOG1 genes. $\Delta noxA$ mutants also displayed increased sensitivity to NADPH oxidase inhibitors [diphenylene iodonium (DPI) and apocynin], NO[•]-generating compounds [sodium nitroprusside (SNP) and hydroxyl amine HCl (HAD)], NO[•] synthase substrate (L-arginine) and NO[•] synthase inhibitor [nitroarginine methyl ester (nitro-arg)]. Similar to $\Delta yap1$ and $\Delta hog1$, $\Delta noxA$ mutants, producing normal ACT toxin, induced significantly smaller and fewer necrotic lesions than the wild type on detached Minneola or calamondin leaves 3 days postinoculation, indicating that NOXA is an important virulence determinant in *A. alternata*.

NOXA, NOXB, and NOXR are core components of the NOX complex, responsible for the production of H₂O₂. All three NOX components are required for vegetative growth, conidiation, resistance to oxidative and nitrosative stress, and full virulence. However, each isoform may independently and cooperatively interact with other yet unidentified components under different environmental conditions and during different developmental stages because the degree of impairment varied considerably among individual Δnox mutants. $\Delta noxA$ mutant was more sensitive to H₂O₂, KO₂, and diamide than $\Delta noxB$ or $\Delta noxR$. In contrast, $\Delta noxB$ or $\Delta noxR$ mutation strain was more sensitive to cumyl H₂O₂ and SDS than $\Delta noxA$. The elevated sensitivity of $\Delta noxB$ and $\Delta noxR$ mutants to ROS was also accompanied by a reduced expression of two redox-responsive genes AaAPI

and AaHOG1. Although expression of AaAPI and HOG1 was upregulated by the NOX system; both AaAPI and HOG1 negatively regulate the expression of NoxB and NoxR. This transcriptional feedback loop might allow fungus to avoid excessive production of toxic ROS. In *A. nidulans*, NOXA is regulated by an HOG1 homolog [189]. Expression of the NOX complex coding genes has been shown to be regulated by FUS3/KSS1 and SLT2 MAP kinases in *N. crassa* and *B. cinerea* [141, 191]. Mammalian p47^{phox} and p67^{phox} are phosphorylated by the p38 HOG1 MAP kinase.

In fungi, expression of NOXA and NOXB is regulated by NOXR and Rac [141, 191, 194]. However, *A. alternata* NOXR negatively regulated the expression of NOXA and had no effects for the expression of NOXB. $\Delta noxB$ mutant was highly resistant to calcofluor white, Congo red and dicarboximide and phenylpyrrole fungicides compared to the wild type or the mutant strain lacking NOXA and NOXR. NOXB seemingly plays a negatively regulatory role in the biosynthesis of chitin because $\Delta noxB$, but not $\Delta noxA$ and $\Delta noxR$, had higher chitin content than the wild type (S.L. Yang, personal communication). As stated above, fungal strains disrupted at any of the AaAPI, AaHSK1, and AaHOG1 genes were all hypersensitive to CHP and TIBA. $\Delta noxA$, $\Delta noxB$, and $\Delta noxR$ mutants also displayed increased sensitivity to these two compounds, suggesting the existence of essential cross-talks between different signaling pathways in the context of multidrug resistance.

The NOX complex has been shown to be required for pathogenicity/virulence in a number of fungal species [141, 167, 188, 196]. Pathogenicity assays revealed that $\Delta noxB$ or $\Delta noxR$ mutant is unable to produce necrotic lesions on unwounded citrus leaves. Both mutants induced wild-type lesions on citrus leaves that were wounded before inoculation, indicating that Δnox mutants are primarily arrested in the penetration stage. $\Delta yap1$ and $\Delta hog1$ mutants are blocked in both penetration and colonization stages.

8. Nonribosomal Peptide Synthetase- (NPS-) Mediated ROS Detoxification

Deletion of an *A. alternata* gene (AaNPS6), encoding a polypeptide analogous to fungal nonribosomal peptide synthetases (NPSs) resulted in fungi that reduced accumulation of host-selective toxin and melanin and displayed increased sensitivity to H₂O₂, superoxide-generating compounds (KO₂ and menadione), and iron depletion (L.-H. Chen, personal communication). $\Delta nps6$ failed to produce siderophore, a low-molecular organic compound involved in acquiring iron from the environment [197, 198]. In nearly all living cells, iron required for numerous metabolic functions and electron transfer processes plays a vital role for cell proliferation and survival [199]. When starved for iron, microorganisms secrete siderophores to solubilize and extract iron. All fungal siderophores (rhodotorulic acid, fusarinines, coprogens, and ferrichromes) that have been identified contain hydroxamates and are synthesized from an unconventional amino acid, L-ornithine [197, 200, 201]. NPSs function to synthesize linear or cyclic peptides without the aid of ribosomes, adding

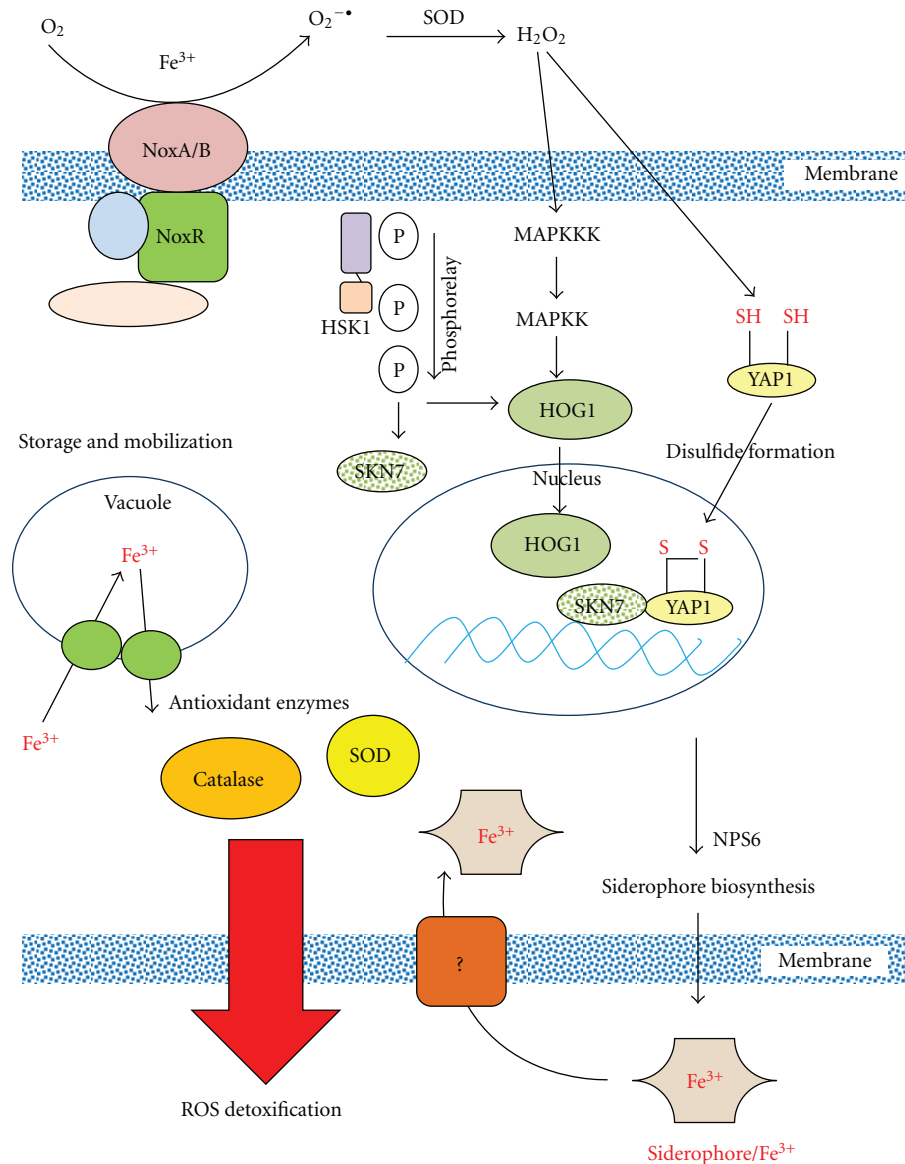


FIGURE 4: Schematic illustration of signaling pathways leading to ROS detoxification in the tangerine pathotype of *A. alternata*. H_2O_2 produced by the membrane-bound NADPH oxidase (NOX) complex plays a central role in the activation of genes responsible for ROS resistance. Upon exposure to ROS, YAP1 forms disulfide bonds between two conserved cysteine residues, undergoes conformation changes, and is transported into the nucleus where YAP1 regulates the expression of numerous genes associated with environmental stress. The YAP1 and SKN7 redox-responsive regulators, the HOG1 mitogen-activated protein (MAP) kinase, the NPS6-mediated siderophore biosynthesis, and the NOX complex are required for ROS detoxification. *NPS6* encoding a nonribosomal peptide synthetase is required for the biosynthesis of siderophores, which can extract environmental iron. Iron is stored in vacuoles. Siderophore-mediated iron acquisition plays a critical role if ROS resistance because iron is a major cofactor for the activities of catalase and SOD. HOG1, in cooperation with unknown regulators, is also required for salt resistance. The two-component histidine kinase (HSK1), likely interacting with SKN7, is primarily used for cellular resistance to sugars. Fungicide sensitivity involves HSK1, HOG1, and SKN7.

D- or L-amino acids, proteins, nonproteins, hydroxyl acids and ornithine into nonribosomal peptides. Many of these peptides have medicinal, pharmaceutical, or agricultural values [202]. AM-toxin produced by the apple pathotype of *A. alternata*, HC-toxin produced by race 1 of the maize pathogen, *Co. carbonum*, and enniatin produced by *Fusarium* spp. are all nonribosomal peptides [7, 203, 204].

Many *Alternaria* species produce and excrete dimethyl coprogen siderophores [205–208]. Coprogen contains a diketopiperazine ring (dimerium acid), in which two N^5 -acyl- N^5 -hydroxy-ornithine units are joined by a peptide bond [198]. The third acyl ornithine unit is linked to the ring via an ester bond. NPSs are involved in assembling three N^5 -acyl- N^5 -hydroxy-ornithine units, which are

the immediate precursors of hydroxamate siderophores. Fungi often have multiple *NPS* genes; each encodes a polypeptide with discrete domains—AMP-binding adenylation (A), thiolation (T) or peptidyl carrier protein (ACP), and condensation (C) domains that are organized as a module [209–212]. The adenylation domain is required for recognition and activation of amino acid substrate. The thiolation domain is involved in 4'-phosphopantetheine binding. The condensation domain is involved in the formation of a peptide bond and elongation and release of the newly synthesized peptide. The number and order of modules in an *NPS* affect the length and structure of nonribosomal peptide.

$\Delta yap1$ and $\Delta nps6$ mutants of *A. alternata* displayed varying levels of hypersensitivity to H_2O_2 and superoxide-generating compounds. $\Delta nps6$ mutant is less sensitive to ROS than $\Delta yap1$, displaying an increased sensitivity to the test oxidants only when applied at higher concentrations: H_2O_2 ($\geq 0.2\%$), KO_2 (≥ 20 mM), and menadione (≥ 5 mM). However, elevated sensitivity to H_2O_2 seen in $\Delta nps6$ or $\Delta yap1$ mutant was alleviated by adding ferric iron into the medium, implicating an important role of iron and siderophore-mediated iron acquisition in the ROS resistance. We have observed that expression of the *AaNPS6* gene was significantly downregulated in fungal mutant lacking *YAP1*. Deletion of *YAP1* in *A. alternata* also reduced the production of siderophores. Moreover, the rescued strain expressing a functional copy of *YAP1* accumulated wild-type level of siderophores and *AaNPS6* gene transcript. Expression of *AaNPS6* and production of siderophores were also downregulated in fungal strain lacking *HOG1* or *NOXA* (L.-H. Chen and S.L. Yang, personal communication), confirming a close linkage between iron acquisition and ROS resistance. The wild-type strain of *A. alternata*, when grown under iron-depleted conditions, produced barely measurable catalase and SOD activities (L.-H. Chen, personal communication). Both antioxidant activities were detectable in *A. alternata* cultured under iron-rich conditions. Hence, we concluded that the increased sensitivity to oxidative stress and the reduced pathogenicity seen in $\Delta yap1$, $\Delta hog1$, $\Delta noxA$, or $\Delta nps6$ were seemingly due to the decreased ability of oxidative stress-detoxifying enzymes.

Genetic analyses revealed that siderophore produced by *NPS6* is required for full virulence of the tangerine pathotype of *A. alternata*. This is likely due to the inability of $\Delta nps6$ mutant to detoxify toxic ROS efficiently. Siderophores are also required for fungal pathogenesis in *A. brassicicola*, *As. fumigatus*, *Co. heterostrophus*, *Co. miyabeanus*, *F. graminearum*, and *M. grisea* [208, 210, 213, 214]. However, siderophore is not required for pathogenesis of the basidiomycete maize pathogen, *U. maydis* [215]. It will be of great interest to determine if *NPS6* is also regulated by *YAP1* and *HOG1* in other fungal species.

9. Conclusions

Based on the observed phenotypes derived from mutants lacking *YAP1*, *HOG1*, *SKN7*, *NOX*, or *NPS6*, a regulatory network is assembled to underscore the intricate interplays

among these signaling pathways in *A. alternata* (Figure 4). The *NOX* complex appears to have an important role in the production of ROS, which may act as secondary messages to regulate various metabolic processes in *A. alternata*. The *NOX* complex is required for transcriptional activation of two important regulators, *YAP1* and *HOG1*, which subsequently regulate the expression of genes encoding the nonribosomal peptide synthetase (*NPS6*) and perhaps other enzymes involved in the biosynthesis of siderophores as well. *SKN7* physically interacts with *YAP1*, regulating the genes involved in ROS detoxification. Maintaining iron homeostasis is critical for ROS detoxification because of the requirement of iron for antioxidant enzymatic activities. Impairment of the *NOX* complex, the *YAP1* regulator, the *HOG1* kinase, or the siderophore-mediated iron acquisition in *A. alternata* impacts its ability to detoxify ROS and to colonize host plant, implicating the importance of ROS detoxification in the successful pathogenesis of *A. alternata*. In addition to detoxifying ROS, *A. alternata* utilizes specialized or synergistically regulated signaling pathways, involved in *HSK1*, *HOG1*, and/or *SKN7*, in response to osmotic stress, fungicides, and other toxic compounds. This cross-interaction between different signaling pathways may have eccentric advantages for integrating cellular responses to a broader spectrum of environmental stimuli.

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