Mitogen-activated protein kinase signaling pathways of the tangerine pathotype of *Alternaria alternata*

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Abstract

Mitogen-activated protein kinase (MAPK)-mediated signaling pathways have been known to have important functions in eukaryotic organisms. The mechanisms by which the filamentous fungus *Alternaria alternata* senses and responds to environmental signals have begun to be elucidated. Available data indicate that *A. alternata* utilizes the Fus3, Hog1 and Slt2 MAPK-mediated signaling pathways, either separately or in a cooperative manner, for conidia formation, resistance to oxidative and osmotic stress, and pathogenesis to citrus. This review provides an overview of our current knowledge of MAPK signaling pathways, in conjunction with the *two-component* histidine kinase and the Skn7 response regulator, in the tangerine pathotype of *A. alternata*.

Introduction

Mitogen-activated protein kinase (MAPK)-mediated signaling cascades in eukaryotic microorganisms are vital for perceiving environmental stimuli at the cell surface and for transmitting these signals to the nucleus to modulate gene expression.[1,2] This signaling pathway comprises three major components: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK, belonging to a family of serine/threonine protein kinases. They function in perceiving environmental stimuli by a phosphorelay mechanism.[3,4] Upon perceiving environmental stimuli, MAPKKK is phosphorylated in the TXY (threonine/x/tyrosine) motif. The phosphorylated MAPKKK phosphorylates the downstream MAPKK, which in turn phosphorylates MAPK.[1,5] The phosphorylated MAPK activates a number of transcription factor-coding genes, whose products eventually make a change in gene expression. Hence, microorganisms are capable of responding and adapting to their environment.

In the budding yeast *Saccharomyces cerevisiae*, phosphorelay systems operated by five MAPK pathways are known to regulate a wide range of cellular functions, including mating, formation of pseudo-hyphae, cell-wall reconstruction, as well as response to osmotic stress.[6] Filamentous fungi have three MAPK protein kinases: the high-osmolarity glycerol (Hog1) homolog, the cell wall integrity (Slt2) homolog and the pheromone response (Fus3/Kss1) homolog.[7,8] MAPK signaling pathways are well conserved among yeasts and fungi, but the biological functions of each component kinase may vary considerably in different species, largely depending on the species' lifestyles and the surrounding environment.[9] Furthermore, synergistic regulations between different transduction pathways further complicate the signaling network.[6,10,11] For example: the genes required for melanin biosynthesis and pigmentation are co-regulated by Fus3 and Slt2 MAPK-mediated pathways in the maize pathogen *Cochliobolus heterostrophus*.[12] In *S. cerevisiae*, Hog1 counteracts the Fus3/Kss1 signaling pathway in response to hyperosmotic stress.[13,14]

Alternaria alternata is a common necrotroph, having at least ten distinct pathotypes. Each of the pathotypes produces a host-selective toxin with a distinct mode of action and induces disease only on susceptible plant species or cultivars.[15-18] The tangerine pathotype of *A. alternata* produces an ACT toxin that is toxic to grapefruit (*C. paradisi* Macfad.), tangerines (*C. reticulata* Blanco), as well as hybrids from grapefruit and tangerine, or tangerine and sweet orange (*C. sinensis* (L.) Osbeck).[19] ACT, containing a 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid structure, causes rapid electrolyte leakage from citrus cells of susceptible cultivars, and is the primary pathogenicity determinant of the tangerine pathotype of *A. alternata*.[15,16,19,20] Signaling pathways that *A. alternata* operates to respond to environmental stress have begun to be elucidated. This article summarizes the current state of knowledge of MAPK-mediated signaling pathways associated with a wide array of developmental, physiological and pathological functions in the tangerine pathotype of *A. alternata*.

The Fus3/Kss1 mitogen-activated protein kinase-mediated signaling pathway in *A. alternata*

The Fus3/Kss1 MAP kinases grouped in the yeast and fungal extracellular signal-regulated kinase (YERK1) subfamily[5] have been intensively studied in *S. cerevisiae*. Fus3 and Kss1 are two closely related MAP kinases, functioning to regulate mating processes and/or filamentous growth. The Fus3 and Kss1 pathways in *S. cerevisiae* are partially redundant in which both share a number of components through the MAPK signaling pathway. However, Fus3 is predominately responsible for mating process, whereas Kss1 is for filamentous growth.[21] In response to availability of nitrogen or pheromone, the Fus3/Kss1 MAP kinase signaling pathway is activated by the G protein/proteinase kinase A (PKA)-mediated signaling pathways and regulates numerous genes required for mating and/or hyphal development. Many filamentous fungi also have the Fus3 homologs. The *Magnaporthe grisea* PMK1 that was able to complement an *S. cerevisiae* mutant defective in mating is essential for appressorium formation and pathogenicity on rice.[22] The Fus3 homolog also plays a critical role in virulence of other phytopathogenic fungi, including *A. brassicicola*, *Bipolaris oryzae*, *Botrytis cinerea*, *Claviceps purpurea*, *C. heterostrophus*, *Colletotrichum lagenarium*, *Cryphonectria parasitica*, *Fusarium* spp., *Mycosphaerella graminicola*, *Pyrenophora teres*, *Stagonospora nodorum* and *Verticillium dahliae*.[23-37] The Fus3 homolog (Cek1) is also an important virulence determinant in the opportunistic human pathogen *Candida albicans*.[38]

The *AaFus3* gene encoding a homolog of the yeast Fus3-like MAP kinase was cloned and

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characterized from the tangerine pathotype of *A. alternata*.[39] The *AaFus3* gene encodes a polypeptide of 352 amino acids, which contain a protein kinase active site, a threonine/glutamic acid/tyrosine (TEY) activation motif and an ATP-binding domain, a characteristic MAP kinase motif. AaFus3 is most similar to the *P. teres* PTK1, the *Bi. oryzae* BMPK1, the *A. brassicicola* AMK1 and the *Co. heterostrophus* ChK1, and is less similar to the *Botryotinia fuckeliana* BMP1, the *M. grisea* PMK1 and the *My. graminicola* MgFus3. Systemic loss-of-function genetics in *A. alternata* reveal that AaFus3 regulates a number of physiological metabolisms and development in *A. alternata* (Figure 1). The *A. alternata* strain lacking *AaFus3* (Δfus3) exhibited growth retardation. Fungal growth could be restored to wild-type levels in a strain that re-acquired a wild-type copy of *AaFus3*. Application of glucose, but not sorbitol, mannitol or sucrose (each at 1.5 M), partially restored vegetative growth of the Δfus3 mutants. The Δfus3 mutants grew faster than wild type or the complementation strains in the presence of potassium chloride (KCl) or sodium chloride (NaCl), implicating the AaFus3-mediated signaling pathway in negative regulation of salt tolerance. The AaFus3 pathway is also involved in fungicide resistance because the Δfus3 mutants are more sensitive to copper fungicides than the wild-type and the complementation strains.[39]

In addition, the Fus3 MAPK–mediated signaling pathway is involved in conidia formation and maturation in *A. alternata*, since Δfus3 mutant does not produce any conidia. The Δfus3 mutant generates dark, aberrant hyphae with distinct septae that occur in chains, but never produces fully mature conidia. Administration of cyclic adenosine monophosphate (cAMP), NaCl, KCl, yeast extracts or various antioxidants such as α-tocopherol (vitamin E), ascorbic acid and proline could not enhance fungal growth or revive conidiation of fungal strains impaired for *AaFus3*. However, application of glucose restored vegetative growth, but not conidia formation. Conidia are vital for completing the *A. alternata* life cycle and for initiating disease on citrus. Similar defects in conidia maturation were also observed in *A. brassicicola* lacking a *Fus3/Kss1* gene homolog.[24]

Conidia formation in fungi is a complicated and tightly regulated process, which is often controlled by different signaling pathways in a given species. Our studies also showed that formation of conidia by *A. alternata* is regulated by the G protein-regulated cAMP level.[40] Mutational inactivation of a Gα subunit-coding gene (*AaGα1*) in *A. alternata* resulted in a severe reduction in conidiation. Exogenous application of cAMP caused decreased conidiation in the wild-type but partially restored conidia formation in the Gα-deficient mutant. In striking contrast, conidiation is negatively controlled by cAMP-dependent PKA.[41] When the cAMP levels are low, the PKA complex, comprising two regulatory and two catalytic subunits, forms a nonfunctional tetramer. The catalytic subunits are separated from the regulatory subunits upon cAMP binds to the regulatory subunits.[42,43] The activated catalytic subunits phosphorylate downstream enzymes or transcriptional regulators. The *A. alternata* strain impaired for PKA catalytic subunit gene (*PKA^{cat}*) produces no detectable PKA activity and produces abundant conidia. In contrast, the strain lacking PKA regulatory subunit gene (*PKA^{reg}*) forms swelling hyphal segments and produces no mature conidia, the phenotypes highly resembling the strain lacking AaFus3. Because impairment of *Fus3* did not affect PKA activity and expression of the *PKA^{cat}* gene and vice versa, it seems that PKA and Fus3 are two different pathways for conidia production. In addition, recent studies revealed that conidiation by *A. alternata* is regulated by the nicotinamide adenine dinucleotide phosphate oxidase complex implicating in the production of reactive oxygen species, the Slt2 MAP kinase, the Skn7 responsive regulator and the siderophore–mediated iron acquisition.[44-46] However, the exact interactions between these pathways leading to conidia formation remain largely unknown.

Northern blot analysis revealed that expression of the *AaFus3* gene in *A. alternata* was upregulated by citrus leaf extracts prepared from host (Minneola) or nonhost (rough lemon) leaves. Pathogenicity assayed on detached Minneola leaves inoculated by placing a mycelial mass revealed that the *A. alternata* strain lacking AaFus3 produced wild-type levels of ACT, yet induced significantly smaller lesions than wild type. The genetically reverted strain produced necrotic lesions comparable to those induced by the wild-type. However, the mutant strain produced wild-type lesions on citrus leaves with wounding prior to inoculation, indicating the requirement of AaFus3 for the penetration process.

Although *AaFus3* is not required for resistance to oxidative stress, our studies suggest a possible linkage between the Yap1-regulated

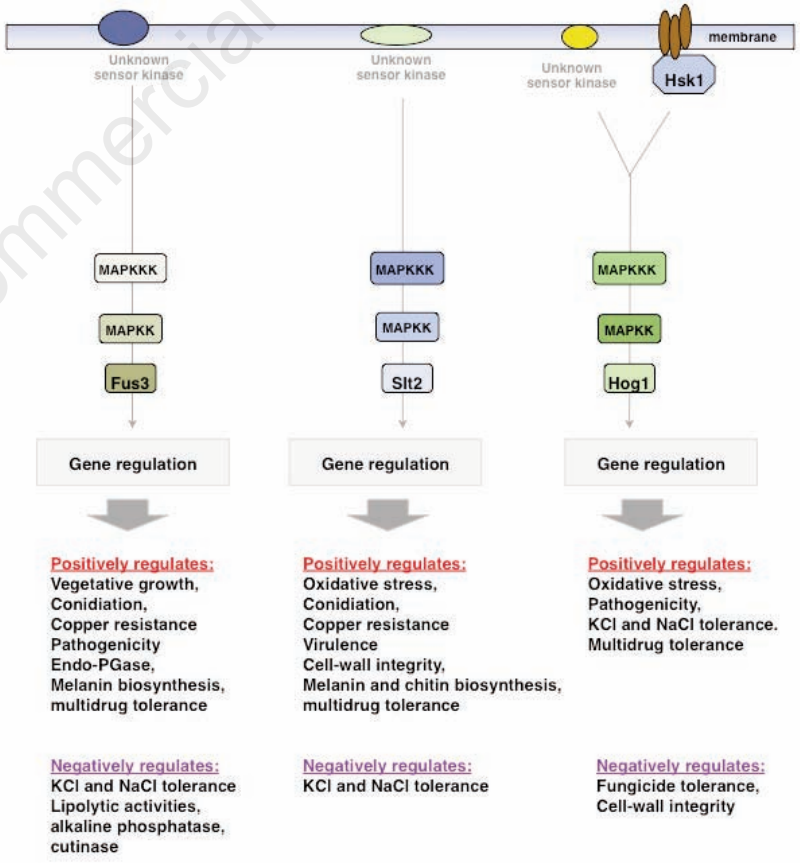

Figure 1. A summary of biological functions of mitogen-activated protein kinase (MAPK)-mediated signaling pathways – the high-osmolarity glycerol (Hog1), the cell wall integrity (Slt2) and the pheromone response (Fus3/Kss1) homologs – in the tangerine pathotype of *Alternaria alternata*.

gene expression and a Fus3 MAPK-mediated signaling pathway in *A. alternata*. Yap1, belonging to the mammalian AP1 family, is a leucine zipper-containing transcriptional activator implicated in cellular responses to stress.[47] Both *AaAP1* (a YAP1 homolog) and *AaFus3* were found to be required for full resistance to 2-chloro-5-hydroxypyridine (CHP), 2,3,5-triiodobenzonic acid (TIBA), diethyl maleate (DEM) and many pyridine-containing compounds. The *AaFus3*, but not *AaAP1*, mutant strain also displayed an increased sensitivity to pyridoxine (vitamin B6) and its derivative pridoxal-5-phosphate. Inactivation of the *AaAP1* gene increased phosphorylation of AaFus3; however, application of hydrogen peroxide (H_2O_2) unchanged phosphorylation of AaFus3.[39] The Δyap1 Δfus3 double mutant strain displayed a greater chemical sensitivity compared to the strains mutated at either *AaFus3* or *AaAP1* alone. Furthermore, TIBA, CHP or DEM promoted accumulation of the *AaAP1* and *AaFus3* gene transcripts and enhanced AaFus3 phosphorylation. The AaAP1::sGFP (*s*ynthetic *g*reen *f*luorescence *p*rotein) fusion protein became localized in the nucleus after the treatment of H_2O_2, TIBA or CHP. Thus, the AaAP1 and AaFus3 signaling pathways are both required for multidrug resistance, probably by regulating common membrane transporters or enzymes that are directly involved in the efflux or detoxification of toxic substances. Recently, expression of two genes encoding putative major facilitator superfamily (MFS) transporters has been demonstrated to be coordinately up-regulated by AaAP1 and AaFus3.[48] Deletion of either of the two MFS transporter-coding genes yielded fungi that displayed an elevated sensitivity to TIBA and CHP (*L.-H Chen and H.-C. Tsai,* 2012, *personal communication*). The AaFus3–mediated signaling pathway down-regulates the alkaline phosphatase, cutinase and lipolytic enzymatic activities,[49] since the impaired strain showed higher levels of those enzymatic activities than the wild-type. However, the *A. alternata* Δfus3 mutant accumulated lower endo-PGase activities. AaFus3 has no impact on pectinase, xylanase and cellulase activities. The Δyap1 mutant strain accumulated wild-type levels of all enzymes tested.

The cell wall integrity-mediated signaling pathway in *A. alternata*

A. alternata has the yeast Slt2 homolog implicating in maintenance of cell wall integrity. The *A. alternata Slt2* (*AaSlt2*) gene encodes a polypeptide of 416 amino acids, containing a serine/X/threonine (TXY) activation site in the N termini, commonly found in the yeast and fungal extracellular signal-regulated kinase (YERK1) subfamily[5] and a protein kinase ATP-binding signature. AaSlt2 is similar to numerous MAP kinases associated with cell wall integrity of fungi. The AaSLT2 MAP kinase protein is most similar to SLT2-like proteins of *A. brassicicola* (AAU11317), *Ajellomyce capsulatus* (XP_001538584) and *C. heterostrophus* (ABM54149) showing 85 to 99% identities. AaSlt2 MAP kinase-mediated signaling pathway has been demonstrated to regulate diverse physiological, developmental and pathological functions in *A. alternata* (Figure 1). Deletion of *AaSlt2* resulted in fungi (Δslt2) showing growth retardation and producing fluffy and white colonies on potato dextrose agar. Unlike the wild-type strain of *A. alternata*, the Δslt2 mutant produces globose and less branching hyphae; some of them form hairpin loops at the end of hyphae, suggesting that AaSlt2 plays a critical role in fungal development.[45]

The *A. alternata Slt2*-impaired mutant reduced conidia formation as much as 95%. Conidia formation was restored in strains by transforming Δslt2 mutant protoplasts with a functional *AaSlt2*. Application of KCl or NaCl restored vegetative growth but not conidiation by Δslt2. In contrast, application of caffeine, sodium dodecyl sulfate, sorbitol, glucose or H_2O_2 did not improve vegetative growth and conidia formation of the mutant. Conidia produced by the wild-type are multicellular, obpyriform and contain both vertical and transverse septae. On the other hand, Δslt2 mutant produces aberrant, less melanized conidia with fewer vertical septae and thinner cell wall.[45] Compared to wild type, Δslt2 mutant has lower melanin content. The size of conidia produced by wild-type and Δslt2 is also very different. Δslt2 mutant produces larger conidia than wild-type. Conidia produced by Δslt2 mutant have less verruculose relative to those of wild type, as examined by scanning electron microscopy. The complementation strain produces conidia similar to those produced by the wild-type.

The Δslt2, but not Δfus3, mutant were hypersensitive to cell-wall destructing compounds, calcofluor white and Congo red, which interfere with chitin polymerization. Δslt2 mutant has lower cell wall chitin content compared to the levels measured in the wild type and the genetically reverted strains. Δslt2 mutant released protoplasts at a rate and magnitude significantly greater than the wild-type and the complementation strains in the presence of an enzymatic mixture containing driselase, lyticase, β-D-glucanase and β-glucuronidase,[45] confirming further that AaSlt2 plays an important role in maintaining cell wall integrity. Unlike *A. alternata*, the *B. cinerea* and the *Col. lagenarium slt2* mutants were unchanged in sensitivity to cell wall-degrading enzymes or Calcofluor white compared to their parental strains.[50,51]

The Slt2 MAPK signaling pathways are well conserved in eukaryotes. However, they may have different functions within and between species. For examples, Slt2 homologs are required for maintaining the integrity of cell walls in *A. brassicicola*, *Cl. purpurea*, *M. grisea*, *Fusarium graminearum* or *Aspergillus nidulans*.[52-56] In contrast, Slt2 plays no roles in cell wall integrity in *B. cinerea*, *Col. lagenarium* or *My. Graminicola*.[50,51,57] The *A. brassicicola* mutant lacking a Slt2 MAP kinase is more sensitive to H_2O_2 than its progenitor;[55,58] the *B. cinerea slt2* mutant is hypersensitive to fludioxonil and paraquat.[51] The *A. alternata* Δslt2 mutant displayed wild-type sensitivity to H_2O_2 and dicarboximide (iprodione and vinclozolin) and phenylpyrrole (fludioxonil) fungicides. Similar to fungal strain lacking *AaFus3* or *AaAP1*, Δslt2 mutant displayed an increased sensitivity to CHP, TIBA or compounds containing pyridine or benzene backbones.[45] Fungal strain lacking a Gα subunit- or a PKA catalytic subunit-coding gene displayed wild-type sensitivity to those compounds. In fungi, the functions of membrane-bound transporters or pumps, such as the ATP-binding cassette transporters and the MFS transporters are often contributable to multidrug resistance.[59] We speculate that those signaling pathways may directly or indirectly regulate some of the transporters and facilitate efflux of the toxic materials in *A. alternata*.

Slt2 homologs have been shown to be required for the production of melanin by *Neurospora crassa* and *C. heterostrophus* and for the production of deoxynivalenol by *F. graminearum*.[12,53,60] We also found that the *A. alternata Slt2* is involved in regulating biosynthesis of secondary metabolites, because Δslt2 mutant accumulated lower levels of ACT toxin and melanin.[45] The levels of ACT toxin and melanin were restored in a strain that expresses a wild-type copy of *AaSlt2*.

AaSlt2 is required for full virulence of *A. alternata* on citrus. Fungal virulence performed on detached Minneola leaves sprayed uniformly with conidial suspension revealed that Δslt2 mutant produced significantly fewer and smaller necrotic lesions than the wild-type. This pathogenic impairment could be in part due to the reduced production of ACT toxin by Δslt2 mutant. The reduction of fungal virulence resulting from the deletion of *AaSlt2* could also be due to slow growth and abnormality of hyphal extension of the mutant. Slt2 homologs also play an important function in pathogenicity/virulence in a number of phytopathogenic fungi. These include *B. cinerea*, *Col. lagenarium*, *Cl. purpurea*, *F. graminearum*, *M. grisea*, *C. heterostrophus*, *My. graminicola* and *A. brassicicola,*[50-58] as well as the human pathogens, *Can. albicans* and *Cryptococcus neoformans*.[61,62]

The high osmolarity glycerol mitogen-activated protein kinase-mediated signaling pathway in *A. alternata*

In *S. cerevisiae*, the activated Hog1 pathway facilitates glycerol accumulation, allowing the yeast to cope with high osmolarity induced by the surrounding environment.[63-66] The function of Hog1 is regulated by phosphorylation via a *two-component* histidine kinase (HSK)-mediated signaling pathway.[64,66,67] *S. cerevisiae* has a single HSK, Sln1p, which is required for osmotic adaption via an Ypd1p containing a histidine (His) phosphotransfer (HPt) domain and two response regulators (RR), Ssk1p and Skn7p.[68-70] All fungal HSKs identified have both a histidine kinase and a RR domains.[63,65,71] Under normal osmolarity, the Sln1p kinase is spontaneously phosphorylated at a conserved histidine residue. The phosphate is passed down to Ypd1p and then Ssk1p or Skn7p in a pattern of His-Asp-His-Asp.[67] The phosphorylated Ssk1p is inactive and fails to activate the Hog1 MAP kinase pathway. When Ssk1p is dephosphorylated, it is able to activate the Hog1 pathway that, in turn, promotes glycerol accumulation, allowing the yeast to counteract high osmolarity. In contrast, the phosphorylated Skn7p is active and capable of regulating the genes whose products are responsible for low osmolarity.[68,70] In addition, *S. cerevisiae* uses a Sho1p protein, independent of HSK, to counteract osmotic stress.[72,73] HSK-mediated signals have long been thought to be transduced primarily via the Hog1 MAP kinase pathway.[74] With the exception of *S. cerevisiae*, all fungi have multiple HSK signaling genes, ranging from three HSK genes in the fission yeast *Schizosaccharomyces pombe* to as many as 21 HSK genes in the filamentous fungus *C. heterostrophus*.[75] This signaling pathway has been implicated in osmotic and oxidative responses, hyphal development, toxin biosynthesis, virulence, as well as sensitivity to dicarboximide and phenylpyrrole fungicides in diverse fungal species, including *N. crassa*, *Bot. fuckeliana*, *A. alternata*, *A. brassicicola*, *Cl. purpurea*, *M. grisea*, *My. Graminicola*, *C. heterostrophus* and *F. graminearum*.[76-89]

The *A. alternata* Hog1 (AaHog1), analogous to the yeast Hog1, has a distinct phosphorylation motif (TGY) required for the hyperosmolarity response.[5] AaHog1 also has a protein kinase ATP-binding region, a MAP kinase and a serine/threonine protein kinase active site. The *A. alternata* Hsk1 (AaHsk1), analogous to fungal group III *two-component* histidine kinase, has a HAMP (histidine kinase, adenylate cyclase, methyl binding protein, and phosphatase) domain and a dimerization/phosphoacceptor domain. Unlike the yeast histidine kinase Sln1p, AaHsk1 has no transmembrane domains.

AaHOG1 plays a critical role in cellular resistance to oxidative and salt stresses in *A. alternata* (Figure 1). Inactivation of the Hog1 homolog by targeted gene disruption in *A. alternata* produced fungi that showed an increased sensitivity to KCl and NaCl salts, menadione, *tert*-butyl-hydroxyperoxide, H_2O_2, TIBA and CHP.[90] Hog1 mutant displayed wild-type sensitivity to glucose, sucrose, sorbitol or mannitol. In contrast, inactivation of the *A. alternata Hsk1* gene produced fungi that showed an increased sensitivity to sucrose, mannitol, glucose, sorbitol, TIBA or CHP. Δhsk1 mutant displayed wild-type sensitivity to *tert*-butyl-hydroxyperoxide, H_2O_2, KCl or NaCl. Hence, AaHsk1 plays a regulatory role in osmotic adaption, specifically to sugar osmoticants, without the involvement of the AaHog1-mediated pathway. AaHog1, independent of AaHsk1, confers cellular resistance to salts and oxidative stress. Although Δ*hog1* mutant displayed wild-type sensitivity to sugars, these osmoticants promoted AaHog1 phosphorylation and subsequently nuclear localization in the Δhsk1 mutant background.[90] In the wild-type background, sugar osmoticants did not impact AaHog1 phosphorylation and did not facilitate nuclear localization of AaHog1. Pathogenicity assays revealed that *AaHog1* is required for fungal pathogenicity, but *AaHsk1* is dispensable for pathogenicity. The *AaHog1*-impaired mutants are non-pathogenic, producing no necrotic lesions on Minneola leaves. Inactivation of the *AaHog1* or *AaHsk1* gene did not influence the production of host-selective ACT toxin by *A. alternata*. It appears that *A. alternata* recruits AaHsk1 and AaHog1 to perform a unique function in resistance to sugar osmoticants and salt stress, respectively. *S. cerevisiae* employs the *two-component* histidine kinase Sln1p and the membrane protein Sho1p, both via regulation of the Hog1 pathway, to counteract osmotic stress.[72] In contrast, the filamentous fungus *A. nidulans* Sho1p homolog is not required for cellular response to osmotic stress.[91] Recently, a *Sho1p* homolog was cloned and inactivated in the tangerine pathotype of *A. alternata*, revealing no role in osmotic adaption (*L.H. Chen, 2012, unpublished*). Disruption of the *Ssk1p* homolog (a responsive regulator upstream of Hog1) in *A. alternata* resulted in fungi that displayed an elevated sensitivity to H_2O_2, *tert*-butyl hydroperoxide, menadione, salts, but not glucose, phenotypes resembling those seen with the Δhog1 mutant (*L.H. Chen, 2012, unpublished*).

Unlike Slt2, AaHog1 appears to have a negatively regulatory role in the maintenance of cell wall integrity. A fungal strain lacking AaHog1, but not AaHsk1, was highly resistant to cell-wall degrading enzymes, such as driselase, β-D-glucanase, β-glucuronidase and lyticase, producing no protoplasts. Moreover, AaHog1 and AaFus3 have an opposite role in terms of KCl or NaCl tolerance (Figure 2). As stated above, Δfus3 mutant displayed an increased resistance to KCl and NaCl, whereas Δhog1 mutant displayed an increased sensitivity to them. In *S. cerevisiae*, Hog1 has also been shown to negatively regulate the Fus3/Kss1 signaling cascade during hyperosmotic stress.[13,14]

AaHog1 and AaHsk1 have shared functions as well, because fungi impaired for AaHog1 or AaHsk1 were more resistant to dicarboximide and phenylpyrrole fungicides than the wild-type. Compared to the resistance seen with the Δhsk1 mutants, the Δhog1 mutant was barely resistant to these fungicides, implicating that AaHsk1 is the key regulator for sensitivity to dicarboximide and phenylpyrrole fungicides.[90] Nuclear localization is important for proper functions of Hog1.[92] Under normal conditions, the AaHog1 protein was phosphorylated at low levels in the wild-type strain of *A. alternata*. Exposure to iprodione or fludioxonil fungicide, TIBA, CHP, NaCl or H_2O_2 enhanced AaHog1 phosphorylation and nuclear localization.

The two component histidine kinase-Skn7 signaling pathway in *A. alternata*

All living cells have different signaling transduction pathways to perceive changes in their environments and to adjust physiological and developmental processes.[93-99] *S. cerevisiae* has two major activation mechanisms – the Sln1p-Ypd1p-Ssk1p-Hog1 and the Sho1p-mediated pathways – in response to osmotic and oxidative stress. Similar to Ssk1 response regulator, Skn7 is a transcription downstream regulator of Sln1p (Figure 2). Skn7p is phosphorylated, specifically occurring at the Asp (D427), under conditions of low osmolarity.[70,100] In contrast, under low turgor conditions, Sln1p kinase is phosphorylated and subsequently activates Ssk1p by a phosphorelay mechanism. The phosphorylated Ssk1p is inactive and unable to activate the Hog1 MAP kinase pathway.

In response to oxidative stress, Skn7p is not modulated by the Sln1p-mediated phosphorylation.[68,101] Under oxidative stress, Skn7p is phosphorylated at serine or threonine residue and forms a heterodimer with the stress responsive transcription regulator Yap1.[100,102,103] Interaction between Skn7 and Yap1 regulates numerous genes associated with oxidative stress response.[101,104-106] However, Yap1 confers resistance to cadmium resistance, apparently bypassing Skn7p.[107]

Skn7p could interact with the calcium responsive activator, the heat-shock transcription factor, the Rho1 GTPase or the cell cycle transcription regulator under different physiological conditions.[108-111] In fungi, Skn7 has been demonstrated to be required for sporulation, fungicide sensitivity, cell wall biosynthesis, oxidative stress adaptation, osmotic stress response, cell cycle, sexual mating and pathogenicity/virulence.[101,109, 112-120] The *A. alternata Skn7* homolog was cloned and functionally inactivated, revealing a close association of Skn7 response regulator and resistance to osmotic and oxidative stress, conidiation, conidial morphology and fungicide sensitivity.[44] The Ssk1-Hog1 pathway, independent of Hsk1 (a Sln1p ortholog), confers resistance primarily to salts and oxidative stress, whereas the Hsk1-Skn7 pathway is responsible for sugar-induced osmotic and oxidative stress (Figure 2).

Genetic analyses reveal that disruption of the *A. alternata Skn7* gene produced fungi that were more sensitive to H_2O_2, *tert*-butyl hydroperoxide and cumyl peroxide, but not to the superoxide-generating compounds (menadione, potassium superoxide and diamide). Δskn7 mutant also displayed an increased sensitivity to glucose, mannitol, sucrose and sorbitol. However, the Δskn7 mutant displayed wild-type sensitivity to NaCl and KCl salts. The Δskn7 mutant displayed an elevated resistance to dicarboximide (iprodione and vinclozolin) and phenylpyrrole fungicides at levels between the *AaHsk1* and the *AaHog1* mutant strains. A fungal strain impaired for *Ssk1*, a responsive regulator upstream of Hog1, displayed an increased sensitivity to these fungicides at levels similar to those seen with the Δskn7 mutant (L.H. Chen, 2012, *unpublished*), indicating that both Ssk1 and Skn7 are involved in fungicide sensitivity. Fungal strain carrying *skn7/hog1* double mutations exhibited fungicide resistance, similar to the strain with a single *AaHsk1* gene mutation.[44] The results indicate that the signals associated with fungicide sensitivity are passed from AaHsk1 down to both Skn7- and Ssk1-HOG-mediated pathways. Pathologically, the *A. alternata* Skn7 and Ssk1-Hog1singlaing pathways are both required for fungal colonization and lesion development in susceptible cultivars of citrus.[44,90] Mutation of the *A. alternata Hsk1* gene did not impact fungal pathogenicity, confirming further that *A. alternata* employs specialized or shared regulatory interactions among different signaling pathways for diverse physiological and pathological functions.

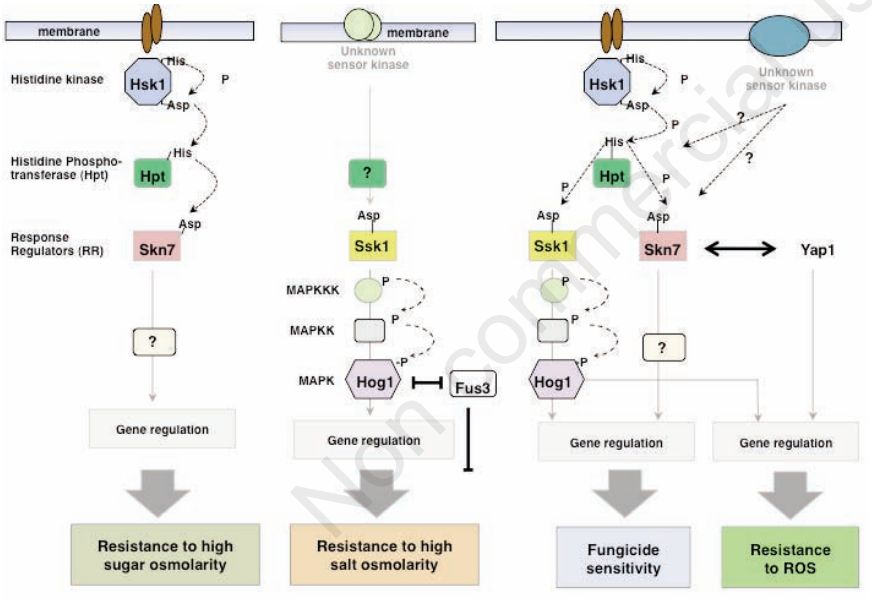

Figure 2. Proposed signaling pathways involving a *two-component* histidine kinase (Hsk1), a histidine phosphotransferase (Hpt) and two responsive regulators (RR), Skn7 and Ssk1, for resistance to high osmolarity and fungicide sensitivity in the necrotrophic fungal pathogen *Alternaria alternata*. Upon sensing high levels of sugars, Hsk1 is phosphorylated and the phosphate is transferred to downstream Hpt and then Skn7p in a pattern of histidine (His)-aspartate (Asp)-His-Asp. Skn7 in turn regulates downstream signaling pathways and eventually produces a change in gene expression. Hsk1 plays no role in resistance to osmotic stress induced by high levels of salts. Unknown kinases, instead of Hsk1, could phosphorylate the Ssk1 regulator via a phosphorelay mechanism and activate the high osmolarity-glycerol 1 (Hog1) MAP kinase pathway. In contrast to Hog1, the Fus3 MAP kinase plays a negative regulatory role for salt tolerance. Hog1 and Hsk1 have shared functions as well, because the *A. alternata* mutant strains lacking Hsk1, Ssk1, Skn7 or Hog1 are more sensitive to dicarboximide and phenylpyrrole fungicides than wild-type. Hsk1 is not required for resistance to reactive oxygen species (ROS). Upon sensing oxidative stress, both Ssk1-Hog1 and Skn7, perhaps activated by unknown kinases, independently regulate the genes required for detoxifying toxic ROS. Skn7 may interact with the redox responsive regulator Yap1 for ROS resistance.

Conclusions

MAPK-mediated signaling cascades play subtle regulatory roles during vegetative growth and conidia formation, for the production of hydrolytic enzymes and melanin, and for resistance to fungicides, osmotic stress and a broad spectrum of structurally diverse compounds in *A. alternata*. Those signaling pathways also have a substantial contribution to fungal pathogenicity and to effective penetration and tissue colonization of citrus hosts. Different MAPK pathways may interact in a cooperative or antagonistic manner, thus diversifying their specificities. The biological roles for MAPK signaling pathways in *A. alternata* are established, but many questions remain to be answered. How *A. alternata* perceives and responds to environmental stimuli will be the key areas for future investigation. Identifying membrane-bound sensor kinases upstream each of the pathways and downstream target proteins will help understand the complex nature of MAPK signaling pathways in relation to various biological and pathological processes in this important fungal pathogen of citrus.

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