Resistance to oxidative stress via regulating siderophore-mediated iron acquisition by the citrus fungal pathogen *Alternaria alternata*

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The ability of the necrotrophic fungus *Alternaria alternata* to detoxify reactive oxygen species (ROS) is crucial for pathogenesis to citrus. We report regulation of siderophore-mediated iron acquisition and ROS resistance by the NADPH oxidase (NOX), the redox activating yes-associated protein 1 (YAP1) regulator, and the high-osmolarity glycerol 1 (HOG1) mitogen-activated protein kinase (MAPK). The *A. alternata* nonribosomal peptide synthetase (NPS6) is essential for the biosynthesis of siderophores, contributing to iron uptake under low-iron conditions. Fungal strains impaired for NOX, YAP1, HOG1 or NPS6 all display increased sensitivity to ROS. Exogenous addition of iron at least partially rescues ROS sensitivity seen for NPS6, YAP1, HOG1, and NOX mutants. Importantly, expression of the NPS6 gene and biosynthesis of siderophores are regulated by NOX, YAP1 and HOG1, supporting a functional link among these regulatory pathways. Although iron fully rescues \( \text{H}_2\text{O}_2 \) sensitivity seen in mutants impaired for the response regulator SKN7, neither expression of NPS6 nor biosynthesis of siderophores is controlled by SKN7. Our results indicate that the acquisition of environmental iron has profound effects on ROS detoxification.

INTRODUCTION

*A. alternata* is a necrotrophic fungal pathogen that kills host cells before invasion and acquires nutrients solely from dead tissues (Akimitsu et al., 2003). Although the early stages of Alternaria brown spot in citrus require the production of host-selective toxin, our previous studies showed that the colonization of *A. alternata* in its host plants relies also on the ability to detoxify reactive oxygen species (ROS) (Lin et al., 2009; Chung, 2012). During the course of host colonization, fungal pathogens of plants often need to overcome an oxidative burst resulting from the production and accumulation of highly toxic ROS (Apel & Hirt, 2004). Indeed, *A. alternata* induces lipid peroxidation, increased accumulation of hydrogen peroxide \( \text{H}_2\text{O}_2 \) and cell death in citrus leaves (Lin et al., 2011). In order to colonize within the oxidative environment of necrotic tissues, *A. alternata* has developed an effective means to cope with ROS toxicity and to thrive in the host plant. The mechanism by which *A. alternata* evades ROS toxicity after penetration has begun to be elucidated. The *A. alternata* YAPI gene, encoding a redox-regulated transcription factor (yes-associated protein 1, YAP1), has been shown to be required for ROS detoxification and fungal pathogenicity in citrus (Lin et al., 2009; Yang et al., 2009). YAP1, analogous with mammalian activator protein 1 (AP-1), has a basic leucine zipper (bZIP) domain and has been shown to be activated by \( \text{H}_2\text{O}_2 \) and various ROS-generating oxidants (Toone & Jones, 1999). Mutational inactivation of the *A. alternata* YAPI gene yielded fungal strains that show elevated sensitivity to oxidizing agents and have lower antioxidant activities compared with wild-type (Lin et al., 2011). YAP1-mediated detoxification of ROS also is a virulence factor in the biotrophic maize pathogen *Ustilago maydis* and the opportunistic human pathogen *Candida albicans* (Enjalbert et al., 2007; Molina & Kahmann, 2007). However, YAP1 plays no virulent role in the plant pathogens *Cochliobolus heterostrophus* and *Botrytis cinerea* and in the animal pathogen *Aspergillus fumigatus* (Lev et al., 2005; Lessing et al., 2007).

The inability of the *A. alternata* Δyapi mutants to induce necrotic lesions on citrus is a consequence of their inability to detoxify ROS (Lin et al., 2009; Yang et al., 2009). Co-application of Δyapi mutants with the NADPH oxidase inhibitor apocynin or diphenylene iodonium partially rescued lesion-producing capability of the mutants. Fungal strains lacking a high-osmolarity glycerol 1 (HOG1)
mitogen-activated protein kinase (MAPK) or a SKN7 regulator also show an increased sensitivity to H₂O₂ and loss of fungal pathogenicity in citrus (Lin & Chung, 2010; Chen et al., 2012; Chung, 2013). The HOG1 MAPK- and SKN7-mediated signalling cascades in fungi play an important role for sensing environmental stimuli, for transmitting these signals to the nucleus to modulate gene expression and for virulence/pathogenicity (Morgan et al., 1997; Li et al., 1998; Gustin et al., 1998; Qi & Elion, 2005). Therefore, we concluded that the ability to detoxify or counteract ROS-mediated plant defence barriers is essential for plant colonization by A. alternata.

A. alternata is capable of producing H₂O₂ through a NADPH oxidase (NOX), which contains two major oxidases, NoxA and NoxB, and one regulatory component NoXR. Both NoxA and NoxB are analogous with mammalian gp91phox and NoxR is analogous with mammalian p67phox (Takemoto et al., 2007). NOX, commonly found in many multi-cellular micro-organisms, is required for cellular differentiation and defence responses (Lambeth, 2004). Genetic analyses revealed that the A. alternata Nox is required for ROS production, cellular resistance to oxidative stress and virulence (Yang & Chung, 2012, 2013). Fungal strains lacking NoxA, NoxB, or NoXR grown on potato dextrose agar (PDA) are hypersensitive to H₂O₂ and many oxidants, even though the levels of sensitivity are less severe compared with those observed in mutants defective at Yap1, Hog1, or Skn7. However, the NoxA NoxB and the NoxA NoXR double-mutant strains display severe hypersensitivity to H₂O₂. Intriguingly, expression of both Yap1 and Hog1 genes was shown to be downregulated in fungi carrying defective NoxA, NoxB or NoXR, implicating a possible link among them. Hence, ROS sensitivity observed for Nox mutants is apparently due to the decreased expression of Yap1 and Hog1. Our recent studies revealed further that deletion of the A. alternata NPS6 gene encoding a nonribosomal peptide synthetase (NPS6) also resulted in increased sensitivity to oxidative stress and decreased virulence to its host plants (Chen et al., 2013). NPS6 is involved in the biosynthesis of dimethyl coprogen siderophores to chelate iron during depleted conditions. The accumulation of the A. alternata NPS6 gene transcript is downregulated in the presence of iron. Compared with Yap1 mutant, Δnps6 mutant of A. alternata is less sensitive to H₂O₂ and superoxide-generating compounds. Mutational inactivation of NPS6 gene homologues also led to increased sensitivity to oxidative stress in various fungi (Lee et al., 2005; Oide et al., 2006). However, the mechanisms underlying oxidative stress resistance, iron homeostasis and fungal pathogenesis remain largely unresolved. In the present study, we provide genetic evidence to establish the direct connection between siderophore-mediated resistance to ROS and reduced virulence of the Δnps6 mutants.

**METHODS**

**Fungal strains and growth conditions.** The wild-type EV-MIL31 strain of Alternaria alternata (Fr.) Keissler was cultured from diseased leaves of Minneola tangelo (Citrus paradisi Macfad. × C. reticulata Blanco) and has been characterized elsewhere (Lin et al., 2009, 2010). The A. alternata mutant strains used in this study and their characteristics are given in Table 1. Fungal strains were cultured on minimal medium (MM), each litre containing 1 mg Ca(NO₃)₂, 4H₂O, 0.2 mg KH₂PO₄, 0.25 mg MgSO₄ .7H₂O, 0.15 mg NaCl and 10 g glucose, at 28 °C. Fungal strains were grown in potato dextrose broth (PDB) for isolation of protoplasts or RNA. Fungi were grown in liquid MM for 5–7 days when protein purification was desired. Sensitivity assays were carried out by transferring agar plugs (2 mm diameter) cut from the colony or by transferring hyphal segments with sterile toothpicks onto MM amended with test compounds. Fungal growth was determined by measuring colony radius. All tests were performed at least twice with three replicates for each treatment.

**Purification and determination of extracellular siderophores.** Fungal strains were grown in 100 ml liquid MM (low-iron) for 7 days. Culture filtrates were separated from fungal masses by vacuum filtration through a pre-weighted filter paper, mixed with FeCl₃ to a final concentration of 1.5 mM, and run through a column packed with Amberlite XAD-16 resin as described by Oide et al. (2006). Iron-binding compounds (siderophores) were eluted with 50 ml methanol. After air-drying, siderophores were dissolved in 1 ml methanol and transferred to a pre-weighed centrifuge tube. Methanol

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Wild-type</td>
<td>Lin et al. (2009)</td>
</tr>
<tr>
<td>Δnps6 (ND2, ND3)</td>
<td>Impaired for NPS6</td>
<td>Chen et al. (2013)</td>
</tr>
<tr>
<td>Δnps6/NPS6 (C12)</td>
<td>NPS6 complementation</td>
<td>Lin et al. (2009)</td>
</tr>
<tr>
<td>Δyap1/YAP1 (YD1, YD2)</td>
<td>Impaired for Yap1</td>
<td>Lin et al. (2009)</td>
</tr>
<tr>
<td>Δnps6</td>
<td>YAP1 complementation</td>
<td></td>
</tr>
<tr>
<td>Δnps6/NOXA (YCP1, YCP2)</td>
<td>Impaired for NOX isoform A (NoxA)</td>
<td>Yang &amp; Chung (2012)</td>
</tr>
<tr>
<td>Δnps6/NOXB (D1, D6)</td>
<td>NoxA complementation</td>
<td>Yang &amp; Chung (2013)</td>
</tr>
<tr>
<td>Δnps6/NOXR (D2, D6)</td>
<td>NoxB double mutant</td>
<td>Chen et al. (2012)</td>
</tr>
<tr>
<td>Δnps6/SKN7 (C33, C87)</td>
<td>SKN7 complementation</td>
<td></td>
</tr>
<tr>
<td>Δhog1 (HD1, HD2)</td>
<td>Impaired for a HOG1 MAPK</td>
<td>Lin &amp; Chung (2010)</td>
</tr>
</tbody>
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was dried and the tube was weighed again to determine the amounts of siderophores. Siderophores were separated by TLC in a solvent system containing methanol/acetic acid/H$_2$O (4:1:5, by vol.) as described previously (Chen et al., 2013).

**Assays for antioxidant enzymic activities.** Fungal strains were grown in liquid MM with or without 0.2 mM FeSO$_4$ for 5 days. Fungal mycelium was harvested by filtering through a layer of Whatman No. 1 filter paper and washed with deionized water. Soluble proteins were purified from fungal mycelium with a buffer containing 10 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM Na$_2$EDTA and 1 % Triton X-100. Protein concentrations were determined using a protein assay kit (Bio-Rad). The integrity and concentration of proteins was examined by fractioning in a 10 % SDS-polyacrylamide gel and staining with Coomassie Brilliant Blue. For enzymic activity, proteins (50 µg) were separated in a 10 % native polyacrylamide gel (minus SDS). To detect catalase activity, gels were immersed in 5 % methanol for 5 min, 0.003 % H$_2$O$_2$ for 10 min, and acrylamide gel (minus SDS). To detect catalase activity, gels were transferred onto positively charged nylon membranes, and hybridized controls). Assays for H$_2$O$_2$ decomposition. Decomposition of H$_2$O$_2$ was determined by measuring a decrease in absorbance at 240 nm (Aebi, 1984). Fungal mycelia cut from agar plugs (2 mm) were immersed in a 2 ml solution containing 20 mM H$_2$O$_2$, with or without 14 mM Fe(III)EDTA and incubated at room temperature (~25 °C). The amounts of H$_2$O$_2$ in the solution were determined spectrophotometrically over time. All treatments also were carried out in water (mock controls).

**Northern blotting.** Fungal RNA was purified with Trizol reagent (Molecular Research Center) and concentrations were determined by spectrophotometry. RNA was denatured in a RNA loading buffer (Li et al., 2003) at 65 °C for 5 min. The integrity and concentration of RNA was examined by denaturing and fractioning in 1.2 % formaldehyde-containing agarose gels and staining with ethidium bromide. For RNA blotting analyses, ~10 µg of total RNA was denatured, electrophoresed, transferred onto positively charged nylon membranes, and hybridized to a DNA probe in a hybridization solution (Roche Applied Science) at 50 °C for 16 h. After hybridization, membranes were washed in 0.1 x SSC and 0.1 % SDS at 50 °C for 1 h. Immunological detection of labelled probe using disodium 3-[4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo (3.3.1.1)dec-an]-4-yl]phenyl phosphate (CSPD) ready-to-use chemiluminescent substrate for alkaline phosphatase was conducted according to the manufacturer’s (Roche Applied Science) recommendations. The molecular probe used for RNA hybridizations was amplified and labelled by PCR to incorporate digoxigenin (DIG)-11-dUTP (Roche Applied Science) into a DNA fragment with two NPS6-specific primers, NP6-2512F (5’-GACTTCCAACATCAGGGA-3’) and NP6-2927R (5’-CTTCCGGATCATCAGGGA-3’).

**RESULTS**

**Siderophore-mediated iron acquisition is required for resistance to ROS**

The *A. alternata* NPS6 gene (AaNPS6) encoding a nonribosomal peptide synthetase has been previously shown to be required for the biosynthesis of dimethyl coprogen siderophores (Chen et al., 2013). Mutational inactivation of the AaNPS6 gene resulted in fungi that, when grown on MM (low-iron), displayed elevated sensitivity to H$_2$O$_2$ and potassium superoxide (KO$_2$) (Fig. 1), suggesting a possible link between iron and cellular resistance to oxidants. The C12 strain expressing the wild-type AaNPS6 gene under control of its endogenous promoter displayed wild-type levels of growth in the presence of H$_2$O$_2$ or KO$_2$. Importantly, exogenous addition of Fe$^{3+}$ in the form of Fe(III)EDTA at 0.2 mM into MM rendered Δnps6 mutants resistant to both H$_2$O$_2$ and KO$_2$ (Fig. 1). On PDA (iron-rich), H$_2$O$_2$ or KO$_2$ had no inhibitory effects on the growth of Δnps6 mutants (data not shown).

**Iron rescues resistance of the Δyap1 mutants to oxidants**

The *A. alternata* strain lacking YAP1 (Δyap1) was highly sensitive to H$_2$O$_2$ (6 mM) and KO$_2$ (20 mM) (Fig. 2). Introduction of a functional copy of YAP1 into a Δyap1

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**Fig. 1.** Siderophore-mediated iron acquisition is required for resistance to ROS. The *A. alternata* NPS6 gene is required for the production of siderophores. Impairment of NPS6 resulted in fungi (ND2 and ND3) that display increased sensitivity to H$_2$O$_2$ and KO$_2$. Exogenous application of Fe$^{3+}$ restores wild-type resistance of Δnps6 mutants to H$_2$O$_2$ and KO$_2$. Reintroduction and expression of a functional NPS6 in the ND2 strain produced a strain (C12) that displays wild-type resistance to oxidants. Fungal strains were grown on MM amended with or without oxidants or Fe(III)EDTA (0.2 mM) for 3 days. Representative replicates are shown.
mutant rescued cellular resistance to H$_2$O$_2$ and KO$_2$. Δyap1 mutants displayed elevated resistance to H$_2$O$_2$ when Fe(III)EDTA (at 0.2 mM), was added to the MM (Fig. 2a). However, KO$_2$ sensitivity observed for Δyap1 mutants was only moderately relieved by the addition of Fe(III)EDTA (Fig. 2b).

Iron protects ROS-sensitive strains against toxicity of H$_2$O$_2$

Similar to the Δyap1 mutant, fungal strains lacking HOG1 MAPK (Δhog1) exhibited increased sensitivity to H$_2$O$_2$ (6 mM). Addition of Fe(III)EDTA slightly enhanced cellular resistance to H$_2$O$_2$ of the Δhog1 mutants (Fig. 3a). Fungal strains lacking the SKN7 response regulator (Δskn7) also displayed increased sensitivity to H$_2$O$_2$. The sensitivity seen for Δskn7 mutants was mitigated considerably by the addition of Fe(III)EDTA or by expressing a wild-type copy of SKN7 (Fig. 3b). The NoxA NoxB and the NoxA NoxR double-mutant strains displayed hypersensitivity to H$_2$O$_2$ (Fig. 3c). However, application of Fe(III)EDTA apparently partially rescued growth of the NoxA NoxB and NoxA NoxR double mutants on H$_2$O$_2$-containing medium.

Regulation of expression of the NPS6 gene by redox-responsive regulators

Northern blotting hybridization of fungal RNA to an AaNPS6-specific probe indicated that accumulation of the AaNPS6 gene transcript decreased markedly in the Δyap1 mutants (YD1 and YD2) lacking the YAP1 gene (Fig. 4a). The YCp1 strain reacquiring and re-expressing a wild-type copy of YAP1 accumulated the AaNPS6 gene transcript to levels comparable to that of wild-type. Similarly, expression of the AaNPS6 gene was downregulated in fungal strains (HD1 and HD2) impaired for HOG1 MAPK (Fig. 4b). The ΔnoxA (A2 and A6) mutants also accumulated lower levels of AaNPS6 transcript than the wild-type (Fig. 4c). The CpA16 strain expressing a wild-type copy of NOXA accumulated AaNPS6 gene transcript levels comparable to that of wild-type. By contrast, the AaNPS6 transcript was detected abundantly in the wild-type, the Δskn7 mutant (D52 and D61), the SKN7 complementation (C33 and C87) strains (Fig. 4d) but not at all in the Δnps6 mutants (Fig. 4e).
Production of dimethyl coprogen siderophores is regulated by redox-responsive regulators

N-dimethyl coprogen siderophores produced by *A. alternata* were purified by passing through a mini-column packed with Amberlite XAD resin and eluted with methanol. Spectrophotometric scanning of the methanol extract from the wild-type and the Δ*hog1* mutant strains revealed a marked difference in the respective absorption spectra (Fig. 5a). The methanol extracts obtained from the cultures of wild-type had a strong absorbance at 290 nm. By contrast, the methanol extracts of the Δ*hog1* mutant had very weak absorbance at 290 nm. The extracts of both wild-type and mutant had a strong absorbance at 240 nm. TLC analysis revealed that accumulation of siderophores decreased drastically in the Δ*hog1* mutant strains (Fig. 5b). Similarly, TLC analysis indicated that deletion of *YAP1* in *A. alternata* nearly abolished siderophore biosynthesis and that its production was rescued in the YCp1 strain expressing the wild-type *YAP1* (Fig. 5c). The Δ*noxAB* and Δ*noxAR* double mutants, defective for a NOX, produced siderophores at levels comparable to the wild-type (Fig. 5d). However, the *A. alternata* Δ*skn7* mutant accumulated siderophores at levels similar to those measured for the wild-type and the complementation strains (Fig. 5e).

Iron impacts antioxidant enzymic activities

The wild-type strain of *A. alternata*, grown in liquid MM, produced barely detectable catalase activities. By contrast, catalase activities were detectable from the wild-type strain cultured in MM amended with 0.2 mM FeSO₄ (Fig. 6 upper panel). The importance of iron for catalase activities

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**Fig. 3.** Iron is required for H₂O₂ resistance in *A. alternata*. (a) Iron partially rescues H₂O₂ resistance of the Δ*hog1* mutant, defective for a HOG1 MAPK. (b) Iron rescues H₂O₂ resistance of Δ*skn7* mutants, defective for a SKN7 response regulator. The C87 strain, regaining a functional copy of SKN7, displays resistance to H₂O₂ regardless of the presence of iron. (c) Iron enhances H₂O₂ resistance of Δ*noxAB* and Δ*noxAR* double mutants, defective for a NOX. Fungal strains were grown on MM amended with or without oxidants or Fe(III)EDTA for 3–5 days. Each column represents mean ± SD radial growth from two independent experiments, with at least three replicates. Asterisks indicate significant differences (Student’s *t*-test; *P* < 0.05).
was also observed in protein samples purified from the \( \Delta \text{nps6} \) mutants and the C12 rescued strain. The addition of iron to the medium also enhanced SOD activity considerably (Fig. 6 middle panel).

**Iron facilitates H\(_2\)O\(_2\) decomposition by \( \text{A. alternata} \)**

Iron can react with superoxide and H\(_2\)O\(_2\) and generate hydroxyl radicals via Fenton chemistry (Halliwell & Gutteridge, 1992). To determine the role of iron in ROS decomposition, the amount of H\(_2\)O\(_2\) in the solution with or without fungal cells was measured over a period of 72 h (Fig. 7). In axenic culture, \( \text{A. alternata} \) was able to degrade H\(_2\)O\(_2\). The addition of iron to fungal culture apparently promoted H\(_2\)O\(_2\) decomposition; decomposition of H\(_2\)O\(_2\) occurred within 60 min. However, in the absence of \( \text{A. alternata} \), H\(_2\)O\(_2\) with added iron decomposed after prolonged incubation (>8 h) suggesting that non-enzymic degradation of H\(_2\)O\(_2\) mediated by iron is a rather slow process.

**DISCUSSION**

The \( \text{A. alternata} \) NPS6 gene, encoding a nonribosomal peptide synthetase, was previously characterized to be required for the biosynthesis of dimethyl coprogen siderophores and as a virulence factor (Chen et al., 2013). The regulation profiles shown here reveal that siderophore biosynthesis is regulated by multiple transcription factors implicated in cellular resistance to ROS. \( \Delta \text{nps6} \) mutant strains display increased sensitivity to H\(_2\)O\(_2\) and other ROS-generating oxidants, suggesting the involvement of NPS6 in resistance to ROS.
However, the mechanism by which siderophore-mediated iron uptake contributes to ROS resistance remains unknown.

In the present study, we provide evidence to define the vital role of siderophore-mediated iron acquisition in antioxidant activities and cellular resistance to oxidative stress (Fig. 8). Under low-iron conditions, the NPS6-catalysed biosynthesis of siderophores may assist the fungus to chelate iron. Iron apparently plays a critical role for antioxidant activities and ROS detoxification. When \( \Delta \text{nps6} \) is impaired, \( A. \text{alternata} \) is likely unable to obtain sufficient iron and displays elevated sensitivity to oxidants at low-iron conditions. However, the wild-type strain of \( A. \text{alternata} \) tolerates ROS under low-iron conditions even though both SOD and catalase activities are barely detectable. It appears that the ROS sensitivity seen for \( \Delta \text{nps6} \) mutants could be only partially attributable to reduced SOD and catalase activities. Iron could be an important cofactor for catalytic activities of antioxidants in addition to SOD and catalase. Iron could also be required for the expression of genes encoding antioxidant enzymes. Lastly, \( A. \text{alternata} \) apparently decomposes \( \text{H}_2\text{O}_2 \) faster in the presence of iron, indicating that iron present in excess could facilitate ROS detoxification via a non-enzymic mechanism.

We also demonstrate that accumulation of the \( \text{NPS6} \) gene transcript and biosynthesis of siderophores is regulated by HOG1, YAP1 and NOXA. Various transcription factors controlling the same target gene in response to environmental changes can result from a variety of mechanisms, including affinity of the binding sites within a regulatory region and association with different co-regulators.

Intriguingly, ROS sensitivity observed in \( \Delta \text{nps6}, \Delta \text{yap1}, \Delta \text{hog1}, \) and \( \Delta \text{noxa} \) could be at least partially mitigated by the addition of \( \text{Fe}^{3+} \) into the medium. However, phenotypic

**Fig. 5.** Production of dimethyl coprogen siderophores is regulated by HOG1, YAP1 and NOXA. (a) Absorption spectrum of dimethyl coprogen siderophores by the wild-type and the strain defective for HOG1, showing that the 290 nm peak is barely detectable in the extracts of the \( \Delta \text{hog1} \) mutant. (b–e) TLC analysis of siderophores produced by the WT and the \( \Delta \text{hog1} \) mutant strains (b), the \( \Delta \text{yap1} \) mutants (YD1 and YD2) and the YAP1-rescued strain (YCp1) (c), the \( \Delta \text{noxa} \) mutants (A2 and A6) and the NoxA-rescued strain (CpA16) (d), and the \( \Delta \text{skn7} \) mutants (D52 and D61) and the SKN7-rescued strains (C33 and C87) (e). Representative replicates are shown.
analyses indicate that NOX, YAP1, SKN7 and HOG1 do not play equivalent roles in siderophore-related iron regulation. We have previously demonstrated that alleviating the toxicity of ROS by *A. alternata* requires YAP1 and HOG1. H$_2$O$_2$ induces increased accumulation of both YAP1 and HOG1 transcripts and increased phosphorylation of HOG1, and facilitates nuclear localization of both YAP1 and HOG1 in *A. alternata* (Lin *et al.*, 2009; Lin & Chung, 2010). This suggests that H$_2$O$_2$ produced by NOX may play a role in signalling and facilitating nuclear localization of YAP1 and HOG1, which activate the genes involved in siderophore biosynthesis for iron acquisition. This is supported further by the fact that siderophore-mediated iron uptake affects SOD and catalase activities. Fungal strains impaired for YAP1 or SKN7 produce relatively lower levels of catalase, SOD, and peroxidases compared with wild-type, even though not all copies of catalase and SOD-coding genes are upregulated by YAP1 (Lin *et al.*, 2009, 2011; Chen *et al.*, 2012). Because NPS6 is expressed primarily under low-iron conditions, *A. alternata* may have other iron-uptake mechanisms besides NPS6-derived siderophores. This pattern of NPS6 transcription is similar to that of the *C. heterostrophus* NPS6 gene (Oide *et al.*, 2006). Under iron-rich conditions, fungi may acquire iron via a reductase-mediated mechanism and/or a direct ferrous influx (Haas *et al.*, 2008).

Δnps6 mutants display elevated sensitivity to ROS-inducing agents under low-iron conditions, whereas Δyap1 or Δhog1 mutants are hypersensitive to ROS when grown on both iron-rich and depleted media. Moreover, iron nearly fully rescues tolerance to H$_2$O$_2$ of the Δyap1 mutant, but has moderate effects for tolerance to KO$_2$ of Δyap1. Iron-dependent

H$_2$O$_2$ + WT

H$_2$O$_2$ + Fe$^{3+}$ + WT

H$_2$O$_2$ + Fe$^{3+}$

H$_2$O$_2$ + WT

Low iron

NPS6

Siderophores

Fe$^{3+}$

SOD, catalase

ROS resistance

Fe$^{3+}$

NOX

H$_2$O$_2$

SKN7 ↔ YAP1

HOG1

Iron-dependent

Iron-independent

Fig. 7. Decomposition of H$_2$O$_2$ by *A. alternata* in the presence or absence of 14 μM Fe(III)EDTA. Agar plugs were prepared from the wild-type strain grown on PDA for 5–7 days. Reactions were carried out in a 20 mM H$_2$O$_2$ solution at room temperature (~25 °C). H$_2$O$_2$ was monitored spectrophotometrically at A$_{240}$ nm over time. The data presented are the mean ± SD of two independent experiments with three replicates.
rescues tolerance to H$_2$O$_2$ of Δhog1 mutants fairly well even though expression of NPS6 and production of siderophores are downregulated in these fungal strains. These observations implicate the existence of an iron-independent ROS-detoxification system that is also regulated by YAP1 and HOG1. It appears that both YAP1 and HOG1 are required for siderophore biosynthesis and for resistance to oxidative stress. However, inactivation of YAP1 does not impact expression of HOG1 and vice versa (Lin & Chung, 2010).

The finding that NOX influences the transcription of NPS6 is less anticipated. NOX is required for the accumulation of intracellular H$_2$O$_2$ in _A. alternata_ because strains mutated in any of the NOX components (NoxA, NoxB and NoxR) accumulate low quantities of H$_2$O$_2$ within hyphae compared with wild-type (Yang & Chung, 2012, 2013). Δnox mutants also are hypersensitive to H$_2$O$_2$ under iron-rich conditions. Under low-iron conditions, sensitivity to ROS of the NOX single mutants is less obvious, occurring only with high concentration of the compounds tested. However, NoxA NoxB and NoxA NoxR double mutants are highly sensitive to H$_2$O$_2$. The involvement of NOX in ROS resistance is at least in part mediated through transcriptional regulation of the genes encoding YAP1 and HOG1.

SKN7 is a putative transcription regulator downstream of the ‘two-component’ histidine kinase- (HSK) mediated signalling pathway (Wurgler-Murphy & Saito, 1997). Mutational inactivation of the SKN7 gene in _A. alternata_ yielded fungal mutants that display increased sensitivity to H$_2$O$_2$ but not to superoxide (Chen et al., 2012). Although iron is able fully to rescue H$_2$O$_2$ sensitivity seen in the Δskn7 null mutants, neither expression of NPS6 nor biosynthesis of siderophores is controlled by SKN7. This suggests that SKN7 regulates other iron-uptake systems instead of siderophores (Fig. 8). _A. alternata_ SKN7 is also required for cellular resistance to sugar-induced osmotic stress via the HSK signalling pathway. In _Saccharomyces cerevisiae_, SKN7 has been demonstrated to interact with YAP1 and other transcriptional regulators associated with heat shock, calcium signalling and the cell cycle (Morgan et al., 1997; Bouquin et al., 1999; Raitt et al., 2000; Williams & Cyert, 2001). However, YAP1-regulated cadmium resistance is independent of SKN7 (Lee et al., 1999).

The requirement of siderophores for iron acquisition and the involvement of iron for tolerance to ROS, and the iron-dependent antioxidant activities suggest a role for iron in _A. alternata_ pathogenesis. At the low- as compared with the high-iron condition, the defect in ROS sensitivity is due to lack of the ability to acquire iron. Not only does iron fully rescue resistance to H$_2$O$_2$ and K$_2$O$_2$ of the Δnps6 mutant of _A. alternata_, but iron is capable of enhancing tolerance to ROS of the Δyap1, ΔnoxA, Δhog1, and Δskn7 mutants to varying degrees. Fungal strains lacking NPS6, YAP1, HOG1, SKN7, or NOX all show increased sensitivity to ROS and reduced virulence in citrus. Thus we conclude that the reduced virulence of the mutants is likely attributable to their inability to deal with toxic ROS in planta (Chung, 2012). Our results support the important role of ROS detoxification during _A. alternata_ pathogenesis in citrus. Further studies will allow us to understand the complex interplay of NOX, YAP1, SKN7 and HOG1 in iron-dependent and -independent gene expression.

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