A nonribosomal peptide synthetase mediates siderophore production and virulence in the citrus fungal pathogen *Alternaria alternata*

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SUMMARY

Alternaria species produce and excrete dimethyl coprogen siderophores to acquire iron. The *Alternaria alternata* gene *AaNPS6*, encoding a polypeptide analogous to fungal nonribosomal peptide synthetases, was found to be required for the production of siderophores and virulence on citrus. Siderophores purified from culture filtrates of the wild-type strain did not induce any phytotoxicity on the leaves of citrus. Fungal strains lacking *AaNPS6* produced little or no detectable extracellular siderophores and displayed an increased sensitivity to H₂O₂, superoxide-generating compounds (KO₂ and menadione) and iron depletion. $\Delta nps6$ mutants were also defective for the production of melanin and conidia. The introduction of a wild-type *AaNPS6* under the control of its endogenous promoter to a $\Delta nps6$ null mutant at least partially restored siderophore production and virulence to citrus, demonstrating a functional link between iron uptake and fungal pathogenesis. Elevated sensitivity to H₂O₂, seen for the $\Delta nps6$ null strain could be relieved by exogenous application of ferric iron. The expression of the *AaNPS6* gene was highly up-regulated under low-iron conditions and apparently controlled by the redox-responsive yeast transcriptional regulator YAP1. Hence, the maintenance of iron homeostasis via siderophore-mediated iron uptake also plays an important role in resistance to toxic reactive oxygen species (ROS). Our results demonstrate further the critical role of ROS detoxification for the pathogenicity of *A. alternata* in citrus.

INTRODUCTION

Iron is a vital element for cell proliferation and survival, because of its role in metabolic functions and electron transfer processes for almost all organisms. Conversely, iron present in excess or as a result of defects in storage can be cytotoxic, because of the production of the highly reactive hydroxyl radical via Fenton chemistry (Halliwell and Gutteridge, 1992). Iron exists in two different

oxidation forms: ferrous (Fe²⁺) and ferric (Fe³⁺). Under aerobic conditions, Fe³⁺ reacts with oxygen, concomitantly reducing to Fe²⁺, to form insoluble ferric hydroxides, which are not available to cells. Fungi, like all free-living organisms, have evolved sophisticated mechanisms to effectively extract iron from their environment (Johnson, 2008). Iron uptake and storage must be carefully regulated to maintain sufficient iron for metabolism and to avoid iron-induced toxicity. Many fungi produce iron-chelating compounds, called siderophores, to acquire ferric iron during iron-depleted conditions (Haas, 2003). Siderophore-bound Fe³⁺ is delivered into the cell via a specific transporter.

There are three main groups of siderophores: aryl caps (catecholates and phenolates), carboxylates and hydroxamates (Haas *et al.*, 2008; Renshaw *et al.*, 2002); the great majority of fungal siderophores identified to date are hydroxamates. Hydroxamate siderophores, which can be divided into four families (rhodotorulic acid, fusarinines, coprogens and ferrichromes), are synthesized from an unconventional amino acid, L-ornithine, catalysed in order by ornithine-N⁵-monooxygenase, transacylase and nonribosomal peptide synthetase (NPS) (Haas, 2003). NPSs are large, multifunctional enzymes that incorporate a wide array of substrates (amino acids, proteins, ornithine and hydroxyl acids) to form linear or cyclic peptides without the involvement of ribosomes. In addition to siderophores, fungal NPSs are responsible for the biosynthesis of nonribosomal peptides with a wide spectrum of biological activities in medicine and agriculture (Kleinkauf and von Doehren, 1996). Fungal toxins, including HC-toxin produced by race 1 of the maize pathogen *Cochliobolus carbonum*, AM-toxin produced by the apple pathotype of *Alternaria alternata* and enniatin produced by *Fusarium* spp., are also the products of NPSs (Haese *et al.*, 1993; Johnson *et al.*, 2000; Panaccione *et al.*, 1992). Filamentous fungi have multiple NPS genes. Disruption of each of the 12 NPS genes in the maize pathogen *Cochliobolus heterostrophus* revealed that only *NPS6* plays a role in fungal virulence (Lee *et al.*, 2005). Further studies revealed that the *C. heterostrophus* *NPS6* gene is required for the biosynthesis of extracellular coprogen siderophores (Oide *et al.*, 2006), providing the first direct evidence for the role of siderophores in the pathogenesis of fungi. Mutational inactivation of the *C. heterostrophus* *NPS6* gene also led to an increased sensitivity to oxidative and low-iron stress (Lee *et al.*,

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2005). *NPS6* is also a virulence determinant in the rice pathogen *Cochliobolus miyabeanus* and in the wheat pathogen *Fusarium graminearum*, implicating a conserved function of extracellular siderophores in fungal virulence (Oide *et al.*, 2006).

Alternaria species are known to produce dimethyl coprogen siderophores to acquire extracellular iron (Jalal *et al.*, 1988; Jalal and van der Helm, 1989; Ohra *et al.*, 1995; Oide *et al.*, 2006). Coprogens contain a diketopiperazine ring (dimerium acid) which is formed by joining two *N*⁵-acyl-*N*⁵-hydroxy-ornithine units via a peptide bond (Jalal *et al.*, 1988; Jalal and van der Helm, 1989). An additional acyl ornithine unit is attached to the ring via an ester bond. *NPS6* is involved in the conjugation of three *N*⁵-acyl-*N*⁵-hydroxy-ornithine units, the immediate precursors of fungal hydroxamate siderophores. Deletion of the *NPS6* homologue in the *Arabidopsis thaliana* pathogen *Alternaria brassicicola* resulted in an elevated sensitivity to H₂O₂ and decreased virulence (Oide *et al.*, 2006).

The tangerine pathotype of *A. alternata* produces a host-selective *A. citri* toxin (ACT), which causes rapid electrolyte leakage from susceptible citrus cells. The production of ACT is absolutely required for *A. alternata* pathogenesis (Miyamoto *et al.*, 2008). Apart from ACT, recent studies have demonstrated that the ability to alleviate reactive oxygen species (ROS) via the yeast transcriptional regulator YAP1, the HOG1 mitogen-activated protein kinase, the SKN7 regulator and the NADPH oxidase complex is required for *A. alternata* pathogenicity/virulence in citrus (Chen *et al.*, 2012; Lin and Chung, 2010; Lin *et al.*, 2009, 2011; Yang and Chung, 2012). Impairment of siderophore synthesis in fungi often results in an elevated sensitivity to oxidative stress (Hof *et al.*, 2009; Oide *et al.*, 2006; Schrettl *et al.*, 2007). To further understand the mechanisms underlying oxidative stress resistance and its role in pathogenesis in citrus, the *A. alternata NPS6* gene (*AaNPS6*), which encodes an apparent orthologue of other fungal *NPS6* siderophore genes, was functionally inactivated by targeted gene disruption. We demonstrated that *AaNPS6* is required for the biosynthesis of dimethyl coprogen siderophores, resistance to oxidative stress and virulence, and that expression of the *AaNPS6* gene is apparently regulated by the redox-responsive regulator YAP1. The results provide further evidence that resistance to oxidative stress in *A. alternata* is necessary for successful colonization of the host plant.

RESULTS

AaNPS6 is involved in conidiation and melanin accumulation

The *A. alternata NPS6* gene contains a 5331-bp intronless open reading frame (ORF) and encodes a 1776-amino-acid polypeptide that shares conserved regions present in *NPS*s of fungi. *AaNPS6* encodes a polypeptide that is highly similar to *A. brassicicola NPS6* (96% similarity and 92% identity) and to *NPS6* of *Cochliobo-*

Fig. 1 (A) Morphology of the *Alternaria alternata* wild-type (WT), *NPS6* disruption (D2 and D3) and genetically complemented (C12) strains grown on potato dextrose agar (PDA). (B, C) Quantitative analysis and comparison of melanin (B) and conidia (C) produced by the *A. alternata* strains. Melanin was purified with 2% NaOH and measured spectrophotometrically for absorbance at 459 nm. Each column represents the mean absorbance or number of conidia $\pm$ standard deviation from two independent experiments, with at least three replicates. Means indicated by the same letter are not significantly different from one another, $P < 0.05$.

lus spp. ($\geq 89\%$ similarity and 83% identity). *AaNPS6* contains one putative adenosine monophosphate (AMP)-binding or adenylation domain (amino acids 210–221), three putative acyl carrier protein (ACP) phosphopantetheine attachment sites or thiolation domains (amino acids 631–646, 1197–1212 and 1268–1283) and two condensation domains (amino acids 712–984 and 1380–1626).

The *AaNPS6* gene was disrupted by transforming two DNA fragments, overlapping within the hygromycin phosphotransferase gene (*HYG*) cassette, into protoplasts of the wild-type strain of *A. alternata* (Fig. S1, see Supporting Information). Polymerase chain reaction (PCR) diagnosis identified two transformants ($\Delta nps6$ -D2 and $\Delta nps6$ -D3) that contain the successful integration of the *HYG* cassette within the *AaNPS6* ORF. Northern blot hybridization of RNA to an *AaNPS6* gene-specific probe detected an expected 5.3-kb transcript from the wild-type, yet failed to identify this transcript from D2 and D3 transformants.

Disruption of *AaNPS6* in the tangerine pathotype of *A. alternata* resulted in mutants producing fluffy, white colonies when grown on potato dextrose agar (PDA) in the light, whereas the wild-type strain produced a dark, pigmented colony (Fig. 1A). *Alternaria alternata* $\Delta nps6$ mutants were pigment deficient, accumulating less melanin than the wild-type (Fig. 1B). Fungal strains (B20, C11

Fig. 2 (A) The chemical structure of the siderophore produced by *Alternaria brassicicola* is a dimethyl coprogen (Oide *et al.*, 2006). (B) Detection of the siderophores produced by the wild-type (WT), *NPS6* disruption (D2 and D3) and complementation (C12) strains of the tangerine pathotype of *A. alternata* grown on agar medium containing chrome azurol S (CAS) for 3 days. The formation of an orange halo around the fungal colony is indicative of siderophore production.

and C12) identified by transformation of $\Delta nps6$ -D2 mutant protoplasts with a functional copy of *AaNPS6* produced wild-type pigmentation (Fig. 1A). Quantitative analyses revealed that $\Delta nps6$ mutants produced significantly fewer conidia than the wild-type under conditions conducive for the formation of conidia. Conidiation was almost fully restored in the complemented strains (Fig. 1C).

AaNPS6 is required for siderophore biosynthesis

Alternaria species produce *N*-dimethyl coprogen siderophores (Fig. 2A). The production of siderophores by the tangerine pathotype of *A. alternata* was evaluated by the growth of fungal strains on medium amended with a chrome azurol S (CAS) dye. The wild-type strain of *A. alternata* produced a distinct orange halo around the fungal colony, which is indicative of the production of siderophores (Fig. 2B). Fungal strains lacking *AaNPS6* failed to generate orange halos, suggesting that *AaNPS6* plays a role in siderophore biosynthesis. The complementation strain induced orange halos on CAS-containing medium (Fig. 2B). Wild-type culture filtrates mixed with FeCl_3 , unlike the *AaNPS6* disruption mutants, displayed red coloration after being passed through Amberlite XAD resins and elution with methanol (Fig. 3A). Culture filtrates obtained from the wild-type, but not $\Delta nps6$ mutants, reacted strongly with iron(III) perchlorate dissolved in perchloric acid (Csaky test), indicating the presence of hydroxamate (Fig. 3B).

Fig. 3 (A) Purification of iron-chelating siderophores from culture filtrates using a column containing Amberlite XAD-16 resins. Siderophores bind to iron, producing red pigmentation after elution with methanol. (B) Quantitative analysis of hydroxamate in the methanol fraction using the Csaky test (Csaky, 1948). (C) Thin-layer chromatography (TLC) analysis of siderophores produced by the tangerine and tomato pathotypes of *Alternaria alternata* and the cabbage pathogen *A. brassicicola*. (D) TLC analysis of siderophores produced by the wild-type (WT), *NPS6* disruption (D2 and D3) and genetically complemented (B20 and C12) strains of the tangerine pathotype of *A. alternata*. Siderophores were purified by Amberlite XAD-16 resins, spotted onto a silica gel plate and separated with methanol-acetic acid- H_2O (4:1:5, v/v).

The methanol extracts obtained from the cultures of the wild-type tangerine pathotype of *A. alternata* resulted in a reddish orange band ($R_f = 0.60$ – 0.62) after thin-layer chromatography (TLC) separation, co-migrating with siderophores purified from culture filtrates of *Alternaria* pathogens of cabbage or tomato (Fig. 3C). Spectrophotometric scanning revealed that the purified pigments displayed a strong absorbance at 290 nm, with a minor peak at 420 nm (data not shown), markedly resembling the siderophores isolated from other *Alternaria* species in a similar manner. *Alternaria* species are known to produce dimethyl coprogen siderophores (Jalal *et al.*, 1988; Jalal and van der Helm, 1989; Ohra *et al.*, 1995; Oide *et al.*, 2006). Coprogen siderophores containing hydroxamate are typically red or orange in coloration and have characteristic absorbance spectra, as described previously, as well as reactivity to iron(III) perchlorate. The tangerine pathotype pigments observed had comparable R_f values on TLC to those of *A. brassicicola* and to the tomato pathotype of *A. alternata*, suggesting that they are, indeed, coprogen siderophores (Fig. 3C, D). Siderophores were barely detectable in culture extracts purified

Fig. 4 Effect of iron and H₂O₂ on the transcription of *NPS6*. Northern blot of fungal RNA purified from the wild-type strain of *Alternaria alternata* grown in minimal medium (MM) amended with or without H₂O₂ and/or FeCl₃ (0.2 mM) or in potato dextrose broth (PDB) amended with or without H₂O₂, and hybridized with the *AaNPS6* gene probe. No extra FeCl₃ was added in PDB. Gels stained with ethidium bromide in the bottom panel indicate the relative loading of the RNA samples. The sizes of hybridization bands are indicated in kilobase pairs (kb).

from two independently isolated $\Delta nps6$ mutants analysed by TLC (Fig. 3D). The introduction of wild-type *AaNPS6* under the control of its endogenous promoter into a $\Delta nps6$ mutant fully restored siderophore production, confirming that *AaNPS6* is required for siderophore biosynthesis (Fig. 3D). A slow-migrating band occasionally seen in some of the samples ($R_f = 0.5$) was probably caused by nonspecific binding or may have formed as a breakdown product of coprogen.

Iron depletion induces the expression of *AaNPS6*

Northern blot analyses revealed that the accumulation of the 5.3-kb *AaNPS6* gene transcript was barely detectable when the fungus was grown in ferrate-containing potato dextrose broth (PDB) (Fig. 4). Regardless of the presence of H₂O₂, the *AaNPS6* gene transcript accumulated to high levels when wild-type *A. alternata* was cultured in minimal medium (MM), but the addition of ferric chloride to MM suppressed its expression considerably.

AaNPS6 plays a role in resistance to hydrogen peroxides

When tested on PDA or MM, the fungal strain defective for the stress-responsive transcriptional regulator YAP1 exhibited an increased sensitivity to H₂O₂, cumyl H₂O₂ (ACROS, Fair Lawn, NJ, USA) and *t*-butyl H₂O₂ relative to its progenitor strain (Fig. S2, see Supporting Information). Unlike the $\Delta yap1$ mutant, $\Delta nps6$ mutants did not exhibit an increased sensitivity to low concentrations of H₂O₂ ($\leq 0.1\%$), cumyl H₂O₂ or *t*-butyl H₂O₂ ($\leq 0.05\%$) on PDA or MM (Fig. S2). When tested on MM (iron depletion conditions), $\Delta nps6$ mutants exhibited an increased sensitivity to H₂O₂ (0.2%), cumyl H₂O₂ (0.1%) and *t*-butyl H₂O₂ (0.1%) at the highest concentrations tested. On PDA, $\Delta nps6$ mutants displayed an increased sensitivity only to 0.1% *t*-butyl H₂O₂. In addition to H₂O₂, $\Delta nps6$

mutants grown on MM were more sensitive than the wild-type and the complemented strains to the superoxide-generating compounds KO₂ (at 20 mM; ACROS) and menadione (MND at 5 mM). Compared with the wild-type, $\Delta nps6$ mutants were more sensitive to the iron chelator bathophenanthroline disulphonic acid (BPTS at 200 μ M), but not to a second iron chelator 2,2'-dipyridyl (2DP at 300 μ M). On MM, the nitric oxide (NO[•])-generating compounds sodium nitroprusside (at 1 mM) and hydroxylamine-HCl (at 5 mM) reduced the radial growth of $\Delta nps6$ mutants by 10%–12% compared with the wild-type and the complemented strains (data not shown). However, $\Delta nps6$ mutants exhibited wild-type tolerance to the singlet oxygen-generating compounds rose bengal (at 50 μ M) and haematoporphyrin (at 0.5 mM).

Exogenous application of iron restores resistance of the $\Delta nps6$ mutants to oxidants

Exogenous application of ferric chloride, ferric sulphate or Fe(III)EDTA (EDTA, ethylenediaminetetraacetate) (each at 0.2 mM) to MM restored the H₂O₂ resistance of $\Delta nps6$ mutants to levels similar to those of the wild-type and the genetically complemented strains (Fig. 5A and data not shown). A fungal strain lacking a YAP1 transcriptional regulator was highly sensitive to H₂O₂ (Fig. S2). Northern blot analyses revealed that the *AaNPS6* gene transcript was present at lower levels in the $\Delta yap1$ null mutants than in the wild-type (Fig. 5B). The fungal strain (YCp1), expressing a copy of wild-type YAP1, accumulated the *AaNPS6* gene transcript at levels similar to those of the wild-type, indicating that the expression of *AaNPS6* is partly regulated by the YAP1 regulator.

AaNPS6 is required for fungal virulence in citrus

The tangerine pathotype of *A. alternata* produces a host-selective toxin ACT. Fungal isolates were grown in culture medium conducive for ACT production to ensure that deletion of *AaNPS6* did not affect the production of secondary metabolites. ACT produced by *A. alternata* strains was tested by the application of culture filtrates directly onto detached Minneola, a hybrid between Duncan grapefruit (*Citrus paradisi* Macfad.) and Dancy tangerine (*C. reticulata* Blanco), leaves. There was no difference in lesion formation among the culture filtrates obtained from the wild-type, $\Delta nps6$ mutant and rescued strains at day 2 (data not shown), suggesting that deletion of *AaNPS6* had no significant effect on ACT production.

The application of siderophores purified from culture filtrates of *A. alternata* onto detached calamondin ($\times$ *Citrofortunella mitis* J. Ingram and H. E. Moore) leaves did not produce any visible lesions (data not shown), indicating no phytotoxicity of siderophores. Pathogenicity assays revealed that $\Delta nps6$ induced fewer necrotic lesions than the wild-type when assessed on detached citrus leaves sprayed uniformly with conidial suspension (Fig. 6A). Statistical analysis using *t*-test ($n = 6$, d.f. = 1, $P < 0.05$) indicated

Fig. 5 (A) Ferric chloride applied at 0.2 mM protects $\Delta nps6$ mutants (D2 and D3) of *Alternaria alternata* from the toxicity of H_2O_2 . The wild-type (WT), $\Delta nps6$ mutant and rescued (C12) strains were grown on minimal medium for 5 days. Each column represents the mean colony diameter $\pm$ standard deviation from two independent experiments with three replicates. Means indicated by the same letter are not significantly different from one another, $P < 0.05$. (B) Northern blot comparing the expression of *NPS6* in the wild-type (WT), *YAP1* disruption strains (YD1 and YD2) and rescued strain (YCp1) after hybridization with an *AaNPS6* DNA probe. Fungal RNA was purified, electrophoresed in a formaldehyde-containing denaturing gel, blotted and washed at high stringency after hybridization with an *AaNPS6* DNA probe. Gels stained with ethidium bromide indicate the relative loading of the RNA samples. The sizes of the hybridization bands are indicated in kilobase pairs (kb).

that the mean area of diseased lesions per leaf induced by $\Delta nps6$ was significantly lower than that of the wild-type (Fig. 6B). Re-introduction of a wild-type *AaNPS6* at least partially restored the virulence of the $\Delta nps6$ mutant to its citrus host.

DISCUSSION

In this study, we characterized an *AaNPS6* gene in the tangerine pathotype of *A. alternata* and provided genetic evidence that *AaNPS6*, encoding a fungal NPS, is involved in the production of siderophore and melanin, iron uptake, oxidative stress resistance, conidiation and virulence. Although the specific structure of the *AaNPS6*-encoded siderophore has not been determined to date, we predicted it to be a dimethyl coprogen siderophore. Like the

dimethyl coprogen siderophores produced by many *Alternaria* spp. (Jalal *et al.*, 1988; Jalal and van der Helm, 1989; Ohra *et al.*, 1995; Oide *et al.*, 2006), the orange/red pigments purified from culture filtrates of the tangerine pathotype of *A. alternata* showed characteristic absorption spectra (290 and 420 nm), reactivity to iron(III) perchlorate and similar R_f values co-migrating with siderophores purified from other *Alternaria* spp. on TLC.

We also showed that the expression of *AaNPS6* is coordinately regulated by the redox-responsive regulator YAP1. The fungal mutant lacking the *YAP1* gene accumulated reduced levels of the *AaNPS6* gene transcript relative to the wild-type and the *YAP1*-complemented strain. The *A. alternata* *YAP1* has been demonstrated to be required for cellular detoxification of toxic ROS (Lin *et al.*, 2009; Yang *et al.*, 2009). Hence, siderophore-mediated iron acquisition plays an important role in ROS detoxification and cellular resistance to oxidative stress.

Alternaria species primarily produce dimethyl coprogen siderophores belonging to the hydroxamate group, and their chemical structures have been determined by 1H and ^{13}C nuclear magnetic resonance and mass spectroscopy (Jalal *et al.*, 1988; Jalal and van der Helm, 1989; Ohra *et al.*, 1995). The *Arabidopsis thaliana* pathogen *A. brassicicola* has also been demonstrated to produce dimethyl coprogen siderophores (Oide *et al.*, 2006). The citrus pathotype of *A. alternata* also produced dimethyl coprogen siderophores, which co-migrate with those purified from *Alternaria* pathogens of cabbage and tomato. Little or no siderophore was detected in culture filtrates prepared from the *A. alternata* mutant strains lacking *AaNPS6*. This phenotype could be restored by introducing a wild-type *AaNPS6* gene into a $\Delta nps6$ mutant, confirming an important role of *AaNPS6* in siderophore biosynthesis. *AaNPS6* was highly expressed in the wild-type strain grown on MM (iron depletion), and its expression was repressed when the fungus was grown in iron-rich medium (e.g. PDB). The results suggest that *NPS6*-mediated siderophore production may assist the fungus to acquire iron under iron-limiting conditions. Because $\Delta nps6$ exhibited similar growth to the wild-type under low-iron conditions, *A. alternata* probably possesses other iron uptake mechanisms in addition to nonribosomal peptide (NRP)-derived siderophores. Some microorganisms are capable of assembling hydroxamate and carboxylate siderophores via NRP-independent mechanisms (Miethke and Marahiel, 2007). Several different mechanisms for iron uptake have also been reported in fungi, including reductase-mediated uptake and direct ferrous uptake (Haas *et al.*, 2008). However, none of the alternative iron uptake systems could fully compensate for the loss of siderophore-associated iron acquisition. $\Delta nps6$ mutants showed increased sensitivity to the iron chelator BPTS, but not 2DP, probably as a result of the different membrane permeabilities of the two iron chelators. Unlike 2DP, which is a membrane-permeable iron chelator, BPTS is unable to pass freely through membranes (Kicic *et al.*, 2001), thus mainly depleting extracellular ions.

Fig. 6 Pathogenicity assays. (A) Necrotic lesions appearing on detached leaves of citrus cv. Minneola inoculated by uniformly spraying with conidial suspension (1×10^4 conidia/mL) prepared from the wild-type (WT), $\Delta nps6$ mutants (D2 and D3) and rescued strain (C12). (B) Disease severity. The inoculated leaves were incubated in a moist chamber for 3 days. The mock controls were treated with water only. The percentage of leaf area containing necrotic lesions was determined on detached Minneola leaves using the software program ImageJ (<http://rsb.info.nih.gov/ij/>). Means indicated by the same letter are not significantly different from one another, $P < 0.01$.

Blocking iron uptake in fungi often reduces conidia formation (Hof *et al.*, 2009; Oide *et al.*, 2006; Schrettl *et al.*, 2007). Siderophore-mediated iron acquisition may also have a role in *A. alternata* development, as $\Delta nps6$ mutants produced significantly fewer conidia and less melanin than the wild-type. We have shown previously that the G-protein and FUS3 and SLT2 mitogen-activated protein kinase-mediated signalling pathways are involved in conidiation in *A. alternata* (Lin *et al.*, 2010; Wang *et al.*, 2010; Yago *et al.*, 2011). However, a recent study has revealed that a cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) represses conidia formation in *A. alternata* (Tsai *et al.*, 2013). It will be of great interest to determine whether these signalling pathways indeed interact with the siderophore-mediated iron uptake leading to conidia

formation. In *A. alternata*, conidia formation is also regulated by NADPH oxidase-mediated ROS production and by a stress-responsive regulator SKN7 (Chen *et al.*, 2012; Yang and Chung, 2012). However, the ROS-responsive regulators YAP1 and HOG1 do not seem to play any role in conidia formation (Lin and Chung, 2010; Lin *et al.*, 2009). It remains to be determined whether the maintenance of redox status has a role in conidia formation.

A fungal strain lacking YAP1 displayed an increased sensitivity to H_2O_2 , cumyl H_2O_2 and *t*-butyl H_2O_2 when tested on both iron-rich and iron-depleted media, and an increased sensitivity to MND and KO_2 when tested on iron-depleted medium. In contrast, the $\Delta nps6$ mutant displayed an elevated sensitivity to ROS-inducing agents primarily under iron-depleted conditions; sensitivity to ROS was

less obvious under iron-rich conditions and was only observed with the highest concentration of *t*-butyl H₂O₂. The ROS sensitivity seen in $\Delta nps6$ could be lessened with the addition of iron to the medium. These results indicate that the bioavailability of iron and siderophore-mediated iron acquisition have a profound impact on ROS resistance. This association, at least in part, is regulated by the redox-responsive transcriptional regulator YAP1. We have shown that the expression of the *AaNPS6* gene was significantly down-regulated in fungal strains lacking *YAP1*. The genetically rescued *YAP1* strain accumulated *AaNPS6* transcript at levels comparable with those seen in the wild-type, implicating a close link between *NPS6*-mediated siderophore synthesis and *YAP1*-mediated ROS resistance.

It remains uncertain whether or not the *A. alternata NPS6* gene plays a general role in the regulation of the biosynthesis of secondary metabolites, even though $\Delta nps6$ mutant strains accumulated lower levels of melanin. *NPSs* are known to be involved in the biosynthesis of a number of fungal metabolites (Eisfeld, 2009). A reduction in melanin biosynthesis by $\Delta nps6$ mutants could be a result of a reduction in iron availability. Some enzymes involved in the biosynthesis of melanin require iron for full activity (Choi *et al.*, 2012; Tangen *et al.*, 2007).

The roles of siderophores in the fungal pathogenicity to plants vary among different pathosystems. The importance of siderophores for fungal pathogenesis was first demonstrated in the maize pathogen *C. heterostrophus* (Lee *et al.*, 2005; Oide *et al.*, 2006). Further investigation by the same groups revealed that siderophore-mediated iron metabolism is required for full virulence in the rice pathogen *C. miyabeanus*, the wheat pathogen *F. graminearum* and the Brassicaceae pathogen *A. brassicicola*. Blocking the synthesis of the ferricrocin siderophore in *Magnaporthe grisea* affects its pathogenicity in rice (Hof *et al.*, 2007). However, siderophore apparently plays no role in the virulence of the basidiomycete maize pathogen *Ustilago maydis* (Mei *et al.*, 1993). Siderophores are also an important virulence determinant of the human pathogen *Aspergillus fumigatus* (Hissen *et al.*, 2005; Schrettel *et al.*, 2007). Our finding indicates that extracellular siderophore is required for full virulence of the tangerine pathotype of *A. alternata*.

In summary, we have shown that *AaNPS6* is a key enzyme for the synthesis of siderophore, which functions to acquire extracellular iron during low-iron conditions in the tangerine pathotype of *A. alternata*. Expression of the *AaNPS6* gene is apparently regulated by the redox-responsive transcriptional regulator YAP1. We have demonstrated that the maintenance of iron homeostasis via siderophore-mediated iron uptake plays an important role in ROS resistance and fungal virulence. The identification of the downstream genes co-ordinately regulated by YAP1 and iron will provide an opportunity to better understand how *A. alternata* remedies oxidative stress and is protected against the deleterious effects of ROS.

EXPERIMENTAL PROCEDURES

Fungal strains and growth conditions

The EV-MIL31 strain of *Alternaria alternata* (Fr.) Keissler, originally single-spore isolated from diseased leaves of Minneola tangelo (*Citrus paradisi* Macfad. $\times$ *C. reticulata* Blanco), was used throughout this study. Fungal mutants, disrupted at the gene encoding a YAP1-like transcriptional regulator, were generated in previous studies (Lin *et al.*, 2009, 2011). An *A. brassicicola* isolate (F0836C) was single-spore isolated from a diseased cabbage leaf; an *A. alternata* isolate (F8838T) was collected from a diseased tomato leaf in Florida. Their identities were confirmed on the basis of ribosomal DNA internal transcribed spacer (ITS) sequences (data not shown). Fungal strains were grown on PDA (Difco, Sparks, MD, USA), MM containing 1 mg Ca(NO₃)₂·4H₂O, 0.2 mg KH₂PO₄, 0.25 mg MgSO₄·7H₂O, 0.15 mg NaCl and 10 g glucose in each liter, or complete medium (CM; MM supplemented with 1 g/L each of yeast extract and casein hydrolysate) at 28 °C. Conidia were collected from fungal cultures grown on PDA under fluorescent light for 4–5 days. Fungal strains were grown in PDB for isolation of protoplasts and ribonucleic acids. For the production of a host-specific ACT, fungal strains were grown in Richard's solution (Kohmoto *et al.*, 1993) for at least 3 weeks. Sensitivity assays were performed by transferring fungal mycelia/conidia as a toothpick point inoculation onto PDA or MM agar medium amended with H₂O₂, MND, KO₂, BPTS, 2DP, rose bengal or haematoporphrin at the appropriate concentrations. Fungal cultures were incubated at room temperature (c. 25 °C) under a constant fluorescent light at an intensity of 40 μ E/m²/s for 3–7 days. Unless otherwise stated, all experiments were repeated at least twice with three replicates. All chemicals used in this work were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in water.

Cloning and sequencing of the *A. alternata NPS6*

The *A. alternata NPS6*-like (*AaNPS6*) gene was cloned from the tangerine pathotype of *A. alternata*. A 3.5-kb DNA fragment was amplified from genomic DNA of *A. alternata* by PCR using the primers Np6-216F (5'-GGCCGACTACGAGCAGTAGTACAGC-3') and Np6-3683R (5'-GAGGCGA GTTCCGAAAGTGATTG-3') which are complementary to a *NPS6* homologue of *A. brassicicola* (Accession No. AB151983). The entire *AaNPS6* ORF and its 5' and 3' untranslated regions were amplified with two inverse *AaNPS6*-specific primers from restriction endonuclease-digested and self-ligated DNA templates, as described previously (Choquer *et al.*, 2005). PCR fragments were purified using a QuickClean 5M PCR Purification Kit (GenScript, Piscataway, NJ, USA) and directly sequenced at Eton Bioscience (Research Triangle Park, NC, USA). The exon/intron positions were determined from comparisons of genomic and cDNA sequences. Double-stranded cDNA of *AaNPS6* was amplified with gene-specific primers from total RNA of *A. alternata* using a SMARTer cDNA Synthesis Kit (BD Clontech, San Jose, CA, USA). Sequence data for *AaNPS6* can be found in the EMBL/GenBank data libraries under accession number JQ973666.

Targeted gene disruption

The *AaNPS6* gene was disrupted and replaced by a *HYG* gene in *A. alternata* using two truncated *AaNPS6::HYG* DNA fragments (Fig. S1). The PCR

fragments overlapping within *HYG* were obtained by PCR, as described previously (You *et al.*, 2009), and were transformed directly into protoplasts prepared from EV-MIL31 strain using CaCl_2 and polyethylene glycol, as described by Chung *et al.* (2002). Fungal transformants were recovered from medium amended with 250 $\mu\text{g}/\text{mL}$ hygromycin (Roche Applied Science, Indianapolis, IN, USA), examined for the integration of *HYG* into *AaNPS6* by PCR using the primers NP6-1011F (5'-CTCAACAACCCGGC GAAGACC-3') and NP6-Gr1 (5'-CTCAACAACCCGGCGAAGACC-3') and further validated by Northern blotting (Fig. S1). Fungal DNA was isolated using a DNeasy Plant Kit (Qiagen, Valencia, CA, USA) and RNA was isolated with Trizol reagent (Molecular Research Center, Cincinnati, OH, USA). An *AaNPS6* probe used for RNA hybridization was amplified and labelled with digoxigenin (DIG)-11-dUTP (Roche Applied Science) by PCR using two *AaNPS6*-specific primers NP6-2512F (5'-GACTTCCAACATCCG AAGCGACG-3') and NP6-2927R (5'-CTTCCGCATCATCAGCAGGGA-3'). The probe was identified by an immunological analysis following the procedures recommended by the manufacturer (Roche Applied Science).

Genetic complementation

The phenotypes seen for a $\Delta nps6$ mutant (D2) were restored by the re-introduction and expression of a functional *AaNPS6* under the control of its endogenous promoter. *AaNPS6* was amplified by a high-fidelity DNA polymerase (Roche Applied Science) with the primers NP6-P3 (5'-CAGACCACACAGCAGCTTGAACGG-3') and NP6-TGA (5'-TCACACAGTCG AAGTTCCAAATGT-3'). The amplified fragment was co-transformed with pCB1532 plasmid, carrying an acetolactate synthase gene (*SUR*) cassette for sulphonylurea resistance (Sweigard *et al.*, 1997), into protoplasts prepared from the *nps6* D2 mutant. Transformants were recovered on medium amended with 5 $\mu\text{g}/\text{mL}$ sulphonylurea (Chem Service, West Chester, PA, USA) and tested for the restoration of siderophore production on an agar plate containing CAS (see below).

CAS assay

The production of extracellular siderophores was evaluated using a CAS agar diffusion assay (Schwyn and Neilands, 1987). CAS binds to an iron-siderophore complex and forms a blue-coloured complex. The formation of an orange halo around the fungal colony (2–3 days) is indicative of the production of siderophores. CAS (60.5 mg; MP Biochemical, Solon, OH, USA) was dissolved in 50 mL of H_2O and mixed with 10 mL of iron solution containing 1 mM FeCl_3 and 6 mM HCl. Hexadecyltrimethyl ammonium bromide (HDTMA) (72.9 mg dissolved in 40 mL of H_2O) was carefully added to the CAS-iron solution with stirring. The solution was autoclaved and mixed with 750 mL of sterile MM prepared in 100 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS) (pH 6.8). Each plate (15 $\times$ 65 mm²) contained 5 mL of CAS agar medium. Fungal mycelium (c. 1 mm²) was placed onto the agar medium and incubated under fluorescent light at room temperature for the development of orange halos.

Production and determination of siderophores

Fungal strains were grown in liquid MM (iron depleted) for 7 days. Culture filtrates were separated from fungal masses by vacuum filtration through filter paper, mixed with FeCl_3 to a final concentration of 1.5 mM and passed through a column packed with Amberlite XAD-16 resin, as described by

Oide *et al.* (2006). Siderophores were eluted with methanol. After methanol was dried, siderophores were dissolved in 1 mL of methanol and analysed by TLC using a 60 F254 silica gel plate (5 $\times$ 20 cm², Selecto Scientific, Suwanee, GA, USA) in a solvent system containing methanol–acetic acid– H_2O (4:1:5, v/v). Siderophore purified from culture filtrates of the cabbage pathogen *A. brassicicola*, in a similar manner, was used as external control because its chemical structure has been determined to be dimethyl coprogen (Oide *et al.*, 2006). A Csaky test was used to confirm the presence of hydroxamate (Csaky, 1948). Culture filtrates (0.5 mL) were mixed with 2.5 mL of iron(III) perchlorate (5 mM) dissolved in 0.1 M perchloric acid and measured spectrophotometrically for absorbance at 480 nm.

Purification and measurement of fungal melanin

Fungal melanin was isolated from 1-week-old cultures with 2% NaOH. Fungal mycelium (c. 100 mg), collected by filtration through a layer of filter paper, was ground with liquid nitrogen, suspended in NaOH solution (1 mL) and boiled at 100 °C for 2 h. The solution was acidified to pH 2.0 with 5 M HCl and centrifuged at 6000 *g* for 15 min. The resulting melanin was dissolved in 2% NaOH, and the solution was measured spectrophotometrically for absorbance at 459 nm (Babitskaya *et al.*, 2000).

Virulence assays

Fungal virulence was evaluated on detached Minneola or calamondin ($\times$ *Citrofortunella mitis* J. Ingram and H.E. Moore) leaves inoculated with conidial suspension (1 $\times$ 10⁴ conidia/mL), as described previously (Lin *et al.*, 2009). Both Minneola and calamondin are highly susceptible to the tangerine pathotype of *A. alternata* (Yago *et al.*, 2011). Minneola produces young leaves primarily in spring; calamondin produces new leaves all year long. Conidial suspension was uniformly sprayed onto detached citrus leaves and the leaves were incubated in a moist chamber for lesion development. Mock control was treated with water only. The toxicity of ACT was determined by a bioassay on detached calamondin ($\times$ *Citrofortunella mitis* J. Ingram and H. E. Moore) leaves. The percentage of diseased lesions on detached Minneola leaves was determined using the ImageJ program (<http://rsb.info.nih.gov/ij/>).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Targeted disruption of *AaNPS6* in *Alternaria alternata* using a split marker approach. (A) Schematic depiction of the generation of truncated, but overlapping, hygromycin phosphotransferase gene (*HYG*) under the control of the *Aspergillus nidulans* *trpC* promoter and gene disruption within *AaNPS6*. Oligonucleotide primers used to amplify each fragment are also indicated. (B) Image of DNA fragments amplified from genomic DNA of fungi with the primers NPS6-1011F and NPS6-Gr1. (C) RNA gel blotting. Gels staining with ethidium bromide for ribosomal RNA (rRNA) indicate relative loading of the RNA samples. Sizes of hybridization bands are indicated in kilobase pairs (kb). WT, wild-type.

Fig. S2 Images of the wild-type (WT), *yap1* strain lacking *YAP1* (ap1), *NPS6* disruption strains (D2 and D3) and genetically complemented strain (B20) of *Alternaria alternata* grown on potato dextrose agar (PDA) or minimal medium (MM) amended with H₂O₂, cumyl H₂O₂, *t*-butyl H₂O₂, menadione (MND), KO₂, bathophenanthroline disulphonic acid (BPTS) or 2,2'-dipyridyl (2DP) for 3–7 days. Experiments were repeated twice with at least three replicates of each treatment. Only some representative replicates are shown. Sensitivity assays were performed by transferring fungal mycelia/conidia as a toothpick point inoculation onto media.