The YAP1 Homolog–Mediated Oxidative Stress Tolerance Is Crucial for Pathogenicity of the Necrotrophic Fungus *Alternaria alternata* in Citrus

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Submitted 26 January 2009. Accepted 23 April 2009.

Citrus brown spot disease is caused by the necrotrophic fungus *Alternaria alternata*. Its pathogenic capability has been thought to depend exclusively on the production of host-selective ACT toxin. However, circumvention of plant defenses is also likely to be important for the disease process. To investigate the fungal response to host-generated reactive oxygen species (ROS), we cloned and characterized the *AaAPI* gene of *A. alternata*, which encodes a polypeptide resembling yeast YAP1-like transcriptional activators implicated in cellular responses to stress. Expression of the *AaAPI* gene in a wild-type strain was primarily induced by H_2O_2 or ROS-generating oxidants. Using a loss-of-function mutation in the *AaAPI* gene, we demonstrated an essential requirement for oxidative tolerance during the host invasion step. Mutants lacking *AaAPI* showed increased sensitivity to H_2O_2 and loss of fungal pathogenicity. The $\Delta AaAPI$ null mutant did not cause any visible necrotic lesions on wounded or unwounded leaves of citrus cv. Minneola. Compared with the wild type, the null mutant displayed lower catalase, peroxidase, and superoxide dismutase activities. All mutant phenotypes were restored to the wild type in fungal strains expressing a functional copy of *AaAPI*. Upon exposure to H_2O_2 , the *AaAPI*::sGFP (synthetic green fluorescent protein) fusion protein became localized in the nucleus. Inoculation of the mutant with NADPH oxidase inhibitors partially restored fungal pathogenicity. Our results highlight the global regulatory role of a YAP1 homolog in response to oxidative stress in *A. alternata* and provide insights into the critical role of ROS detoxification in the pathogenicity of *A. alternata*.

All organisms with an aerobic lifestyle produce harmful reactive oxygen species (ROS), primarily superoxide (O_2^-) and hydrogen peroxide (H_2O_2), as an inevitable consequence of physiological metabolism (Apel and Hirt 2004). Thus, activation of detoxification systems that scavenge ROS, maintenance of reduced redox states within subcellular microenvironments, and repair of ROS-triggered damage are absolutely required to ensure survival of cells under aerobic conditions (Mittler 2002). Among ROS, H_2O_2 is less toxic but stable and able to pass freely through membranes (Branco et al. 2004). Hydro-

gen peroxide can further react with O_2^- via the Haber-Weiss reaction or with metal ions via the Fenton pathway (Imlay 2003) to generate the extremely toxic hydroxyl radical, thus exacerbating the cellular toxicity of H_2O_2 . During plant–microbe interactions, the production of H_2O_2 by a specific plasma membrane NADPH oxidase of plants is termed the hypersensitive reaction (HR), leading to programmed cell death and cellular defense against pathogen attack (Greenberg 1997). When produced in excess, ROS can also serve as secondary messengers in the pathogen-response signal transduction pathway (Neill et al. 2002; Veal et al. 2007). HR plays a critical role in plant defense against saprophytic microorganisms and biotrophic phytopathogens, yet it is less effective against necrotrophic phytopathogens (Glazebrook 2005). Most necrotrophic fungi produce toxins or cell-wall-degrading enzymes that kill host cells prior to invasion and obtain nutrients exclusively from dead tissues (Divon and Fluhr 2007). Indeed, ROS have been proposed to facilitate plant colonization by necrotrophic pathogens such as *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Govrin and Levine 2000).

Alternaria alternata is a ubiquitous necrotroph comprising at least seven pathogenic variants, each producing unique host-selective toxins and causing disease on different host plants (Hatta et al. 2002; Ito et al. 2004; Kohmoto et al. 1991). On citrus, *A. alternata* has two distinct pathotypes. The rough lemon pathotype, producing the host-selective ACRL toxin, is pathogenic only to lemon (*Citrus jambhiri* Lush) and Rangpur lime (*Citrus × limonia* Osbeck). By contrast, the tangerine pathotype of *A. alternata*, which produces the host-selective ACT toxin with a core 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid structure (Kohmoto et al. 1993), causes brown spots on citrus leaves and fruit. ACT toxin causes rapid electrolyte leakage from citrus cells and is highly toxic to tangerine (*Citrus reticulata* Blanco) and grapefruit (*Citrus paradisi* Macfad.), as well as hybrids from grapefruit and tangerine or tangerine and sweet orange (*Citrus sinensis* (L.) Osbeck). ACT toxin does not affect rough lemon or Rangpur lime (Kohmoto et al. 1993). As with many *Alternaria* spp., the genes encoding polypeptides for biosynthesis of ACT toxin likely reside on a dispensable chromosome (Hatta et al. 2002).

The relative roles of ROS in host defense against pathogenic fungi may depend on the lifestyle of the pathogen (Glazebrook 2005). Furthermore, the relative sensitivity of the fungal pathogen to ROS may depend on the effectiveness of its own ROS detoxification machinery. To thrive within the oxidative environment of necrotic tissues, pathogenic fungi have evolved multiple defense systems, both enzymatic and nonenzymatic (Cessna et al. 2000; Mayer et al. 2001; Moye-Rowley 2003).

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In *Saccharomyces cerevisiae*, the YAP1 transcription factor is one of the most important determinants of the yeast's response to chemical stress, responsible for transcriptional activation of genes involved in ROS detoxification as well as drug and heavy metal resistance (Toone et al. 2001). YAP1 is a leucine zipper-containing transcriptional activator belonging to the mammalian AP1 family (Moye-Rowley 2003). YAP1 is localized in the cytoplasm under normal conditions. Upon exposure to oxidative stresses, YAP1 undergoes a conformational change mediated by the formation of disulfide bonds (Wood et al. 2003; Yan et al. 1998) and is transported into the nucleus where it activates the expression of oxidative stress-associated genes (Toone and Jones 1999). The amino terminal and carboxyl terminal cysteine-rich domains are essential for formation of the disulfide bonds, nuclear localization, and transcriptional activation function of YAP1. Mutation of YAP1 gives rise to fungi that are sensitive to a variety of oxidizing agents, drugs, and xenobiotic compounds (Toone and Jones 1999). YAP1-mediated detoxification of ROS has recently been identified as being an essential virulence determinant in the biotrophic maize pathogen *Ustilago maydis* (Molina and Kahmann 2007) and the opportunistic human pathogen *Candida albicans* (Enjalbert et al. 2007). However, YAP1 plays no roles for virulence in the plant pathogen *Cochliobolus heterostrophus* or the animal pathogen *Aspergillus fumigatus* (Lessing et al. 2007; Lev et al. 2005).

The mechanism by which phytopathogenic fungi avoid killing by ROS after invasion remains poorly understood. Pathogenic fungi may rely on various strategies to protect themselves from ROS toxicity and survive in the host plant. The objective of this work was to identify the YAP1 homolog in *A. alternata*, to determine whether the gene is involved in the oxidative stress response, and to evaluate whether fungal antioxidant systems are required for *A. alternata* pathogenicity.

RESULTS

Cloning and characterization of AaAPI.

Two degenerate primers deduced from the conserved N-terminal cysteine-rich domain of YAP1-like proteins were synthesized and used to amplify a 600-bp DNA fragment from the genome of *A. alternata*. Sequence analysis confirmed that the predicted open reading frame (ORF) of the amplified DNA displayed high similarity to numerous API proteins of fungi and yeasts (Supplementary Fig. 1). The gene was designated *A. alternata* API-like gene (*AaAPI*). The entire *AaAPI* ORF and its 5' and 3' nontranslated regions were amplified with two inverse *AaAPI*-specific primers from restriction endonuclease-digested and self-ligated DNA templates. Further analysis and comparison of the assembled sequences from cDNA and genomic DNA revealed that the *AaAPI* gene has a 2,021-bp ORF interrupted by two small introns of 50 and 81 bp. Analysis of sequences 832 bp upstream from the putative ATG translational initiation codon of *AaAPI* identified a putative stress responsive element (STRE: AGGGG) found in genes induced by various stresses such as oxidative damage in yeasts (Marchler et al. 1993), one putative binding site (GATA) for the nitrogen-regulated AreA and the light-induced WC1/WC2 complex, an ambient pH-regulated PacC-binding motif (GCCAGG), and three cAMP-induced C/EBP-DNA binding motifs (CAAT or CCAAT).

The predicted *AaAPI* polypeptide contains 629 amino acids and exhibits substantial similarity (44 to 87%) and identity (33 to 81%) to a number of API homologs and hypothetical proteins identified in fungi and yeasts; *AaAPI* is most similar to the API-like proteins of *Pyrenophora tritici-repentis* (XP_001931984) and *Cochliobolus heterostrophus* (AAS64313).

Analysis of the predicted polypeptide of *AaAPI* identified several conserved domains of YAP1 orthologs (Fig. 1A): a basic leucine zipper (bZIP) DNA-binding domain and a nuclear localization domain (amino acids 161 to 224), an N-terminal cysteine-rich domain (n-CRD; amino acids 387 to 429), and a car-

Fig. 1. Functional domains of *AaAPI* identified in the tangerine pathotype of *Alternaria alternata*. **A**, Physical map of *AaAPI* showing conserved functional domains: a basic leucine zipper (bZIP), a cysteine-rich N-terminal domain (n-CRD), a carboxyl-terminal domain (c-CRD), and nuclear export sequence (NES). **B**, Alignment of the bZIP domain of *AaAPI* from *A. alternata* with those identified in other phytopathogenic fungi. Identical (asterisks), close similar (colons), and more distant similar (periods) amino acid residues are indicated. The conserved bZIP domains are shaded. **C**, Phylogenetic relationships of *AaAPI*, inferred from the conserved amino acids in the bZIP domain, in yeast and fungal AP-1 families: *Cochliobolus heterostrophus* Chap1 (AAS64313), *Pyrenophora tritici-repentis* PtAPI (XP_001931984), *Phaeosphaeria nodorum* Nod (EAT86127), *Aspergillus oryzae* AorAPI (BAE92562), *A. fumigatus* AfAPI (EAL88844), *Neosartorya fischeri* NF1A (EAW16217), *Sclerotinia sclerotiorum* SSAPI (EDN93694), *A. nidulans* AnAPI (EAA62093), *A. niger* AnigAPI (CAK45991), *A. clavatus* ACLA (EAW06606), *Botryotinia fuckeliana* BC1G (EDN20443), *A. terreus* ATEG (EAU33261), *Coccidioides immitis* CIMG (EAS36028), *Afollomyces capsulatus* HEAG (EDN09983), *Gibberella zeae* GzAPI (XP_388976), *Podospora anserina* PaAPI (CAP66611), *Yarrowia lipolytica* YLAPI (CAG77752), *Magnaporthe grisea* MgAPI (EDK00544), *Chaetomium globosum* CHGG (EAQ85132), *Neurospora crassa* NCap1 (CAB91681), *Kluyveromyces fragilis* KLULA (AAC39320), *Pichia guilliermondii* PGUG (EDK36566), *Pichia jadinii* CUYAPI (BAE48266), *Schizosaccharomyces pombe* SpYAP1 (CAB66170), *Saccharomyces cerevisiae* ScYAP1 (CAA41536), *Candida glabrata* CaGLOP (CAG59929), and *C. albicans* CALB1 (EAK94712). The phylogram was created with program PHYLP (Saitou and Nei 1987). Each of the branch lengths are proportional to the number of differences in amino acids compared. The horizontal bar indicates the relative distance in the phylogenetic tree.

boxyl-terminal cysteine-rich domain (c-CRD; amino acids 572 to 605). The strongest similarity was identified in the bZIP, n-CRD, and c-CRD domains (Fig. 1B). A putative nuclear export sequence (NES) within the c-CRD (amino acids 564 to 577) is a possible binding site for the Crm1p-like exporter (Yan et al. 1998). Three bipartite nuclear localization signals (NLS) are present between amino acids 142 to 156, 152 to 169, and 166 to 183. AaAP1 contains regions that are rich in aspartic acid (within amino acids 93 to 137), serine (amino acids 336 to 386), and asparagine (amino acids 458 to 467). Phylogenetic relationships of AaAP1 to other fungal and yeast AP1 proteins, inferred from the conserved bZIP domain, revealed that AP1-like proteins are diverse among filamentous fungi yet apparently separated from yeast AP1 orthologs (Fig. 1C).

Targeted disruption of *AaAP1*.

A targeted gene-disruption approach (Fig. 2A) was used to determine the function of the *AaAP1* gene in *A. alternata*. Two

Fig. 2. Targeted disruption of *AaAP1* in *Alternaria alternata*. **A**, Schematic illustration of a split-marker strategy for disruption of *AaAP1* (indicated by shaded columns) by inserting a hygromycin phosphotransferase B gene (*HYG*) under control by the *Aspergillus nidulans* *trpC* promoter. **B**, Autographic image of a DNA gel blot of *SpeI* genomic digests of *Alternaria alternata* wild type (WT) and $\Delta AaAP1$ deletion strains D1 and D2 hybridized with a digoxigenin (DIG)-labeled *AaAP1* fragment as indicated in A. Hybridizing patterns indicate successful disruption at the *AaAP1* locus. **C**, Image of an RNA gel blot, probed with a DIG-labeled *AaAP1*, showing a 2-kb *AaAP1* transcript from the wild type but not from two $\Delta AaAP1$ deletion strains, D1 and D2. Ribosomal RNA stained with ethidium bromide gives relative loading of the samples. **D**, Southern blot hybridization of *SpeI*-digested genomic DNA isolated from the wild type, transformants (T3 to T8) displaying resistance to both hygromycin and H_2O_2 , and an *AaAP1*-complemented strain (Cp1) to an *AaAP1* probe.

hybrid DNA fragments containing a split and truncated hygromycin phosphotransferase B gene (*HYG*) marker fused with 5' or 3' *AaAP1* sequences were amplified from the disruption construct, T-yapHyg, and directly transformed into protoplasts prepared from the wild-type strain of *A. alternata*. Fungal transformants were recovered from media containing hygromycin and tested for H_2O_2 sensitivity. Of 35 transformants screened, two exhibited reduced growth in the presence of 0.1% H_2O_2 and were considered putative *AaAP1* disruptants. Southern blot hybridization of *SpeI*-digested wild-type genomic DNA to an *AaAP1* probe identified the expected 2.4-kb hybridizing band (Fig. 2B). However, a 4.0-kb band was detected in DNA of two putative transformants (D1 and D2), resulting from integration of the 1.6-kb *HYG* gene cassette at the *AaAP1* locus. Northern blot hybridization of total RNA prepared from the wild type and the two putative *AaAP1* disruptants revealed that the two transformants were *AaAP1*-null mutants because they failed to accumulate any detectable *AaAP1* transcript (Fig. 2C). Southern blot hybridization of genomic DNA prepared from six transformants that were resistant to both hygromycin and H_2O_2 to an *AaAP1* probe detected the 2.4-kb band of the wild type (Fig. 2D).

AaAP1 contributes to resistance to oxidative stress.

The *AaAP1* null mutant reduced growth on regular potato dextrose agar (PDA) by 30 to 33% compared with the wild type (Fig. 3). Thus, growth reduction greater than 33% was considered to be indicative of hypersensitivity to the compounds tested. Growth of the *AaAP1* mutant was inhibited by 82 to 100% in the presence of H_2O_2 or menadione (a superoxide generator) and by 42 to 55% in the presence of tert-butyl hydroperoxide, sodium dodecyl sulfate (SDS), or potassium superoxide (KO_2 ; 1,2,3-benzotriol [ACROS]) (Fig. 3; Supplementary Fig. 2). However, the *AaAP1* mutant was not sensitive to osmotic and salt stress-related compounds (e.g., sorbitol, mannitol, NaCl, and KCl). Beyond hypersensitivity to H_2O_2 or menadione, the *AaAP1* null mutant retained normal formation and germination of conidia at a rate and magnitude similar to the wild type (data not shown).

The involvement of the *AaAP1* gene in H_2O_2 resistance was further confirmed by genetic complementation. Co-transformation of a copy of the *AaAP1* gene under the control of its native promoter with the pCB1532 plasmid (which carries a sulfonylurea resistance (*SUR*)-selectable marker; discussed below) into protoplasts of the *AaAP1* null mutant (D1) resulted in genetically reverted strains with fully restored H_2O_2 resistance and other phenotypes comparable with those of the wild type (Fig. 3), indicating that *AaAP1* is responsible for resistance to oxidative stress in *A. alternata*. Southern blot hybridization of *SpeI*-digested genomic DNA prepared from the complementation strain Cp1 identified the expected 2.4-kb band, resembling that of the wild type (Fig. 2D). A 4-kb hybridizing band, likely representing the disrupted copy of *AaAP1*, was also detected in the Cp1 genomic DNA.

Expression of *AaAP1* is responsive to oxidative stress.

Northern blot analyses were carried out to assess whether expression of the *AaAP1* gene responds to H_2O_2 and other compounds related to oxidative stress in axenic culture. Accumulation of the 2-kb *AaAP1* gene transcript was barely detectable when the fungus was grown on PDA but was elevated in the presence of H_2O_2 , menadione, and tert-butyl hydroperoxide (Fig. 4A). A similar change in expression of *AaAP1* was also observed following a change of growth medium in which the fungal culture was first grown on PDA for 3 days; shifted to a medium containing H_2O_2 , KO_2 , menadione, tert-butyl hydroperoxide, SDS, rose Bengal, or methylthiazolyl-diphenyl-tetra-

zolium bromide (MTT); and incubated for an additional 24 h prior to RNA purification. The *AaAPI* gene transcript was barely detectable when the fungal culture was replated on the same medium (PDA) but accumulated to high levels when shifted to a stressor-containing medium, primarily in response to H₂O₂, menadione, or tert-butyl hydroperoxide, and to a lesser extent in response to KO₂ (Fig. 4B). Expression of *AaAPI* was not affected by SDS, rose Bengal, or MTT. Accumulation of catalase, glutathione reductase, or glutathione peroxidase coding gene transcript among the wild-type, *AaAPI* disruptant, and genetically complemented strains was similar in the presence or absence of H₂O₂, as assessed by semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) (Supplementary Fig. 3).

Nuclear localization of AaAPI::sGFP upon exposure to H₂O₂.

To investigate whether or not AaAPI protein is localized in the nucleus under oxidative stress conditions, the *AaAPI* gene cassette was translationally fused with a *synthetic green fluo-*

rescent protein (sGFP) gene. To ascertain that the AaAPI::sGFP fusion protein is functioning correctly, we first tested the construct for complementation of the D1 null mutant for H₂O₂ resistance. Only strains displaying H₂O₂ resistance were chosen for microscopic analysis. Expression of *AaAPI*::sGFP under the control of endogenous *AaAPI* promoter in the D1 null mutant restored H₂O₂ resistance and produced bright green fluorescence in the hyphal cytoplasm under unchallenged conditions (Fig. 5). In contrast, upon exposure to H₂O₂, green fluorescence was observed as distinct patches distributed in scattered hyphae exactly resembling those from the 4',6-diamidino-2-phenylindole-stained nuclear DNA, implicating nuclear localization of AaAPI::sGFP in response to oxidative stress (Fig. 5).

Downregulation of oxidative stress-related enzymes in the *AaAPI* null mutant.

We measured the overall activities of catalase, peroxidase, and superoxide dismutase (SOD) produced by the *AaAPI* null mutant and compared the observed activities with those of the wild type. We found a marked reduction in catalase, peroxidase, and SOD activities in the *AaAPI*-disrupted mutant (Fig. 6), suggesting that AaAPI is required for the fungal response to oxidative stress. Indeed, expression of a wild-type *AaAPI* gene in the D1 null mutant restored the activities of the three antioxidant enzymes to near wild-type levels, further supporting the theory that AaAPI plays a profound role for oxidative stress resistance and that *A. alternata* is able to detoxify ROS.

AaAPI plays an essential role during fungal pathogenesis.

Fungal pathogenicity assayed on detached Minneola leaves was evaluated using various inoculation techniques. To determine whether the *AaAPI* gene is required for fungal patho-

Fig. 3. *Alternaria alternata* $\Delta AaAPI$ mutant is hypersensitive to oxidants. Sensitivity of *A. alternata* wild type (WT), $\Delta AaAPI$ deletion strains D1 and D2, and two complementation strains Cp1 and Cp2 was determined by radial growth on potato dextrose agar (PDA) supplemented with oxidants or compounds as indicated. Sensitivity (percentage growth reduction) was calculated as a cumulative percentage of growth of WT and $\Delta AaAPI$ deletion strains grown on the same plate. Transformation and expression of a functional *AaAPI* gene in the D1 mutant resulted in Cp1 and Cp2 strains with restored phenotypes. Only one representative replicate is shown. MTT, methylthiazolyl-diphenyl-tetrazolium bromide; SDS, sodium dodecyl sulfate.

Fig. 4. Accumulation of the *AaAPI* transcript of the *AaAPI* protein in response to oxidative stress in *Alternaria alternata*. **A**, RNA gel blots of wild-type *A. alternata* grown on potato dextrose agar (PDA) with or without H₂O₂, menadione (MND), or tert-butyl hydroperoxide (t-BHP) for 5 days, and hybridized with a digoxigenin-labeled *AaAPI* fragment. Gel stained with ethidium bromide indicates relative loading of the RNA samples. **B**, RNA gel blot of wild-type *A. alternata* grown on PDA with a layer of cellophane for 3 days and shifted to media containing oxidants or chemicals as indicated. The mock treatment contains RNA obtained from fungal mycelia shifted to the nonamended PDA. MTT, methylthiazolyl-diphenyl-tetrazolium bromide; RB, rose Bengal; SDS, sodium dodecyl sulfate.

genicity and lesion development, conidial suspensions prepared from the wild type, the *AaAPI* null mutant, and the genetically reverted Cp1 strain were inoculated onto detached Minneola leaves. Leaves inoculated with the wild-type suspension developed lesions at 1 or 2 days postinoculation (dpi), appearing as small black spots surrounded by yellow halos. Lesions incited by the wild type continuously expanded, merged, and became irregular black patches of necrotic tissue at 4 dpi (Fig. 7A and B). Necrosis also often occurred along the leaf veins, due to translocation of the host-selective toxin produced by the wild type into the vascular system. By contrast, inoculation of the *AaAPI* null mutant using conidial suspension failed to incite necrotic lesions. Occasionally, the *AaAPI* null mutant induced small lesions at 4 dpi (Fig. 7A, indicated by arrows); however, this occurred for only 18% of the total spots inoculated, and such lesions did not expand in size (Fig. 7B). The small lesions were likely caused by limited secretion of the host-selective ACT toxin produced by the *AaAPI* mutant. In separate studies, the *AaAPI* mutant was capable of producing ACT toxin in axenic culture (Ching-Hsuan Lin, *unpublished data*). The *AaAPI* mutant secreted ACT toxin that induced necrosis along the leaf veins on detached Minneola leaves when inoculation was conducted using agar plugs cut from the colony. Pathogenicity assays using conidial suspension revealed that the genetically reverted strains incited necrotic lesions at rates and magnitudes indistinguish-

able from those incited by the wild type. Infection assessed on detached leaves sprayed uniformly with conidial suspension also confirmed that the *AaAPI* mutant is nonpathogenic to Minneola leaves (Fig. 7C). Moreover, the *AaAPI* null mutant was unable to cause necrotic lesions even when the Minneola leaves were wounded prior to inoculation (Fig. 7D).

The *AaAPI* null mutant is impaired in penetration and colonization stages.

Observations by light microscopy revealed that wild-type *A. alternata* induced necrotic lesions at 2 to 3 dpi, resulting in

Fig. 5. Expression of the *AaAPI::sGFP* (synthetic green fluorescent protein) fragment under endogenous *AaAPI* promoter in the D1 null mutant restored H_2O_2 resistance and produced bright green fluorescence in the hyphal cytoplasm under normal conditions. In contrast, upon exposure to H_2O_2 for 60 min, green fluorescence was observed as distinct foci distributed in scattered hyphae, implicating nuclear localization of *AaAPI::sGFP*. 4',6'-diamidino-2-phenylindole (DAPI) fluorescence indicates distribution of nuclei (indicated by arrows). Fungal isolate was grown in liquid potato dextrose broth for 16 h, treated with or without H_2O_2 , and examined by fluorescence microscopy.

Fig. 6. *AaAPI* regulates the production of antioxidant activities in *Alternaria alternata*. **A**, Catalase activities in protein crude extract obtained from *A. alternata* wild type (WT), $\Delta AaAPI$ deletion strains D1 and D2, and two complementation strains Cp1 and Cp2, were assessed by determining the decomposition of H_2O_2 in the presence of the colorimetric reagent purpald, and measured spectrophotometrically at absorbance at 550 nm. **B**, Peroxidase activities were assessed based on the oxidation of pyrogallol in the presence of H_2O_2 to form purpurogallin displaying absorbance at 420 nm. **C**, Superoxide dismutase (SOD) activity was determined by inhibition of the reduction of nitroterazolum blue chloride (NTB). The data presented are the mean and standard deviation of two independent experiments with at least three replicates.

collapse of host tissues in which epidermal layers were disrupted and cellular organelles deformed (Fig. 8A). Germination of conidia and penetration of fungal hyphae occurred within host tissues. By contrast, inoculation with the *AaAPI*-disrupted mutant did not produce necrotic lesions and did not cause degradation of host organelles (Fig. 8B). Conidia of the mutant germinated well on the leaf surface, indistinguishable from those of the wild type. However, the *AaAPI* null mutant apparently failed to penetrate the epidermal layer because fungal hyphae were not seen within host tissues. Inoculation of the *AaAPI* complementation strain (Cp1) produced necrotic lesions and resulted in degradation of host tissues similar to the effects produced by the wild type (data not shown).

NADPH oxidase inhibitors restore pathogenicity of the *AaAPI* null mutant.

Plants produce H_2O_2 and superoxide primarily by plasma membrane-bound NADPH oxidases (Doke et al. 1996). A

pharmacological approach using NADPH oxidase inhibitors was used to determine whether production of H_2O_2 in citrus cells is the primary barrier contributing to the impaired pathogenicity of the *AaAPI* null mutant. We observed that co-application of apocynin (hydroxy-3-methoxyacetophenone) or diphenylene iodonium (DPI), both NADPH oxidase inhibitors, with an *AaAPI* null mutant induced necrotic lesions at 5 dpi, and the lesions continued to expand at 8 dpi (Fig. 9). However, application of apocynin, DPI, or the *AaAPI* mutant alone did not cause any visible lesions on Minneola leaves. The rate of lesion formation induced by the *AaAPI* null mutant was much lower than that of the wild type. The wild-type isolate of *A. alternata* usually incited necrotic lesions on Minneola leaves at 2 dpi; thus, the NADPH oxidase inhibitors only partially restored virulence of the *AaAPI* null mutant.

DISCUSSION

ROS production occurs universally in eukaryotic cells, having roles in cellular defense, cell differentiation, and cell signaling (Aguirre et al. 2005; Apel and Hirt 2004; Mittler 2002; Neill et al. 2002). ROS also are involved in nonhost resistance in

Fig. 7. *AaAPI* is indispensable for pathogenicity of *Alternaria alternata* to citrus cv. Minneola. **A**, Fungal pathogenicity assayed on detached Minneola leaves inoculated with 10 μ l of conidial suspension (10^4 conidia/ml) prepared from *A. alternata* wild type (WT), $\Delta AaAPI$ deletion strains D1 and D2, and two complementation strains Cp1 and Cp2. The inoculated leaves were incubated in a mist chamber for lesion development. Photos were taken 4 days postinoculation (dpi). The mock controls were treated with water only. Inoculation of the $\Delta AaAPI$ deletion strain (D1) occasionally produced pinpoint lesions (indicated by arrowheads). Only some representative replicates are shown. **B**, Quantitative analysis of lesion formation on Minneola leaves inoculated with conidia suspension of *A. alternata* strains. Lesion diameters were determined at 2, 3, and 4 dpi. **C**, Fungal pathogenicity assayed on detached Minneola leaves uniformly sprayed with conidial suspension of *A. alternata* strains. Lesions were recorded at 4 dpi. **D**, Development of necrotic lesions by *A. alternata* WT and $\Delta AaAPI$ deletion strains D1 and D2 on detached Minneola leaves with wounding prior to inoculation.

Fig. 8. Light microscopy of Minneola leaves inoculated with *Alternaria alternata* strains. **A**, Destruction of Minneola leaf tissues inoculated with wild type (WT) at 2 days postinoculation, showing deformed epidermal layers and cellular organelles and fungal infection hyphae (Hp) and conidia (Cn). **B**, Inoculation of the $\Delta AaAPI$ deletion D1 strain onto Minneola leaves shows no signs of tissue deformation and fungal hyphae within the host.

plants (Narusaka et al. 2005). ROS are toxic to virtually all types of biological macromolecules, including fatty acids, proteins or enzymes, sugars, and nucleic acids; thus, excessive exposure to ROS often results in cell death (Daub et al. 2005). However, all organisms have evolved complex mechanisms to protect against ROS-induced damage (Apel and Hirt 2004; Miller and Britigan 1997; Moye-Rowley 2003; Toone and Jones 1999). Nonetheless, only a few phytopathogenic fungi have been investigated in this respect, and the precise role of antioxidant activity in relation to pathogenicity or virulence of fungal species remains controversial (Giesbert et al. 2008; Keon et al. 2007; Lev et al. 2005; Mayer et al. 2001; Molina and Kahmann 2007; Tanaka et al. 2006).

The role of ROS in host resistance and pathogen invasion is highly dependent on the type of plant-microbe interaction (Hückelhoven and Kogel 2003; Mellersh et al. 2002; Shetty et al. 2007; Takemoto et al. 2007) and may also be influenced by the balance between ROS-generating and ROS-detoxifying systems. Such balance can change quickly owing to changes in host physiology and environmental stimuli. Furthermore, the production of ROS by plants may have different effects against pathogens with different lifestyles (biotrophs versus necrotrophs) (Glazebrook 2005; Spoel et al. 2007). For example, disruption of the *CHAP1* homolog of the necrotrophic fungus *Cochliobolus heterostrophus*, which causes southern corn leaf blight, resulted in mutants that are hypersensitive to H_2O_2 but of normal virulence (Lev et al. 2005). Similarly, deletion of the *Aspergillus fumigatus* transcriptional regulator AfYap1 did not affect the pathogenicity of the fungus in an animal model (Lessing et al. 2007). However, recent studies on the biotro-

phic fungal pathogen *U. maydis* showed that the YAP1-controlled H_2O_2 detoxification system plays a role in fungal infection of maize (Molina and Kahmann 2007). Interestingly, the *NPS6* gene encoding a nonribosomal peptide synthase has been demonstrated to be involved in siderophore-mediated iron metabolism, resistance to H_2O_2 , and virulence in several ascomycetous fungi (Lee et al. 2005; Oide et al. 2006). By contrast, ROS acts as a negative regulator in the fungal endophyte *Epichloë festucae*, likely by prohibiting excessive fungal proliferation and, thus, allowing the fungus to maintain a mutualistic relationship with its host plant (ryegrass, *Lolium perenne*) (Takemoto et al. 2006). Mutation of an NADPH oxidase gene, *noxA*, in *E. festucae* yielded fungal strains that are defective in H_2O_2 production yet highly pathogenic to the host plant (Tanaka et al. 2006). By contrast, deletion of the *nox*-like gene in the ergot pathogen *Claviceps purpurea* reduced both conidial formation and pathogenicity (Giesbert et al. 2008). NADPH oxidases have also been genetically demonstrated to be critical for pathogenicity both in the rice blast fungus *Magnaporthe grisea* and in the gray mold fungus *B. cinerea* (Egan et al. 2007; Segmüller et al. 2008).

For *Alternaria alternata*, *B. cinerea*, *Mycosphaerella graminicola* (anamorph: *Septoria tritici*), and many necrotrophic fungi that obtain nutrients primarily from dead cells, it appears that ROS generation or induction of the HR by the host may actually facilitate colonization (Govrin and Levine 2000; Keon et al. 2007). However, the necrotrophic fungus *Sclerotinia sclerotiorum* produces oxalic acid that effectively suppresses the ROS produced by the host plant, thus facilitating fungal penetration and colonization (Cessna et al. 2000). To survive in harsh environments, necrotrophic fungi have evolved sophisticated mechanisms for cellular protection against the toxicity of ROS.

Similar to other homologs of the API family (Moye-Rowley 2003; Toone and Jones 1999), AaAPI contains a conserved leucine zipper (bZIP) domain, a nuclear localization motif, and two regions rich in cysteine residues (n-CRD and c-CRD) that are vital for both cellular localization of YAP1 and YAP1-mediated resistance to oxidative damage (Toone et al. 2001). In yeasts, nuclear localization is a critical step for the function of YAP1 in transcriptional regulation (Coleman et al. 1999). The YAP1 transcription factor, in response to redox or other stress-related stimuli (e.g., UV, cadmium, and cytotoxic drugs), forms disulfide bonds between intracysteine residues (Coleman et al. 1999). Formation of disulfide bonds in YAP1 leads to the change in protein configuration which disguises the nuclear export motif and prevents binding by the nuclear export protein, Crm1P, thus resulting in nuclear location of YAP1 (Wood et al. 2003; Yan et al. 1998). Substitution of two cysteine residues with arginines within the c-CRD region resulted in cytoplasmic localization of YAP1, due to its inability to form disulfide bonds (Kuge et al. 2001). Nuclear localization of the YAP1-like proteins responding to oxidative stress has been well established in yeasts (Alarco and Raymond 1999; Toone et al. 2001) and *Aspergillus fumigatus* (Lessing et al. 2007), as well as in phytopathogenic fungi (Lev et al. 2005; Molina and Kahmann 2008). In view of its conserved domains and cysteine residues, it is likely that AaAPI also fulfills its function by forming disulfide bonds and undergoing appropriate nuclear localization. Indeed, nuclear localization of AaAPI was also observed in *Alternaria alternata* in response to H_2O_2 stress.

AaAPI-disrupted mutants failed to induce necrotic lesions on detached Minneola leaves even though they retain the ability to synthesize ACT toxin. Wounding the leaves prior to inoculation did not facilitate infection and lesion formation. This nonpathogenic phenotype of the *AaAPI* null mutant could be

Restoration of pathogenicity by NADPH oxidase inhibitors

Fig. 9. Application of NADPH oxidase inhibitors restored pathogenicity of the *Alternaria alternata* $\Delta AaAPI$ mutant. Pathogenicity and lesion formation were assessed on detached Minneola leaves inoculated with the $\Delta AaAPI$ mutant, NADPH oxidase inhibitors apocynin (APC), and diphenylene iodonium (DPI). The controls were treated with APC or DPI dissolved in dimethyl sulfoxide.

associated with reduced activities of fungal antioxidants, including catalase, peroxidase, and SOD, and down-regulation of other antioxidant proteins such as laccase and polyphenol oxidase is also likely (Mayer et al. 2001). Northern blot and semiquantitative RT-PCR analyses, however, revealed no differences for the expression of a copy of catalase, glutathione reductase, and glutathione peroxidase coding genes between the wild-type strain, the *AaAP1* disruptant, and the genetically complemented strains in the presence or absence of H_2O_2 . This is likely due to multiple copies of these genes in *A. alternata*. However, it was observed that this nonpathogenic phenotype of the *AaAP1* null mutant is closely associated with reduced activities of fungal antioxidants, including catalase, peroxidase, and SOD. Nonetheless, the inability of the *AaAP1* null mutants to incite necrotic lesions is apparently a consequence of their inability to detoxify ROS. Support for this hypothesis was obtained by chemical inhibition of NADPH oxidase, an ROS generator: co-application of *AaAP1*-disrupted mutants with the inhibitor apocynin or diphenylene iodonium restored lesion-forming capability to the mutants. Thus, in a low-ROS setting, *AaAP1*-disrupted mutants were able to proliferate and exert pathogenicity.

To provide a framework to better understand the interaction between citrus and *A. alternata* pathogenicity, a hypothetical model describing the intertwined regulatory controls of *AaAP1* is proposed (Fig. 10). In this model, the conidia of *A. alternata* germinate on the host plant surface and secrete ACT toxin, which quickly kills host cells. The dead cells coalesce, forming necrotic lesions, from which the fungal pathogen acquires nutrients and proliferates in the host. Degradation of cell organelles followed by nutrient release may be beneficial to the invading pathogen. Translocation of ACT toxin via the vascular system further exacerbates the necrotic lesions. In response to *A. alternata* invasion, citrus cells produce ROS mainly via a plasma membrane-anchored NADPH oxidase. Hydrogen peroxide can also be generated from diverse compartments in plant cells (Fig. 10) and has been proposed to play a crucial role in HR and plant resistance against a diverse range of phytopathogens (Greenberg 1997). As with many fungi (Takemoto et al. 2007), *A. alternata* may contain an NADPH oxidase-coding gene, which likely produces a low level of H_2O_2 during early infection. Cell death induced by ACT toxin may release a considerable amount of H_2O_2 in the vicinity of the infection court, leading to elevated expression of *AaAP1* and nuclear localization of the *AaAP1* transcription factor in *A. alternata*. Subsequently, *AaAP1* activates genes whose products are involved in H_2O_2 detoxification and cellular protection. Numerous genes involved in the detoxification of ROS have been reported to be regulated by YAP1, including those for catalase, peroxidase, SOD, glutathione reductase, glutathione synthase, thioredoxin reductase, and multidrug resistance transporter (Godon et al. 1998; Toone et al. 2001). Overall, our studies reveal that effective detoxification of ROS via an *AaAP1*-mediated pathway is critical for successful colonization of citrus by *A. alternata*. Thus, not only is the host-selective toxin produced by *A. alternata* necessary for fungal pathogenicity but so too is *AaAP1* as a global regulator of genes involved in ROS resistance.

MATERIALS AND METHODS

Fungal strains and culture conditions.

The wild-type EV-MIL31 strain of *A. alternata* (Fr.) Keissl. used in this study as both a recipient host for transformation and in the mutagenesis experiments was single-conidium cultured from diseased leaves of Minneola tangelo, a hybrid between Duncan grapefruit (*Citrus paradisi* Macfad.) and Dancy

tangerine (*Citrus reticulata* Blanco) in Florida. Fungal strains were cultured on PDA (Difco, Detroit) at 28°C. For DNA or RNA purification, fungal strains were grown on agar medium with a layer of sterile cellophane. For preparation of fungal protoplasts, fungal strains were grown in 50 ml of potato dextrose broth (PDB) for 4 to 5 days, blended with 200 ml of freshly prepared PDB, and incubated for an additional 16 h. Conidia were harvested from fungal cultures grown on PDA under a fluorescent light for 3 to 4 days. Fungal transformants were regenerated from RMM medium containing sucrose at 3.42 g/ml as an osmotic reagent as described (Chung et al. 2002). Sensitivity assays to H_2O_2 or other chemicals were conducted by transferring fungal mycelia as a toothpick point inoculation onto PDA containing compounds at appropriate concentrations and incubating the mycelia under constant fluorescent light. Fungal radial growth was measured at 4 days. Unless otherwise indicated, all chemicals used in this study were purchased from Sigma-Aldrich-Fluka.

Cloning of *AaAP1*.

To obtain a YAP1 homolog, two degenerate primers, AP-1F (5'-AARGARAARCAAYTNAARGAY-3') and AP-1R (5'-GGYTCNGGNSWNGTNCC-3'), that are complementary to the conserved N-terminal cysteine-rich domain were synthesized and used for PCR amplification from genomic DNA of the *A. alternata* EV-MIL31 strain using a Go-Taq DNA polymerase (Promega, Madison, WI, U.S.A.). A 0.6-kb DNA fragment was amplified and cloned into a pGEM-T easy vector (Promega) for sequencing from both directions at Eton Bioscience (San Diego, CA, U.S.A.). The similarity of the amplified fragment was determined by searching against the databases at the

Fig. 10. Proposed regulatory interactions between citrus and *Alternaria alternata*. Model for *AaAP1*-mediated regulation and interaction leading to successful colonization and lesion formation in citrus.

National Center for Biotechnology Information using the BLASTX program. The 5'- and 3'-end sequences of the *AaAPI* gene were obtained by sequencing a DNA fragment amplified by PCR with two inverse primers (5'-ccaggagtcgcccacacg-3' and 5'-ccccaggcgctatgacaggca-3') from endonuclease-clipped and self-ligated genomic DNA pools of *A. alternata*. The promoter region was analyzed using regulatory sequence analysis tools. ORF and exon or intron positions were predicted using Softberry gene-finding software and verified by comparisons of genomic and cDNA sequences. Functional domains were predicted according to the PROSITE database using ExPASy or Motif/ProDom and Block programs. Sequence data from this article can be found in the EMBL/GenBank data libraries under accession number FJ376607.

Creation and identification of the *AaAPI* null mutants.

For targeted gene disruption, a 1.7-kb DNA fragment, encompassing the entire *AaAPI* ORF and its 5' and 3' nontranslated region, was amplified with primers yap1DF2 (5'-ggctcagacgagcgacacca-3') and yap1DR2 (5'-ggagaacgctatcagtggtgag-3') and cloned into pGEM-T easy vector to become T-yapDFR2. The *AaAPI* disruption construct, T-yapHyg, was prepared by inserting a 1.6-kb *Bam*HI fragment of the *HYG* cassette under the control of the *Aspergillus nidulans trpC* gene promoter from pUCATPH at the *Nru*I site of T-yapDFR2. Two split *HYG* fragments fused independently with the truncated 5' or 3' end of *AaAPI* were amplified from T-yapHyg to facilitate double crossing-over recombination. A 1.5-kb fragment containing the truncated 5' *AaAPI* and 3' *HYG* was obtained by PCR with primers yap1DF2 and hyg3 (5'-ggatgcctcgtcgaagta-3'); a 2.1-kb fragment containing 3' *AaAPI* and 5' *HYG* was amplified with primers yapDR2 and hyg4 (5'-cgttgcaagaactcctgaa-3') (Fig. 2). PCR fragments were directly transformed into protoplasts prepared from the wild-type EV-MIL31 strain of *Alternaria alternata*, using CaCl₂ and polyethylene glycol as previously described (Chung et al. 2002). The *HYG* gene is not functional unless homologous recombination takes place between the truncated *HYG* DNA fragments. Fungal transformants growing on RMM medium supplemented with hygromycin at 200 µg/ml (Roche Applied Science, Indianapolis, IN, U.S.A.) were picked and tested for H₂O₂ sensitivity.

Genetic complementation of an *AaAPI*-disrupted mutant.

A functional copy of *AaAPI* under control of its endogenous promoter was amplified by a high-fidelity DNA polymerase (Roche Applied Science) using gene-specific primers hypo1 (5'-cgctcagcatatctgtgcgcat-3') and yap-taa (5'-ttatccgaggagcttatcttggg-3'). The amplified 3.8-kb fragment was co-transformed with the pCB1532 plasmid harboring the *Magnaporthe grisea* acetolactate synthase gene cassette that confers sulfonyleurea resistance (Sweigard et al. 1997) into protoplasts prepared from an *AaAPI* null mutant as described (Chung et al. 2002). For GFP expression, the *AaAPI* fragment containing its endogenous promoter was translationally in-frame fused with the sGFP coding gene and co-transformed with pCB1532 into protoplasts prepared from a null mutant. Transformants were selected on RMM medium containing sulfonyleurea (chlorimuron ethyl) (Chem Service, West Chester, PA, U.S.A.) at 5 µg/ml and examined for H₂O₂ sensitivity and for green fluorescence.

Manipulation of nucleic acids.

Plasmid DNA was propagated in *Escherichia coli* DH5α cells and extracted using a Wizard DNA purification kit (Promega). Fungal DNA was purified using a DNeasy plant kit (Qiagen, Valencia, CA, U.S.A.); RNA was obtained with Trizol reagent (Molecular Research Center, Cincinnati, OH, U.S.A.). Double-stranded cDNA of *AaAPI* was amplified with gene-

specific primers from total RNA of *A. alternata* using a SMARTER cDNA synthesis kit (BD Clontech, Palo Alto, CA, U.S.A.) and cloned into pGEM-T easy vector for sequence analysis. Standard procedures were used for digestion of DNA with restriction endonucleases, electrophoresis, DNA ligation, transformation of bacterial cells, Southern and Northern blot hybridizations, and post-hybridization washing. Molecular probes used for DNA and RNA hybridizations were amplified and labeled by PCR to incorporate digoxigenin-11-dUTP (Roche Applied Science) into a DNA fragment with two *AaAPI*-specific primers, yap-31 (5'-ccaggagtcgcccacacg-3') and yap-atg (5'-atggccggaactaccaacgac-3'). Detection of the probe was performed by an immunological assay using CSPD as a chemofluorescent substrate (Roche Applied Science) for alkaline phosphatase.

Enzymatic assays.

Fungal proteins were extracted by grinding fungal mycelia in liquid nitrogen mixed with ice-cold potassium phosphate buffer (250 mM, pH 7.0) and collecting the supernatant after centrifugation at 10,000 × *g* for 15 min at 4°C. The concentration of crude proteins was determined by a protein assay kit (Bio-Rad, Hercules, CA, U.S.A.). The overall catalase activities were determined by measuring the decomposition of H₂O₂ in the presence of a colorimetric reagent, Purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazol), dissolved in 480 mM hydrochloric acid (Johansson and Håkan Borg 1988). The reaction mixture containing 50 µg of crude protein extract (in 100 µl of potassium phosphate buffer), 0.3% H₂O₂ (10 µl), and methanol (50 µl) was incubated at room temperature (25°C) for 20 min, stopped by adding 7.8 M potassium hydroxide (50 µl) (Fisher Scientific, Pittsburgh) and 100 µl of 34.2 mM Purpald solution, incubated for an additional 10 min, and mixed with 50 µl of potassium periodate (65.2 mM dissolved in 470 mM potassium hydroxide). The reaction was centrifuged at 9,500 × *g* for 10 min to remove purple particulates and the supernatant was measured spectrophotometrically with absorbance at 550 nm (A₅₅₀). One unit of catalase is defined as that required to decompose 1 mM H₂O₂ per minute at pH 7.0 at 25°C.

Cellular peroxidase activity was assessed using a method based on the oxidation of pyrogallol (ACROS) in the presence of H₂O₂ to form purpurogallin (2,3,4,6-tetrahydroxy-5H-benzocycloheptene-5-one), showing A₄₂₀ (Abrash et al. 1989). The reaction containing 50 µg of fungal crude protein extract (1 µl), 0.5% H₂O₂, and 5% pyrogallol dissolved in 100 mM potassium phosphate buffer (pH 6.0) was incubated at 25°C for 1 min and measured at A₄₂₀. The regression line was established using horseradish peroxidase (Sigma). One unit of peroxidase is defined by formation of 1 mg of purpurogallin in 20 s at pH 6.0 at 20°C.

SOD activity was determined by inhibition of the reduction of nitrotertrazolium blue chloride to nitroblue tetrazolium (NBT)-diformazan by superoxide radical that is generated by xanthine oxidase during conversion of xanthine to uric acid and H₂O₂ (Giannopolitis and Ries 1977). Enzymatic reaction containing 50 µg of fungal protein extract, 0.75 mM NBT (80 µl), 3 mM xanthine (10 µl), and 4.4 units of xanthine oxidase was measured at A₅₅₀, 30 s after incubation. A standard curve was constructed using pure SOD (Sigma). One unit of SOD inhibits 50% of NBT-diformazan formation under the conditions of the assay. All experiments were carried out two times with at least three replicates.

Determination of fungal pathogenicity.

Evaluation of fungal pathogenicity was carried out on detached Minneola leaves (4 to 6 days after emergence and

approximately 3 to 4 cm in length) inoculated with conidial suspension (1×10^4 conidia/ml). Conidia from fungal isolates grown on PDA at 28°C in the light for 5 to 6 days were collected by flooding with sterile water and low-speed centrifugation ($5,000 \times g$). Conidial suspension was pipetted (10 µl) or sprayed with a minisprayer onto detached *Minneola* leaves and the inoculated leaves were incubated in a mist chamber for 5 to 8 days for lesion development.

Microscopy.

For light microscopy, plant tissues were stained with methylene blue-Azure A at 65°C and examined using a Leitz Laborlux phase-contrast microscope (Leica Microsystems, Exton, PA, U.S.A.). GFP fluorescence was detected using a 450 to 490-nm excitation filter and a 520-nm barrier filter. The images were photographed at 1,024-by-1,024 pixel resolution and exported as TIF files.

ACKNOWLEDGMENTS

We gratefully acknowledge D. Henderson for initial editing. This research was supported by the Florida Agricultural Experiment Station and grants from the Florida Citrus Production Research Advisory Council.

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