A Major Facilitator Superfamily Transporter-Mediated Resistance to Oxidative Stress and Fungicides Requires Yap1, Skn7, and MAP Kinases in the Citrus Fungal Pathogen *Alternaria alternata*

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Abstract

Major Facilitator Superfamily (MFS) transporters play an important role in multidrug resistance in fungi. We report an *AaMFS19* gene encoding a MFS transporter required for cellular resistance to oxidative stress and fungicides in the phytopathogenic fungus *Alternaria alternata*. *AaMFS19*, containing 12 transmembrane domains, displays activity toward a broad range of substrates. Fungal mutants lacking *AaMFS19* display profound hypersensitivities to cumyl hydroperoxide, potassium superoxide, many singlet oxygen-generating compounds (eosin Y, rose Bengal, hematoporphyrin, methylene blue, and cercosporin), and the cell wall biosynthesis inhibitor, Congo red. *AaMFS19* mutants also increase sensitivity to copper ions, clotrimazole, fludioxonil, and kocide fungicides, 2-chloro-5-hydroxypyridine (CHP), and 2,3,5-triiodobenzoic acid (TIBA). *AaMFS19* mutants induce smaller necrotic lesions on leaves of a susceptible citrus cultivar. All observed phenotypes in the mutant are restored by introducing and expressing a wild-type copy of *AaMFS19*. The wild-type strain of *A. alternata* treated with either CHP or TIBA reduces radial growth and formation and germination of conidia, increases hyphal branching, and results in decreased expression of the *AaMFS19* gene. The expression of *AaMFS19* is regulated by the Yap1 transcription activator, the Hog1 and Fus3 mitogen-activated protein (MAP) kinases, the ‘two component’ histidine kinase, and the Skn7 response regulator. Our results demonstrate that *A. alternata* confers resistance to different chemicals via a membrane-bound MFS transporter.

Introduction

Major Facilitator Superfamily (MFS) transporters have been demonstrated to be involved in multidrug resistance in fungi [1,2]. MFS transporters are capable of transporting small
molecules in response to ion gradients or function as drug:H\(^+\) antiporter in microorganisms. Mounting evidence indicates that MFS transporter may also indirectly control membrane potential by changing membrane lipid homeostasis, and regulate internal pH and the stress response machinery in fungi [2]. Many MFS transporters are required for microorganisms to grow under stress conditions. In the budding yeast, MFS transporters containing either a 12- or 14-transmembrane domain have been demonstrated to confer resistance to a wide array of chemicals and drugs [3] and their regulation has been found to be controlled by several stress related transcription factors including Yap1, Msn2, Msn4, and Sfp1 [2]. In phytopathogenic fungi, MFS transporters have been shown to be involved in resistance to toxins and fungicides [4–8].

The tangerine pathotype of *Alternaria alternata* produces a host selective (HS) toxin that kills host cells prior to colonization. The ability to produce the HS toxin is required for *A. alternata* pathogenesis [9]. *Alternaria alternata* infection in citrus leaves triggers rapid lipid peroxidation and accumulation of hydrogen peroxide (H\(_2\)O\(_2\)), which eventually leads to cell death [10]. Experiments have demonstrated that the ability to detoxify toxic reactive oxygen species (ROS) is too required for *A. alternata* pathogenesis [11–17].

*Alternaria alternata* is capable of detoxifying toxic ROS via multiple regulatory pathways. *A. alternata* mutant strains lacking the Yap1 transcription activation, the Hog1 mitogen-activated protein (MAP) kinase, the Skk1 regulator, the Skn7 response regulator, the NADPH oxidase (Nox), or the Gpx3 glutathione peroxidase all displayed hypersensitivity to oxidants and reduced lesion formation on citrus [11,12,14–18]. Exogenous addition of iron partially rescued H\(_2\)O\(_2\) sensitivity seen for Yap1, Hog1, Skn7, and Nox mutants [19], indicating the important role of iron uptake in ROS resistance. This could be attributable to the fact that iron is an important cofactor for antioxidant activities and that iron itself could promote ROS detoxification through a non-enzymatic mechanism. Further studies revealed that the expression of the non-ribosomal peptide synthetase coding gene (*Nps6*) and the production of siderophore were regulated by Yap1, Hog1, and Nox, but not by Skn7 [15]. The ability to synthesize siderophore via the Nps6 and to chelate iron has also been demonstrated to be required for ROS detoxification and fungal pathogenesis. Although H\(_2\)O\(_2\) sensitivity seen in the Skn7 mutant could be resorted by addition of iron, Skn7 apparently could regulate non-siderophore iron acquisition. Experiments have also demonstrated that mutational inactivation of Yap1 or Skn7 resulted in fungal strains that had lower glutathione reductase, catalase, glutathione peroxidase, superoxide dismutase (SOD), glutathione-S-transferase, and ligninolytic peroxidase activities. In addition, the glutathione system played a vital role in ROS detoxification. The expression of the Gpx3 glutathione peroxidase gene has been shown to be coordinately regulated by Yap1, Hog1, and Nox and inactivation of Gpx3 results in decreased sensitivity to oxidants. Taken collectively, it is tempting to speculate that low-level H\(_2\)O\(_2\) generated by Nox may likely act as a signaling molecule to activate transcriptional expression and/or nuclear localization of Yap1, Hog1, Skn7, and perhaps many other regulators, which leads to the further activation of downstream genes under oxidative stress conditions in *A. alternata*.

Yap1 is a leucine zipper-containing transcriptional activator, which has been demonstrated to be responsible for transcriptional activation of genes involved in ROS detoxification as well as drug and heavy metal resistance in fungi [20]. Yap1 has also been known to act as an important regulator of major facilitator superfamily (MFS) gene expression in yeasts [21–24]. In addition to ROS sensitivity, *A. alternata* strains lacking Yap1 or Hog1 were hypersensitive to 2-chloro-5-hydroxyypyridine (CHP) and 2,3,5-triiodobenzoic acid (TIBA). The toxicity of CHP or TIBA to *A. alternata* remains to be determined.

Suppressive subtractive hybridization had identified two genes encoding putative major facilitator superfamily (MFS) transporters that were co-ordinately regulated by the Yap1
transcription regulator in *A. alternata* [10]. In the present study we report a functional characterization of a 12-spanner MFS transporter to explore its roles in resistance to oxidants and fungicides, and in virulence. We also determine the toxic effects of CHP or TIBA to *A. alternata*.

**Results**

**Characterization of an AaMFS19 gene encoding a major facilitator superfamily transporter (MFS)**

The *A. alternata* MFS gene (*AaMFS19*, accession # GS597470) encoding a major facilitator superfamily transporter was originally identified from the wild-type cDNA library after subtraction with that of a *Yap1* mutant. Alignment of *AaMFS19* sequence with other fungal MFS transporter sequences in databases available at the National Center for Biotechnology Information (NCBI) revealed that *AaMFS19* was predicted to contain a 1558-bp open reading frame separated by three small introns (61, 55, and 56 bp) that encodes a polypeptide of 461 amino acids. Hydropathy analysis revealed that *AaMFS19* contains 12 putative transmembrane domains. The *AaMFS19* protein has a predicted molecular mass of 50.2 kDa and a predicted pI of 9.26.

**AaMFS19** is required for tolerance to oxidants, fungicides and xenobiotics

Previous studies have demonstrated that *A. alternata* mutant impaired for the stress-responsive transcription regulator *Yap1* increased hypersensitivity to a wide spectrum of oxidants as well as to both 2,3,5-triiodobenzoic acid (TIBA) and 2-chloro-5-hydroxypyridine (CHP) compared to its progenitor strain [10]. To test whether or not *AaMFS19* plays a role in resistance to oxidants, CHP, and TIBA, *A. alternata* mutant defective for *AaMFS19* was created by targeted gene disruption. Using a split marker gene deletion approach, two *A. alternata* mutants (D27 and D64) defective at *AaMFS19* locus were identified after screening 5 independently selected transformants by PCR (S1 Fig). The *AaMFS19* impaired mutants reduced radial growth by 5 to 8% compared to wild-type cultured on PDA for 3 days. When culturing on PDA in light, both D27 and D64 strains produced ovoid, dark-pigmented conidia with both cross and longitudinal septae similar to those produced by wild-type. Chemical sensitivity assayed on PDA revealed that both the *AaMFS19* deficient mutants (D27 and D64) were more sensitive to CHP, TIBA, clotrimazole, fludioxonil (a phenylpyrrole fungicide), and copper fungicides, CuCl$_2$ (7.5 mM), and CuSO$_4$ (5 mM) than wild-type (Fig 1). Both D27 and D64 also increased sensitivity to cumyl H$_2$O$_2$ and the superoxide-generating compound, potassium superoxide (KO$_2$) (Fig 2). Both mutants also increased sensitivity to various singlet oxygen-generating chemicals, including eosin Y, rose Bengal (RB), hematoporphyrin (HP), methylene blue (MB), and cercosporin (Fig 2). Compared with wild-type, both D27 and D64 mutants were more sensitive to the cell wall perturbing agent, Congo red. In contrast, toluidine blue (TB) promoted radial growth of both D27 and D64 strains. All observed phenotypes were restored in the CP3 strain by transforming the D27 mutant protoplasts with a wild-type copy of *AaMFS19*. Both D27 and D64 displayed wild-type sensitivity to H$_2$O$_2$ (0.05%), diamide (a thiol-oxidizing agent; 2.5 mM), menadione (a superoxide-generating compound; 2 mM), and tert-butyl hydroperoxide (0.05%) (S2 Fig).

**Expression of AaMFS19 is suppressed by xenobiotics**

Northern blot hybridization revealed that expression of *AaMFS19* was down-regulated in the wild-type strain grown on PDA amended with TIBA, CHP, vinclozolin (a dicarboximide fungicide), clotrimazole, or H$_2$O$_2$ for 3 days (Fig 3A). When wild-type was cultured on PDA for
2 days, shifted to medium amended with TIBA, CHP, vinclozolin, or clotrimazole, and incubated for an additional 24 h, expression of AaMFS19 was down-regulated as examined by semi-quantitative RT-PCR (Fig 3B). Expression of both actin- and β-tubulin-coding genes was also decreased in the wild-type shifted to medium containing CHP, vinclozolin, or clotrimazole. Shifting to a TIBA-containing medium reduced expression of the β-tubulin-coding gene but did not significantly change the transcript levels of the actin-coding gene in the wild-type. However, the transcript levels of the GPx3 gene encoding a glutathione peroxidase in the wild-type were not affected by test chemicals.

Expression of AaMFS19 is regulated by stress-responsive regulators, MAP kinases and histidine kinase

Northern blot hybridization revealed further that the accumulation of the AaMFS19 transcript was decreased in fungal strains defective for the stress-responsive transcription regulator Yap1 (Fig 4A), consistent with previous findings [10]. The AaMFS19 transcript was detected at similar levels in the wild-type and the Yap1 complementation strains. Inactivation of a Hog1 MAP...
kinase-coding gene also resulted in a decrease of the AaMFS19 transcript (Fig 4B). Fungal strains inactivated at a "two-component" histidine kinase (Hsk1)-coding gene accumulated much lower levels of the AaMFS19 transcript than wild-type. Similar reduction of the AaMFS19 transcript was also observed in fungal strains impaired for a Fus3 MAP kinase or a Skn7 response regulator. However, mutation at a Slt2 MAP kinase did not affect the expression of AaMFS19.

AaMFS19 plays a role in fungal virulence

Pathogenicity tests assayed on detached calamondin leaves using a point inoculation (10 μl, 10^5 conidia/ml) method revealed no significant differences in lesions induced by the wild-type, the AaMFS19 mutant, and the CP3 complementation strains (data not shown). However, pathogenicity assessed further on detached citrus leaves sprayed uniformly with conidial suspension (10^5 conidia/ml) revealed a reduction of necrotic lesions induced by the AaMFS19 mutants, as compared to those induced by the wild-type and the Cp3 strains (Fig 5).
Pleiotropic effects of TIBA and CHP on *A. alternata*

As described above, *A. alternata* impaired at *AaMFS19* increased sensitivity to both 2-chloro-5-hydroxypyridine (CHP), 2,3,5-triiodobenzoic acid (TIBA), vinclozolin (Vin), clotrimazole, and $H_2O_2$. The wild-type strain was grown on a layer of cellophane overlaid on PDA for 3 days and shifted to PDA amended with chemicals as indicated for an additional 24 h. Fungal RNA was purified, electrophoresed on a formaldehyde-containing gel, blotted to membrane, and hybridized with an *AaMFS19*-specific probe. Ethidium bromide (EtBr)-stained rRNA is shown to ensure equal loading of the samples. **B** Semi-quantitative reverse transcription PCR analysis of *AaMFS19*, the actin and β-tublin-coding genes, and the *GPx3* gene encoding a glutathione peroxidase. PCR products were visualized on 1.2% agarose gel staining with EtBr.

Pleiotropic effects of TIBA and CHP on *A. alternata*

As described above, *A. alternata* impaired at *AaMFS19* increased sensitivity to both 2-chloro-5-hydroxypyridine (CHP, 3 mM) and 2,3,5-triiodobenzoic acid (TIBA, 5 mM) compared to wild-type. The toxicity of CHP and TIBA to *A. alternata* remains elusive. The wild-type strain of *A. alternata* displayed sensitivity to both compounds in a dosage-dependent manner (**Fig 6A** and **6B**). The effective concentrations of TIBA and CHP resulting in 50% inhibition ($IC_{50}$) of *A. alternata* were around 3.2 and 2.6 mM, respectively. Treatment of *A. alternata* with CHP or TIBA resulted in a marked reduction in the production and germination of conidia (**Fig 6C** and **6D**). Treatment of *A. alternata* with CHP or TIBA also resulted in an increase of hyphal branching (**Fig 7**).
Discussion

AaMFS19 encoding a MFS transporter was first identified from a cDNA library prepared from the wild-type strain of *A. alternata* after subtraction with cDNA from a Yap1 mutant [10]. In the present study, AaMFS19 containing 12 transmembrane helices has been shown to be required for cellular resistance to cumyl H$_2$O$_2$, KO$_2$, and many singlet oxygen-generating compounds including eosin Y, rose Bengal, hematoporphyrin, methylene blue, and cercosporin [25]. Although toluidine blue (TB) has also been known to generate singlet oxygen [26], the compound promoted radial growth of the AaMFS19 mutant strains (D27 and D64). Furthermore, accumulation of the AaMFS19 gene transcript was shown to be regulated by the stress-responsive regulators Yap1, Hog1, and Skn7, which confirms further the involvement of AaMFS19 in resistance to ROS-producing compounds. The results also indicate that ROS resistance in *A. alternata* is, at least in part, mediated by membrane-bound transporters. Despite the fact that the function of AaMFS19 is associated with ROS resistance, it seems unlikely that AaMFS19 is specifically functioning at ROS, as it is not required for resistance to H$_2$O$_2$ or other ROS-generating compounds such as diamide, menadione,
tert-butyl hydroperoxide, and toluidine blue. In the budding yeast Saccharomyces cerevisiae, the involvement of MFS transporters in multidrug resistance and oxidative stress response, likely either through metabolic regulation or change of the plasma membrane compositions, has also been observed [27–29]. The expression of many MFS transporter coding genes has been found to be regulated by oxidative stress-related transcription factors including Yap1, Msn2, Msn4, and Sfp1 [2].

It appears that AaMFS19 is required for resistance to coppers (CuCl$_2$ and CuSO$_4$) and copper fungicides, indicating that AaMFS19 could serve as a copper exporter. Experimental results also have shown that fungal strains with AaMFS19 deficiency increase sensitivity to clotrimazole and fludioxonil fungicides. The diversity of AaMFS19 function in cellular tolerance to fungicides, oxidants, and xenobiotics indicates the important role of active efflux systems in multidrug resistance. The results also show that AaMFS19 has a general function capable of exporting structurally diverse chemicals. Fungi have many membrane-bound transporters, all

![Fig 5. Virulence assays on detached calamondin leaves. Conidial suspension (10$^4$ conidia/ml) collected from the wild type, the strains lacking AaMFS19 (D27 and D63), and the CP3 strain expressing a copy of AaMFS19 were sprayed uniformly onto detached calamondin leaves. The mock controls were treated with water only. The leaves were incubated in a moisture chamber for lesion development. Photos were taken 2 days post inoculation (dpi). Experiment was repeated three times with at least 10 leaves. Only representative replicates are shown.](doi:10.1371/journal.pone.0169103.g005)
of which could function synergistically against toxic chemicals [2,30,31]. Thus, it is not surprising that AaMFS19 plays a moderate role in multidrug resistance.

Pyridine (Fig 6A) composed of an aromatic ring with five carbons and one nitrogen atom can be found in many natural products and could react with H$_2$O$_2$ to form superoxide and hydroxyl radicals in the presence of Cu$^{2+}$ [32,33]. Pyridine and its derivatives are essential parts of RNA, DNA, NADP/NADPH, flavin nucleotides (FAD/FADH), ATP, and GTP in the biological systems. The compound 2,3,5-triiodobenzoic acid (Fig 6B) is an inhibitor of indole-3-acetic acid (IAA) transportation and often used as an herbicide [34,35]. However, their toxicity toward fungal pathogens has never been determined. In the present study, we have shown that TIBA and CHP are toxic to the citrus fungal pathogen Alternaria alternata by suppressing radial growth and by reducing conidial formation and germination. Wild-type strain treated with either CHP or TIBA displayed an abnormal formation of hyphae, frequently producing short branches. Both CHP and TIBA also interfere with the expression of the AaMFS19 gene in A. alternata, even though AaMFS19 plays a role in CHP and TIBA resistance. CHP and, to a lesser extent, TIBA also impact the expression of actin and β-tubulin coding genes. Similar transcriptional inhibition is also observed in A. alternata treated with fludioxonil, vinclozolin, and clotrimazole fungicides as well as with H$_2$O$_2$. However, CHP, TIBA, and fungicides have no effects on the expression of Gpx3 encoding a glutathione peroxidase, indicating that CHP and TIBA selectively suppress gene regulation.

Mutational inactivation of AaMFS19 resulted in two fungal strains that increase sensitivity to CHP and TIBA. Expressing a functional copy of AaMFS19 in one of the mutants restored
wild-type levels of resistance, confirming the involvement of AaMFS19 in cellular resistance to CHP and TIBA. However, AaMFS19 is apparently unable to fully protect A. alternata from the toxicity of CHP and TIBA. AaMFS19 likely acts to efflux CHP and TIBA from the fungal cells and prevent an excessive accumulation of these toxic chemicals. It is also likely that in addition to multiple drug and stress resistance, AaMFS19 could play an indirect role in stress resistance. A correlation between multidrug resistance and membrane lipid homeostasis has been established in fungi [36,37]. Nevertheless, AaMFS19 is able to permit normal grow at lower concentrations of chemicals. Alternaria alternata mutant impaired for Yap1 increased hypersensitivity to TIBA and CHP [10] and thus Yap1-mediated resistance to these compounds is likely operated through activation of AaMFS19.

In addition to Yap1, A. alternata strains impaired for Hsk1, Skn7, Hog1, or Fus3 increased sensitivity to TIBA and CHP [12,15,38] and decreased the expression of AaMFS19. The results suggest that Hsk1, Skn7, Hog1, and Fus3-mediated signaling pathways conferring TIBA and CHP resistance are closely associated with the function of AaMFS19. However, increased cellular sensitivity to fludioxonil fungicide seen in the AaMFS19 mutants is unlikely mediated by Hsk1, Skn7, and Hog1, because mutation of Hsk1, Skn7, or Hog1 results in fungi that are resistant to fludioxonil [12,15]. As with CHP and TIBA, fludioxonil, vinclozolin, and clotrimazole fungicides suppress the expression of genes encoding AaMFS19, actin, and β-tubulin. In addition, Hsk1, Skn7, and Hog1 play an important role in osmotic stress induced by salts and sugars while AaMFS19 plays no role at all (Pei-Ling Yu, unpublished). Increased sensitivity to copper fungicides seen in the AaMFS19 mutants is not regulated by Fus3 because inactivation of Fus3 leads to resistance to copper fungicides in A. alternata [38]. The expression of
AaMFS19 is apparently differentially regulated in response to different environmental stimuli. Our results demonstrate that AaMFS19 plays a complex role in physiological and pathological functions.

Pathogenicity tests reveal that AaMFS19 plays a role in fungal virulence because gene deletion mutants of AaMFS19 are reduced in their ability to induce necrotic lesions on detached calamondin leaves after uniformly spraying with conidial suspensions. The results suggest an important biological role of AaMFS19 during pathogenesis in planta. Whether or not AaMFS19 plays a role in resistance to natural plant toxins awaits to be determined. Lastly, because the AaMFS19 mutant strains also increase sensitivity to many ROS-generating compounds, the results derived from the present study confirm previous finding that the ability to deal with ROS plays an important role on A. alternata pathogenesis in citrus.

Material and Methods

Fungal strains and growth conditions

The wild-type strain (EV-MIL31) of A. alternata was cultured from diseased leaves of Minneola tangelo and has been previously characterized [14,18]. Fungal strains defective for a Yap1 regulator (Δyap1), a Skn7 response regulator (Δskn7), a high osmolarity glycerol (Δhog1) mitogen-activated protein kinase (MAPK), a cell wall integrity MAPK (Δslt2), a Fus3 MAPK (Δfus3), and a “two component” histidine kinase (Δhsk1) were generated from the EV-MIL31 strain in previous studies [11,12,15,38,39]. YCp1 strain was previously created by expressing a functional copy of Yap1 in a Δyap1 mutant [11]. Fungal strains were cultured on potato dextrose agar (PDA; Difco, Sparks, MD) at 28˚C with constant fluorescent light. For DNA and RNA purification, Alternaria strains were cultured on PDA covered with a sterile cellophane membrane. For protoplast isolation, fungi were cultured in potato dextrose broth (PDB) on a shaker for 3 to 4 days. Fungal transformants were recovered from a regeneration medium [40] amended with hygromycin (250 μg/ml, Roche Applied Science, Indianapolis, IN) or sulfonylurea (5 μg/ml, chlorimuron ethyl; Chem Service, West Chester, PA).

Sensitivity tests

Chemical sensitivity was assayed on PDA plates supplemented with a test compound. Fungal hyphae and conidia from 5 to 7 day-old culture were picked with sterile toothpicks and transferred onto the test medium. Fungal cultures were incubated at 28˚C in constant fluorescent light at intensity of 40 μE/m²/sec and colony radius was measured at 5 to 9 days. Each treatment contained four replicates and all experiments were performed at least two times. The difference of radial growth of the disruption mutants relative to the wild-type cultured on the same plate was measured. Percentage change was determined by dividing the relative difference of the growth by the wild-type growth and then multiplying by 100. All test chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Production and germination of conidia

Fungal mycelium was ground in sterile water using a disposable pestle and sprayed evenly onto PDA. Plates without sealing were placed in a plastic box and incubated at 28˚C in constant fluorescent light for 3 to 5 days. Conidia were harvested by scraping with sterile water and low-speed centrifugation (5000 x g) and examined microscopically with the aid of a hemacytometer. Conidia were germinated on glass slide incubated in a moist chamber and examined with a Leitz Laborlux phase contrast microscope (Leitz Wetzlar, Germany). Germination
of conidia was determined by placing them on glass slides and incubating in a plastic box for 6 h and examined microscopically.

Cloning, targeted gene disruption and genetic complementation

The *A. alternata* major facilitator superfamily (AaMFS) gene fragment (clone #19; accession no. GS597470) was previously identified from a wild-type cDNA library after subtraction with that of a *YAP1* null mutant [10]. The full-length *AaMFS19* gene sequence was identified from the completed genome sequence of *A. alternata* [41] and was amplified by PCR with the primers from genomic DNA. Fungal DNA was purified using a DNeasy Plant kit (Qiagen). Open reading frame (ORF) and exon/intron positions were predicted using Softberry gene-finding software.

*AaMFS19* gene was inactivated by inserting a bacterial phosphotransferase B gene (*HYG*) cassette under the control of the *Aspergillus nidulans* *trpC* gene promoter and terminator, conferring resistance to hygromycin in the genome of *A. alternata*. Truncated but overlapping *HYG* fragments (M13R19/hyg3 and hyg4/M13F) were amplified by PCR and fused with truncated *AaMFS19* fragments by two-round PCR (S1 Fig). A 5’*AaMFS19*:5’*HYG* fusion fragment (1.5 + 1.2 kb) was amplified by two-round PCR with the primers 19F, M13R19, M13R and hyg3. A 3’*AaMFS19*:3’*HYG* fusion fragment (1.3 + 1.8 kb) was amplified with primers hyg4, M13F, M13F19N, and 19R. The primers M13R19 and M13F19N contain the oligonucleotides completely complementary to the sequence of primers M13R and M13F, respectively. Amplicons (10 μl each) were mixed and introduced into fungal protoplasts prepared from the EV-MIL31 strain using polyethylene glycol and CaCl₂ as previously described [40,42]. Fungal transformants growing on a regeneration medium amended with 250 μg/ml hygromycin (Roche Applied Science) were selected and examined by PCR with an *AaMFS19*-specific primer pairing with a *HYG*-specific primer as indicated in S1 Fig. The 473F and 5KR primers that are not present in the split marker fragments, respectively, were paired with the hyg3 and hyg4 primers and used to examine for the occurrence of homologous integration within *AaMFS19*. A 3.0-kb fragment was amplified with the primers 473F and hyg3 from genomic DNA prepared from transformants D27 and D64 and no product was amplified from that of wild-type. A 2.7-kb fragment was amplified with the primers 5KR and hyg4 from genomic DNA of D27 and D64 but not wild-type. For genetic complementation, a full-length *AaMFS19* fragment under the control of its native promoter (3.8 kb) was amplified by PCR with two *AaMFS19*-specific primers (19F and 19R) and co-transformed with the pCB1532 plasmid carrying a sulfonylurea-resistant (*Sur*) gene [43] into protoplasts of an *AaMFS19* mutant (D27). Transformants appearing from medium amended with 5 μg/ml sulfonylurea were tested for restoration of cellular resistance to 3 mM 2-chloro-5-hydroxypyridine (CHP).

Gene expression

Gene expression was assessed by Northern-blot hybridization and semi-quantitative RT-PCR. Fungal strains were grown on PDA covered with a layer of cellophane for 3 to 5 days. Fungal RNA was isolated from mycelium using a TriZol reagent (Invitrogen, Carlsbad, CA). For Northern-blot hybridization, RNA was electrophoresed in a formaldehyde-containing agarose gel, blotted onto a nylon membrane, and hybridized to an *AaMFS19* DNA probe. The probe was amplified and labeled with a digoxigenin (DIG)-11-dUTP by PCR with the *AaMFS19* gene-specific primers according to the manufacturer’s recommendation (Roche Applied Science). The probe was detected by an immunological assay using CSPD as a chemiluminescent substrate for alkaline phosphatase.

For medium shift experiments, the wild-type strain was cultured on PDA covered with a layer of cellophane for 2 days. The cellophane membranes containing fungal mycelium were
lift and transferred onto a PDA supplemented with 0.1% dimethyl sulfoxide (DMSO), 5 mM 2,3,5-triiodobenzoic acid (TIBA), 2.5 mM CHP, 10 μM vincozolin, or 80 μM clotrimazole. After incubation for an additional 24 h, mycelium was harvested and subjected to RNA isolation. First-strand cDNA was synthesized using a MMLV High Performance Reverse Transcriptase (Epicentre) and used for PCR amplification with AaMFS19-specific primers. Amplicons were electrophoresed in 1% agarose gel and stained with ethidium bromide.

**Virulence assays**

Fungal virulence was assessed on detached calamondin (*Citrus mitis* Blanco) leaves inoculated by placing 10 μl of conidial suspension (10^5 conidia per ml) on each spot as described previously [14,16,17]. Alternatively, detached leaves were uniformly sprayed to run-off with conidial suspensions. The inoculated leaves were incubated in a plastic box for lesion formation. Each fungal strain was tested on at least 10 leaves and experiments were repeated three times.

**Ethics Statement**

No ethical permissions were required for this work which involved no experimentation involving animals or human samples.

**Supporting Information**

S1 Fig. Targeted disruption of AaMFS19 using a split marker approach and PCR confirmation of disruption.

(DOCX)

S2 Fig. AaMSF19 deletion mutants show wild-type resistance to H_2O_2, tert-butyl hydroperoxide, diamide, and menadione.

(DOCX)

**Author Contributions**

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Formal analysis: LHC HCT PLY.

Funding acquisition: KRC.

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References

1. Gulshan K, Moye-Rowley WS. Multidrug resistance in fungi. Eukaryot Cell 2007; 6: 1933–1942. doi: 10.1128/EC.00254-07 PMID: 17873085

2. dos Santos SC, Teixeira MC, Dias PJ, Sá-Correia I. MFS transporter required for multidrug/multixenobioc (MD/MX) resistance in the model yeast: understanding their physiological function through post-genomic approaches. Front Physiol. 2014; 5: 180. doi: 10.3389/fphys.2014.00180 PMID: 24847282

3. Sá-Correia I, dos Santos SC, Teixeira MC, Cabrito TR, Mira NP. Drug:H⁺ antiporters in chemical stress response in yeast. 2008; Trend Microbiol. 17: 22–31.

4. Callahan TM, Rose MS, Meade MJ, Ehrenshaw M, Upchurch RG. CFP, the putative cercosporin transporter of Cercospora kikuchii, is required for wild type cercosporin production, resistance, and virulence on soybean. Mol Plant-Microbe Interact. 1999; 12: 901–910. doi: 10.1094/MPMI.1999.12.10.901 PMID: 10517030

5. Del Sorbo G, Schoonbeek H, De Waard MA. Fungal transporters involved in efflux of natural toxic compounds and fungicides. Fungal Genet Biol. 2000; 30: 1–15. PMID: 10955904

6. Hayashi K, Schoonbeek H, De Waard MA. Bcmfs1, a novel major facilitator superfamily transporter from Botrytis cinerea, provides tolerance towards the natural toxic compounds camptothecin and cercosporin and towards fungicides. Appl Environ Microbiol 2002; 68: 4986–5004. doi: 10.1128/AEM.68.10.4986-5004.2002 PMID: 12324349

7. Roohparvar R, De Waard MA, Kema GHJ, Zwiers L. MgMfs1, a major facilitator superfamily transporter from the fungal wheat pathogen Mycosphaerella graminicola, is a strong protectant against natural compounds and fungicides. Fungal Genet Biol. 2007; 44: 378–388. doi: 10.1016/j.fgb.2006.09.007 PMID: 17107817

8. Roohparvar R, Mehrabi R, Van Nistelrooy JGM, Zwiers L, De Waard MA. The drug transporter MgMfs1 can modulate sensitivity of field strains of the fungal wheat pathogen Mycosphaerella graminicola to the strobilurin fungicide trifloxystrobin. Pest Manag Sci. 2008; 64: 685–693. doi: 10.1002/ps.1569 PMID: 18366066

9. Akimitsu K, Peever TL, Timmer LW. Molecular, ecological and evolutionary approaches to understanding Alternaria diseases of citrus. Mol Plant Pathol. 2003; 4: 435–446. doi: 10.1046/j.1364-3703.2003.00189.x PMID: 20569403

10. Lin CH, Yang SL, Chung KR. 2011. Cellular responses required for oxidative stress tolerance, colonization and lesion formation by the necrotrophic fungus Alternaria alternata in citrus. Curr Microbiol. 62: 807–815. doi: 10.1007/s00284-010-9795-y PMID: 20978890

11. Lin CH, Yang SL, Chung KR. The YAP1 homolog-mediated oxidative stress tolerance is crucial for pathogenicity of the necrotrophic fungus Alternaria alternata in citrus. Mol Plant Microbe Interact. 2009; 22: 942–952. doi: 10.1094/MPMI-22-8-0942 PMID: 19589070

12. Lin CH, Chung KR. Specialized and shared functions of the histidine kinase- and HOG1 MAP kinase-mediated signaling pathways in Alternaria alternata, a filamentous fungal pathogen of citrus. Fungal Genet Biol. 2010; 47: 818–827. doi: 10.1016/j.fgb.2010.06.009 PMID: 20601043

13. Yang SL, Lin CH, Chung KR. Coordinate control of oxidative stress, vegetative growth and fungal pathogenicity via the AP1-mediated pathway in the rough lemon pathotype of Alternaria alternata. Physiol Mol Plant Pathol. 2009; 74: 100–110.

14. Yang SL, Yu PL, Chung KR. The glutathione peroxidase–mediated reactive oxygen species resistance, fungicide sensitivity and cell wall construction in the citrus fungal pathogen Alternaria alternata. Fungal Genet Biol. 2016; 18: 923–935. doi: 10.1111/1462-2920.13125 PMID: 26567914

15. Chen LH, Lin CH, Chung KR. Roles for SKN7 response regulator in stress resistance, conidiation and virulence in the citrus pathogen Alternaria alternata. Fungal Genet Biol. 2012; 49: 802–813. doi: 10.1016/j.fgb.2012.07.006 PMID: 22902811

16. Yang SL, Chung KR. The NADPH oxidase-mediated production of hydrogen peroxide (H₂O₂) and resistance to oxidative stress in the necrotrophic pathogen Alternaria alternata of citrus. Mol Plant Pathol. 2012; 13: 900–914. doi: 10.1111/j.1364-3703.2012.00799.x PMID: 22435666

17. Yang SL, Chung KR. Similar and distinct roles of NADPH oxidase components in the tangerine pathotype of Alternaria alternata. Mol Plant Pathol. 2013; 14: 543–556. doi: 10.1111/mpp.12026 PMID: 23527595

18. Yu PL, Chen LH, Chung KR. How the pathogenic fungus Alternaria alternata copes with stress via the response regulators SKS1 and SHO1. PLoS One 2016; 11(2): e0149153. doi: 10.1371/journal.pone.0149153 PMID: 26863027

19. Chen LH, Yang SL, Chung KR. Resistance to oxidative stress via regulating siderophore-mediated iron-acquisition by the citrus fungal pathogen Alternaria alternata. Microbiology 2014; 160: 970–979. doi: 10.1099/mic.0.076182-0 PMID: 24586035
20. Toone WM, Morgan BA, Jones N. Redox control of AP-1-like factors in yeast and beyond. Oncogene 2001; 20: 2336–2346. doi: 10.1038/sj.onc.1204384 PMID: 11402331

21. Alarco A, Balan I, Talibi D, Mainville N, Raymond M. AP1-mediated multidrug resistance in *Saccharomyces cerevisiae* requires *FLR1* encoding a transporter of the major facilitator superfamily. J Biol Chem. 1997; 272: 19304–19313. PMID: 9235926

22. Coleman ST, Tseng E, Moye-Rowley WS. *Saccharomyces cerevisiae* basic region-leucine zipper protein regulatory networks converge at the *ATR1* structural gene. 1997; J Biol Chem. 272: 23224–23230. PMID: 9287330

23. Alarco A, Raymond M. The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response. J Bacteriol. 1999; 181: 700–708. PMID: 9922320

24. Rognon B, Kozovska Z, Coste AT, Pardini G, Sanglard D. Identification of promoter elements responsible for the regulation of *MDR1* from *Candida albicans*, a major facilitator transporter involved in azole resistance. Microbiology 2006; 152: 3701–3722. doi: 10.1099/mic.0.29277-0 PMID: 17159223

25. Daub ME, Herrero S, Chung KR. Reactive oxygen species in plant pathogenesis: the role of perylenequinone photosensitizers. Antioxid Redox Signal. 2013; 19: 970–989. doi: 10.1089/ars.2012.5080 PMID: 23259634

26. Pottier R, Bonneau R, Joussot-Dubien J. pH dependence of singlet oxygen production in aqueous solutions using toluidine blue as a photosensitizer. Photochem Photobiol 1975; 25: 59–61. PMID: 242021

27. Dhaoui M, Aucere F, Blaiseau PL, Lesuisse E, Landoulsi A, Camadro JM, et al. *Gex1* is a yeast glutathione peroxidase and spermidine export controls cell cycle delay and times antioxidant protein expression during oxidative stress response. EMBO Rep 2013; 14: 1113–1119. doi: 10.1038/embor.2013.165 PMID: 24136413

28. Kruger A, Vowinckel J, Mulleder M, Grote P, Capuano F, Bluemlein K, et al. Tup1-mediated perminde and spermidine export controls cell cycle delay and times antioxidant protein expression during oxidative stress response. FEMS Yeast Res. 2013; 13: 97–106. doi: 10.1111/1567-1364.12013 PMID: 23106982

29. Rios G, Cabedo M, Rull B, Yenush L, Serrano R, Mulet JM. Role of the yeast multidrug transporter Qdr2 in cation homeostasis and the oxidative stress response. FEMS Yeast Res. 2013; 13: 97–106. doi: 10.1111/1567-1364.12013 PMID: 23106982

30. Coleman JJ, Mylonakis E. Efflux in fungi: La Pi and spermidine export controls cell cycle delay and times antioxidant protein expression during oxidative stress response. EMBO Rep 2013; 14: 1113–1119. doi: 10.1038/embor.2013.165 PMID: 24136413

31. Paul S, Moye-Rowley WS. Multidrug resistance in fungi: regulation of transporter-encoding gene expression. Front Physiol. 2014; 5: 143. doi: 10.3389/fphys.2014.00143 PMID: 24795641

32. Wateratore T, Koller K, Messner K. Copper-dependent depolymerisation of lignin in the presence of fungal metabolite, pyridine. J Biotechnol. 1998; 62: 221–230. PMID: 9729805

33. Nerud F, Baldrin P, Gabriel J, Ogbeifun D. Decolorization of synthetic dyes by the Fenton reagent and the Cu/pyridine/H2O2 system. Chemosphere. 2001; 44: 957–961. PMID: 11513429

34. Katekar GF, Geissler AE. Auxin transport inhibitors. Plant Physiol. 1980; 66: 1190–1195. PMID: 18661601

35. Lahey KA, Yuan R, Ueng PP, Timmer LW, Chung KR. Induction of phytohormones and differential gene expression in citrus flowers infected by the fungus *Colletotrichum acutatum*. Mol Plant-Microbe Interact. 2004; 17: 1394–1401. doi: 10.1094/MPMI.2004.17.12.1394 PMID: 15597741

36. Panwar SL, Pasrija R, Prasa R. Membrane homeostasis and multidrug resistance in yeast. Biosci Rep. 2008; 28: 217–228. doi: 10.1042/BSR20080071 PMID: 18754755

37. Shahi P, Moye-Rowley WS. Coordinate control of lipid composition and drug transport activities is required for normal multidrug resistance in fungi. Biochem Biophys Acta 2009; 1794: 852–859. doi: 10.1016/j.bbabap.2008.12.012 PMID: 19150612

38. Lin CH, Yang SL, Wang N, Chung KR. The Fus3 MAPK signaling pathway of the citrus pathogen *Alternaria alternata* acts independently and cooperatively with the fungal redox-responsive AP1 regulator for diverse developmental, physiological and pathogenic functions. Fungal Genet Biol. 2010; 47: 381–391. doi: 10.1016/j.fgb.2009.12.009 PMID: 20036749

39. Yago J, Lin CH, Chung KR. The SLT2 mitogen-activated protein kinase-mediated signalling pathway governs conidiation, morphogenesis, fungal virulence and production of toxic and melanin in the tangerine pathotype of *Alternaria alternata*. Mol Plant Pathol. 2011; 12: 653–665. doi: 10.1111/j.1364-3703.2010.00701.x PMID: 21726368

40. Chung KR, Lee MH. Split marker-mediated transformation and targeted gene disruption in filamentous fungi. *In: Genetic transformation systems in fungi*. Vol. 2. van den Berg MA, Maruthachalam K. (eds.), 2015; pp. 175–180. Springer International Publishing Switzerland.

41. Wang M, Sun X, Yu D, Xu J, Chung K, Li H. Genomic and transcriptomic analyses of the tangerine pathotype of *Alternaria alternata* in response to oxidative stress. Sci Rep 2016; 6:32437.
42. Chung KR, Shilts T, Li W, Timmer LW. Engineering a genetic transformation system for *Colletotrichum acutatum*, the causal fungus of lime anthracnose and postbloom fruit drop. FEMS Microbiol Lett. 2002; 213: 33–39. PMID: 12127485

43. Sweigard JA, Chumley FC, Carroll AM, Farrall L, Valent B. A series of vectors for fungal transformation. Fungal Genet Newsl. 1997; 44: 52–53.