JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Monomeric L-Amino Acid Oxidase-Induced Mitochondrial Dysfunction in *Rhizoctonia solani* Reveals a Novel Antagonistic Mechanism of *Trichoderma harzianum* ETS 323

Chia-Ann Yang,[†] Chi-Hua Cheng,[‡] Jeng-Woei Lee,[§] Chaur-Tsuen Lo,[∥] Shu-Ying Liu,[⊥] and Kou-Cheng Peng*[,‡]

[†]Institute of Medical Science and [§]Institute of Life Science, Tzu Chi University, Hualien 97004, Taiwan, Republic of China

[‡]Institute of Biotechnology, National Dong Hwa University, Hualien, 97401, Taiwan, Republic of China

[∥]Department of Biotechnology, National Formosa University, Yunlin 63208, Taiwan, Republic of China

[⊥]Department of Molecular Biotechnology, Da-Yeh University, Changhua 51591, Taiwan, Republic of China

Supporting Information

ABSTRACT: The monomeric L-amino acid oxidase (mTh-LAAO) of *Trichoderma harzianum* ETS 323 has been suggested to antagonize *Rhizoctonia solani* by an unknown mechanism. Here, the mTh-LAAO-treated *R. solani* exhibited hyphal lysis and apoptotic characteristics such as DNA fragmentation, reactive oxygen species (ROS) accumulation, lipid peroxidation, and mitochondrial membrane potential depolarization. This hyphal lysis was suppressed by the mitochondria-dependent apoptosis inhibitor oligomycin while accompanied by reduction of ROS accumulation. This result suggested that mitochondria-mediated apoptosis in *R. solani* was involved in mTh-LAAO-induced growth inhibition, which was supported by the evidence of cytocheome *c* release and activation of caspases 9 and 3. Furthermore, the data indicated that the mTh-LAAO-induced fungal cell death was also closely interrelated with the interaction of mTh-LAAO with *R. solani* hyphal cell wall proteins. These results illuminate the biological function and mechanism underlying the antagonistic action of *T. harzianum* mTh-LAAO against fungal pathogens.

KEYWORDS: *L-amino acid oxidase, Trichoderma harzianum, Rhizoctonia solani, biocontrol, apoptosis*

INTRODUCTION

L-Amino acid oxidase (LAAO, E.C.1.4.3.2), a homodimeric flavoprotein, oxidatively deaminates an L-amino acid substrate to the cognate α-keto acid, accompanied by the production of hydrogen peroxide and ammonia.[1] This enzyme has been reported to induce apoptosis in mammalian cell lines[2,3] and possess antibacterial activity,[4] but the biological purpose of this enzyme in the producing host itself is not fully understood.

Trichoderma harzianum, a filamentous fungus, has been used agriculturally as a biocontrol agent against plant pathogens such as the soil-borne *Rhizoctonia solani* that causes major losses of agricultural crops such as tobacco and potatoes.[5] *T. harzianum* parasitizes *R. solani* via high-level coiling and then penetrates *R. solani* by releasing glucanases and chitinases, which hydrolyze *R. solani*'s cell wall.[6]

An L-amino acid oxidase (Th-LAAO) was isolated from proteins secreted by *T. harzianum* ETS 323 and is thought to be involved in antagonizing *R. solani*.[7] Th-LAAO is a homodimeric glycoprotein like other LAAO family members, composed of 67.9 kDa monomers, contains a flavin adenine dinucleotide (FAD) cofactor, and phenylalanine is its best substrate.[7] Studies on the antagonistic effects of Th-LAAO against *R. solani* indicate that the secreted monomeric form predominates when *T. harzianum* ETS 323 is grown in the presence of deactivated *R. solani* hyphae. Moreover, in vitro, monomeric Th-LAAO (mTh-LAAO) induces hyphal lysis corresponding to growth inhibition of *R. solani* to a greater extent than the homodimeric form.[7] However, the mechanism has not yet been elucidated. Therefore, in the present study, we attempted to decipher the mechanisms underlying the ability of monomeric Th-LAAO to inhibit the growth of fungal pathogen *R. solani*.

MATERIALS AND METHODS

Chemicals. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Culture Conditions. *T. harzianum* ETS 323, accession number BCRC 930081 (Bioresource Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan) was maintained on potato dextrose agar (PDA) (Difco Laboratories) plates at 25 °C. *R. solani*, isolated in Taiwan, was also maintained on PDA plates at 25 °C. *T. harzianum* ETS 323 and deactivated *R. solani* hyphae were cocultivated as described previously.[6,7]

Purification of Th-LAAO. Monomeric Th-LAAO was purified as described previously.[7] The resulting Th-LAAO was identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Phylogeny Analysis. LAAO sequences from *Viridovipera stejnegeri* (GenBank AAQ56232), *Crotalus adamanteus* (GenBank AAC32267), *Bothrops moojeni* (GenBank AAR31183), *Bothrops jararacussu* (GenBank AAR31182), *Daboia russellii siamensis* (GenBank

Received: September 24, 2011
Revised: February 20, 2012
Accepted: February 21, 2012
Published: February 21, 2012

ACS Publications © 2012 American Chemical Society

AAZ08620), *Naja atra* (GenBank ABN72546), *Bungarus multicinctus* (GenBank ABN72539), *Demansia vestigiata* (GenBank A6MFL0), *Ophiophagus hannah* (GenBank ABN72538), *Meleagris gallopavo* (GenBank ACA64754), *Monodelphis domestica* (GenBank P_001381664), *Rattus norvegicus* (GenBank P_001100152), *Canis familiariz* (GenBank XP_539553), *Bos taurus* (GenBank XP_002686575), *Mus musculus* (GenBank NP_034345.2), *Platichthys stellatus* (GenBank BAI66017), *Sebastes schlegeli* (GenBank BAF43314), (GenBank BAG72078), *Myoxocephalus polyacanthocephalus* (GenBank BAG72078), *Aplysianin kurodai* (GenBank Q17043), *Aplysia punctata* (GenBank CAH59505), *Aplysia californica* (GenBank AAT12273), *Emericella nidulans* (GenBank AAT84085), *Rhodococcus opacus* (GenBank AAL14831), *Trichoderma harzianum* ETS 323 (GenBank GU902953), *Siganus canaliculatus* (GenBank ADW77183), *Streptococcus oligofermentans* (GenBank ACA52024), *Bacillus* sp. BT1B (GenBank ZP_08000130), and *Aspergillus oryzae* (GenBank BAC55901) were used for phylogeny analysis. Phylogenetic trees were constructed by the neighbor-joining (NJ) method, using the MEGA 4 program, to analyze the phylogenetic evolution of L-amino acid oxidase sequences.

DNA Fragmentation Analysis. Two-millimeter-diameter discs of *R. solani* hyphae were obtained from the edges of a 5-day-old PDA (potato dextrose agar) fungal culture. Afterward, a fungal disk was placed at the center of a 9-cm PDA plate to which a cellophane membrane had been attached. The cellophane membrane provides support for hyphae attachment as well as making it easy to harvest. A sterile filter paper disk (6 mm) impregnated with 3 μg of mTh-LAAO was placed on the fungal hyphae, while a control disk was saturated with distilled water. The hyphae were harvested by scraping from the cellophane membrane after 1.5 days of cultivation at 28 °C. The DNA was extracted as described.[8] To detect DNA fragmentation, samples were electrophoresed through a 1% (w/v) agarose gel and visualized under ultraviolet light after being stained with 0.5% (w/v) ethidium bromide. All experiments were performed in triplicate.

Detection of Reactive Oxygen Species in Fungal Hyphae. A thin layer of PDA was spread on a sterile microscope slide coverslip. One disk (2 mm in diameter) of *R. solani* hyphae was cut from the edges of a 5-day-old fungal culture and transferred to the center of the coverslip. One sterile filter paper disk impregnated with 3 μg of mTh-LAAO was placed on the fungal hyphae and further incubated for 4 days at 28 °C. Sterile distilled water was used as a negative control. Intracellular reactive oxygen species (ROS) accumulation was determined by use of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA). The coverslip cultures were incubated with 1 μM H_2DCF-DA for 2 h at 37 °C, washed 3 times with 20 mM phosphate buffer (pH 7.4), and observed with a fluorescence microscope (IX70; Olympus, Tokyo, Japan). All experiments were performed at least in triplicate.

Lipid Peroxidation Assay. The hyphae of mTh-LAAO-treated *R. solani* were harvested as described above. Lipid peroxidation was detected by measuring malondialdehyde (MDA), an oxidation product of polyunsaturated fatty acids generated by ROS damage. The hyphae were incubated in deionized distilled water (ddH_2O) with gentle shaking for 10 min. After centrifugation for 20 min at 4000*g*, a supernatant aliquot was collected and used to determine the MDA concentration via a thiobarbituric acid (TBA) assay.[9]

Analysis of Mitochondrial Membrane Potential. The center of the PDA-treated coverslip was inoculated with a 2-mm diameter plug of *R. solani* hyphae. One paper disk impregnated with mTh-LAAO (3 μg) was placed on the fungal hyphae and incubated for 2 days at 28 °C. The depolarization of mitochondrial membrane potential was determined by incubating the fungal culture coverslip with the MitoCapture cationic dye (Mitocapture apoptosis detection kit, BioVision, Mountain View, CA). The intact mitochondria of a normal cell fluoresce red, whereas an apoptotic cell retains the cationic dye in the cytoplasm and emits green fluorescence.

Effect of Oligomycin on Mitochondria-Dependent Growth Inhibition. A mixture of oligomycin (15 μM) and mTh-LAAO (3 μg·disk^{-1}) was added to *R. solani* and incubated at 28 °C for 5 days. Sterile distilled water and mTh-LAAO served as the positive and negative controls, respectively. The percentage of hyphal growth reduction was calculated using the following equation: hyphal growth reduction (%) = $[(D_c - D_t)/D_c] \times 100$, where D_c and D_t are the radii of *R. solani* growth on control and mTh-LAAO-containing plates, respectively.

Western Blotting Analysis. Two-day-old fungal hyphae of mTh-LAAO-treated *R. solani*, treated with or without oligomycin (15 μM), were harvested as described above. Cytochrome *c* release was measured in cytosolic and mitochondrial samples.[10] Cytosolic and mitochondrial proteins were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) and transferred to a poly(vinylidene difluoride) (PVDF) membrane (PerkinElmer, Waltham, MA). After nonspecific binding was blocked with 5% (w/v) bovine serum albumin, cytochrome *c*, caspase 3, caspase 9, and β-actin were detected by use of mouse monoclonal anti-cytochrome *c*, rabbit anti-caspase 3 polyclonal antibody, mouse anti-caspase 9 monoclonal antibody (1:1000; StressGen Biotechnologies Corp., Victoria, BC, Canada), and mouse anti-β-actin polyclonal antibody (1:1000; Millipore Corp., Bedford, MA), respectively. Immunoblot analysis was performed with horseradish peroxidase-conjugated anti-mouse immunoglobulin G by using the Western Lighting Plus-ECL detection reagent (PerkinElmer, Waltham, MA).

Interaction between mTh-LAAO and *R. solani* Hyphae. We conjugated purified mTh-LAAO with fluorescein isothiocyanate (FITC) as previously described.[11] The unbound dye was washed away with ddH_2O by using an Amicon UltraCentrifugal Filter Unit with a 50 kDa cutoff. A 3-day-old *R. solani* culture on a PDA coverslip was incubated with 1 mg·mL^{-1} FITC-mTh-LAAO at 28 °C for 2 h and then washed 3 times with ddH_2O. Images were acquired by fluorescence microscopy (IX70; Olympus, Tokyo, Japan).

Extraction of *R. solani* Cell Wall Protein. *R. solani* hyphae from 5-day-old liquid cultures were collected and washed three times with ddH_2O. The fungal cell walls were collected as previously described.[12] Briefly, the fungal hyphae were pulverized in liquid nitrogen by using a mortar and pestle. The resulting hyphal powder (2.5 g) was incubated in 100 mL of ddH_2O containing 0.3% SDS (w/v) at 4 °C for 17 h. After centrifugation for 20 min at 6000*g*, the hyphal cell wall materials were recovered and washed three times with ddH_2O. Cell wall proteins were prepared from cell wall materials by suspending 1 g of cell walls in 20 mL of ddH_2O and autoclaving for 20 min at 15 psi, 121 °C, to disintegrate the cell walls and release the proteins.[13] The cell wall protein fractions were collected by centrifugation at 6000*g* for 10 min and filtration through a 0.22-μM filter (Millipore, Cork, Ireland).

Isolation of *R. solani* mTh-LAAO-Binding Cell Wall Proteins. Glyco-affinity chromatography was used to isolate Th-LAAO binding cell wall proteins (LBCWPs). Purified mTh-LAAO, a glycoprotein that can bind to concanavalin (ConA) resin, was added to a 5-mL ConA–Sepharose column (GE Healthcare Bio-Science, Piscataway, NJ), which was then washed with wash buffer (1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.5 M NaCl, and 0.02 M Tris-HCl, pH 7.4). *R. solani* cell wall protein fractions were loaded onto the mTh-LAAO-bound ConA column, which was then washed with wash buffer. The LBCWP–mTh-LAAO complexes were eluted with methyl α-D-mannopyranoside (0.25 M) in 0.02 M Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl at 1 mL·min^{-1}. The LBCWP–mTh-LAAO complexes were separated by SDS–10% PAGE and electroblotted onto a PVDF membrane and stained with Ponceau S, and the bands of interest were excised and subjected to Edman N-terminal sequencing by the Department of Life Science, National Taiwan University, TechComm.

Effect of Binding of mTh-LAAO to LBCWPs on Growth of *R. solani*. A 30-μg·mL^{-1} mTh-LAAO solution was first bound to the ConA column, and then the cell wall protein extracts were loaded onto the mTh-LAAO-bound ConA–Sepharose column prior to saturation of the specific mTh-LAAO contact sites (i.e., LBCWP binding site) on the *R. solani* hyphal cell wall. The LBCWP–mTh-LAAO complexes were then eluted with methyl α-D-mannopyranoside (0.25 M) in 0.02 M Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl. The LBCWP–mTh-LAAO complexes were desalted and concentrated with an Amicon Ultra centrifugal filter device (3 kDa cutoff, Ultra-4; Millipore,

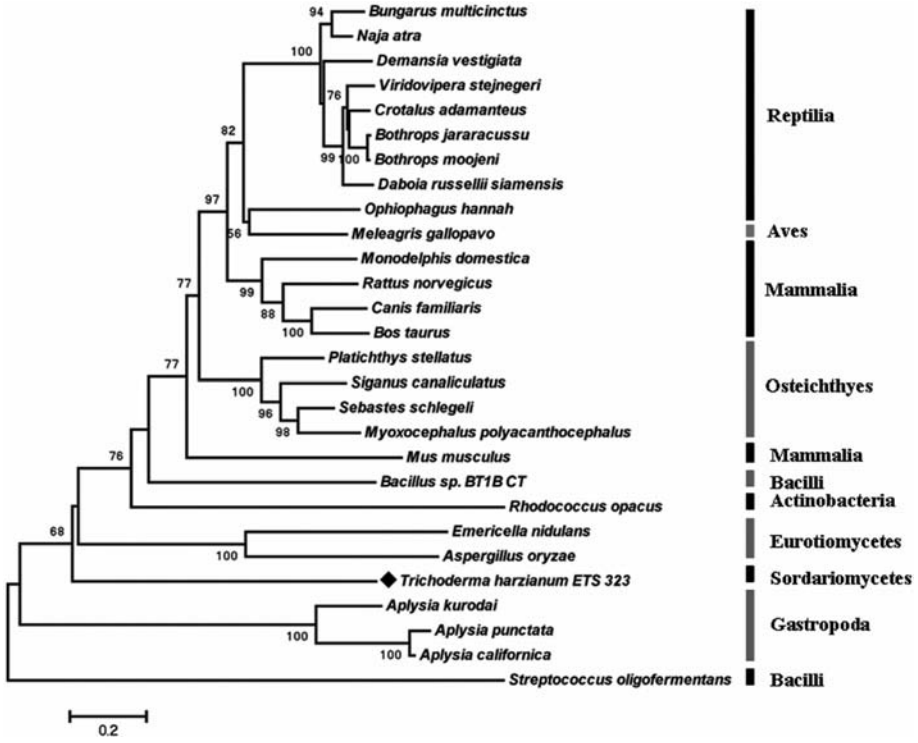

Figure 1. Phylogenetic tree of L-amino acid oxidase family members. Numbers on the branches are percentages of 1000 bootstrap samples. Bootstrap values greater than 50% are reported on the nodes. The bootstrap iterations were 0.2.

Billerica, MA). As a control, mTh-LAAO was not subjected to this procedure.

RESULTS AND DISCUSSION

Phylogenetic Analysis of LAAOs. Th-LAAO, an LAAO family member, has recently been identified among the secreted proteins of *T. harzianum* ETS 323.[7] The monomeric Th-LAAO (mTh-LAAO) has been suggested to exert an antagonistic effect against a fungal pathogen, *R. solani*. This enzyme, at a concentration of 3 μg·disk^{-1}, resulted in a 50% reduction of *R. solani* hyphae growth by the fifth day of incubation.[7]

On the basis of the phylogenetic tree of LAAOs (Figure 1), LAAOs form four major clusters, including sequences from both vertebrate (Reptilia, Mammalia, and Osteichthyes) and invertebrate (Gastropoda) animals. Class Aves was found to contain a member of the Reptilia-related subfamily, although the bootstrap support for this was not strong (82%). In general, the phylogenetic relationship of LAAOs in bacteria or fungi did not form a monophyletic group, despite the clustering of the sequence from *Emericella nidulans* with that of *Aspergillus oryzae*, with 100% bootstrap support. However, the sequence from *T. harzianum* ETS 323 fell outside of most of the clusters. This may indicate the phylogenetic independence and limited homology of the LAAO of *T. harzianum* to other LAAO family members.

mTh-LAAO-Induced DNA Damage. DNA fragmentation, a marker of apoptosis,[14] was detected in mTh-LAAO-treated *R. solani* cultures (Figure 2). *R. solani* hyphae were treated with mTh-LAAO that caused DNA strand breaks (Figure 2, lane T), whereas there was none caused by the control (ddH_2O) (Figure 2, lane C).

ROS Accumulation in mTh-LAAO-treated *R. solani*. During the interaction between *T. harzianum* ETS 323 and *R. solani*, reactive oxygen species (ROS), another fungal apoptotic

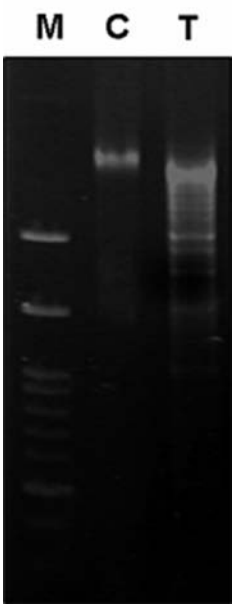

Figure 2. DNA strand breaks in mTh-LAAO-treated *R. solani*. M, molecular weight marker; C, *R. solani* only; T, *R. solani* incubated with mTh-LAAO.

marker,[15,16] were detected by H_2DCF-DA assay. *R. solani* hyphae strongly fluoresced, in contrast to those of *T. harzianum* ETS 323 (Figure S1, Supporting Information). This result raised the possibility that ROS accumulated in mTh-LAAO-treated *R. solani*.

The mTh-LAAO-treated *R. solani* hyphae significantly exhibited malformations on day 2 (Figure S2, Supporting Information) and day 3 (Figure 3A, panel b), and lysis began on day 4 (Figure 3A, panel c). However, control hyphae

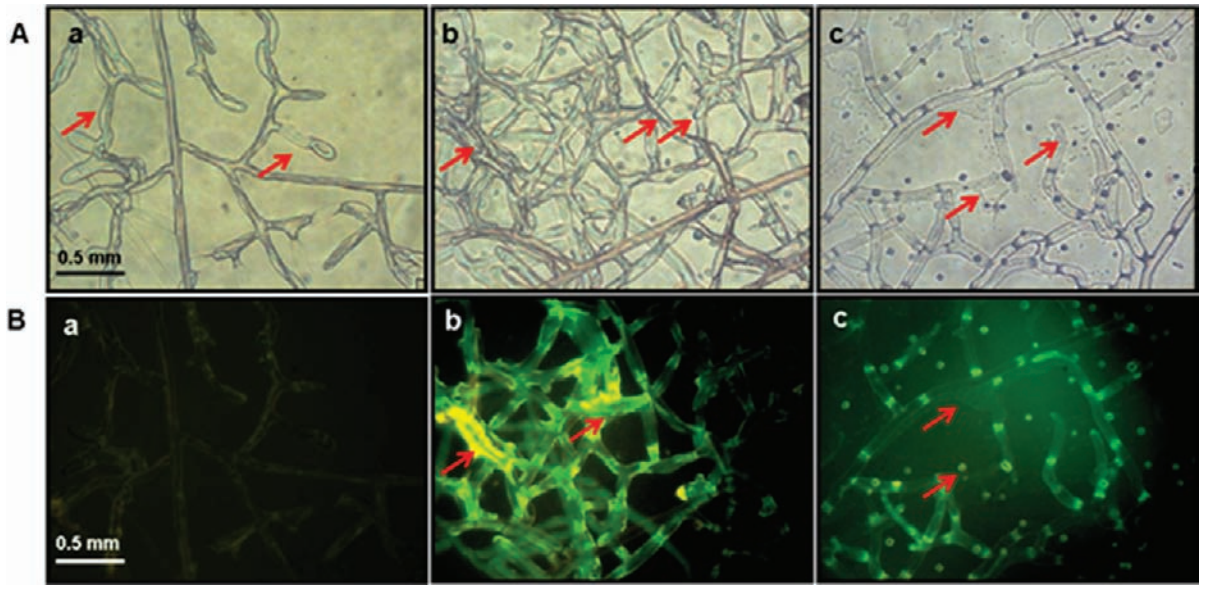

Figure 3. ROS accumulation in mTh-LAAO-treated *R. solani*. (A) Light microscopy. (a) Control treatment after 4 days. Arrows in the panel indicates the smooth (left) and extensive hyphae (right, hyphae penetrating agar). (b, c) mTh-LAAO-treated *R. solani* on (b) day 3 and (c) day 4. (B) Fluorescence microscopy: arrows indicate green fluorescence in hyphae and cell lysates, which corresponds to ROS accumulation, for (b) day 3 and (c) day 4 of incubation with mTh-LAAO. (a) No appreciable fluorescence by 4-day-old hyphae controls.

maintained their normal smooth and extended morphology on day 4 (Figure 3A, panel a). After mTh-LAAO treatment, hyphae fluoresced green on day 2 (Figure S2, Supporting Information) and day 3 (Figure 3B, panel b) and cell lysis occurred on day 4 (Figure 3B, panel c). There was no detectable fluorescence on day 4 in untreated samples (Figure 3B, panel a). These data support a mechanism involving ROS production resulting from the interaction of mTh-LAAO with *R. solani*.

Lipid Peroxidation in mTh-LAAO-Treated *R. solani*. Lipid peroxidation, a result of ROS-mediated damage, was detected in mTh-LAAO-treated *R. solani* at day 5 (Figure 4). Increased MDA concentrations were apparent in mTh-LAAO-treated *R. solani*, consistent with lipid peroxidation. The controls were negative.

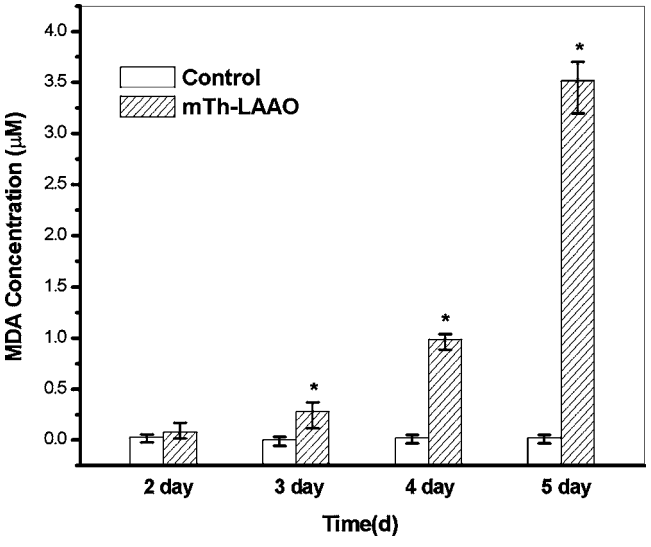

Figure 4. Lipid peroxidation in mTh-LAAO-treated *R. solani*. An increase of lipid peroxidation was observed after 5 days. (*) Significantly different from control group ($P < 0.05$).

Depolarization of Mitochondrial Membrane Potential Induction by mTh-LAAO. The intriguing data presented above led us to determine whether ROS accumulation was associated with mitochondrial dysfunction. For this purpose, we determined the mitochondrial depolarization of transmembrane potential. The mTh-LAAO-treated *R. solani* exhibited green hyphal fluorescence corresponding to mitochondrial alterations, whereas control hyphae emitted red fluorescence as expected for intact mitochondria (Figure 5).

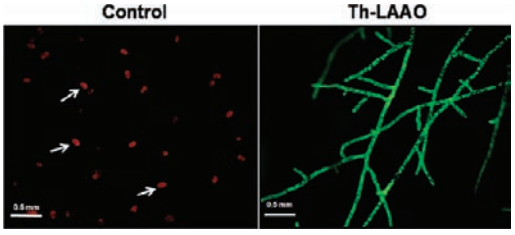

Figure 5. Mitochondrial membrane depolarization potential of *R. solani* treated with mTh-LAAO. (left) Control-treated and (right) mTh-LAAO-treated *R. solani*. Arrows indicate intact mitochondria of hyphae.

Oligomycin Suppresses mTh-LAAO-Induced Growth Inhibition of *R. solani*. Oligomycin inhibits mitochondrial F_oF_1-ATPase and has been reported to suppress mitochondria-dependent apoptosis in fungi.[15] Here, we examined the ability of oligomycin to block Th-LAAO-induced hyphal lysis and growth inhibition. Figure 6A and Figure S3 (Supporting Information) show that oligomycin attenuated mTh-LAAO-induced hyphal lysis and growth inhibition of *R. solani* during 5 days of incubation. In the presence and absence of oligomycin, growth was reduced by 50% and 97%, respectively, on the second day of incubation. On day 4, oligomycin was effective in suppressing Th-LAAO-induced growth inhibition by 92%.

dx.doi.org/10.1021/jf203883u | J. Agric. Food Chem. 2012, 60, 2464–2471

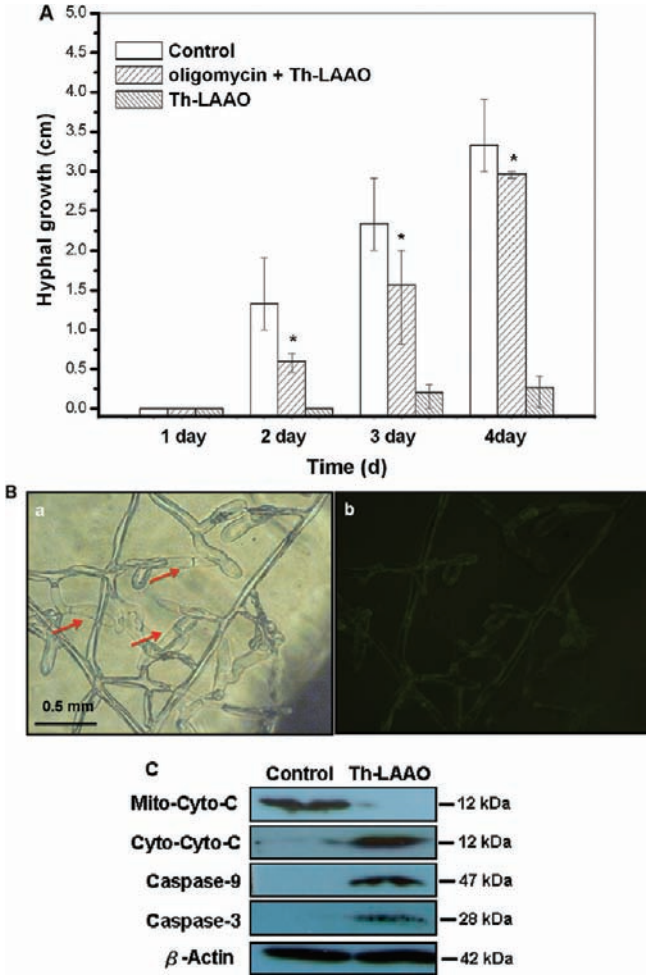

Figure 6. Effect of oligomycin on mTh-LAAO-induced growth inhibition and cytochrome *c* release by *R. solani*. (A) *R. solani* treated with mTh-LAAO in the presence or absence of oligomycin over 4 days. (B) mTh-LAAO-induced ROS accumulation in *R. solani* was suppressed by oligomycin on day 3. (a) Light microscopy: healthy, smooth *R. solani* hyphae. (b) No detectable green fluorescence emitted by *R. solani* hyphae, (C) Western blots of cytochrome *c* and caspases 9 and 3 in *R. solani* cytosol extracts. Cyto-Cyto-C, cytochrome *c* in cytosol extracts; Mito-Cyto-C, cytochrome *c* in mitochondrial extracts.

Oligomycin also reduced ROS accumulation in mTh-LAAO-treated *R. solani*. Thus, DCF-DA fluoresced to a much lower extent in hyphae, corresponding to inhibition of ROS accumulation by oligomycin (Figure 6B). ROS accumulation plays an important role in fungal apoptosis.[15−17] Likewise, mTh-LAAO-induced hyphal lysis and growth inhibition were blocked when ROS accumulation was inhibited. Therefore, it was reasonable to deduce that ROS accumulation was essential for the mTh-LAAO-induced apoptotic response in *R. solani*, mediated by mitochondrial dysfunction.

mTh-LAAO Induces Apoptosis in *R. solani* by Mitochondria-Mediated Caspase Pathway. In previous reports,[18,19] oligomycin exerts its anti-apoptotic effects by blocking the oligomerization of Bax, a pro-apoptotic factor that promotes mitochondrial membrane permeability by altering inner mitochondrial membrane potential. This effect inhibits the release of mitochondrial cytochrome *c* into the cytosol and initiates apoptosis. Therefore, we investigated the effect of mTh-LAAO on mitochondrial cytochrome *c* release of *R. solani* (Figure 6C). The data indicated that cytochrome *c* levels in the cytosol were increased significantly in mTh-LAAO-treated *R. solani* but not in the control treatment. The caspase cascade (caspases 3 and 9) is known for its essential role in the downstream execution of apoptosis pathway in eukaryote cells and is activated by mitochondrial cytochrome *c* release.[20−22] Caspase 3 is reported to mediate the end of the apoptosis pathway. Caspase 9 contributes to apoptosis induced by cytochrome *c* release from mitochondria. Here, the results showed that the caspase pathway was activated in mTh-LAAO-treated *R. solani*, with induction of caspases 3 and 9 (Figure 6C). In contrast, control hyphae showed no detectable signal of these apoptosis pathway components.

In this study, cytochrome *c* was found to be released from mitochondria after treatment by mTh-LAAO, supporting the notion that caspases 9 and 3 were activated by the mitochondria-mediated intrinsic apoptosis pathway for triggering mTh-LAAO-induced growth inhibition in *R. solani*.

Binding of mTh-LAAO to Hyphal Surfaces of *R. solani*. The interaction between mTh-LAAO and *R. solani* was analyzed by use of FITC−mTh-LAAO. This modified enzyme exerted similar inhibitory effects as mTh-LAAO on *R. solani* growth (data not shown). The results demonstrated that mTh-LAAO bound to *R. solani* hyphal surfaces (Figure 7A). Signals were not detected upon incubation with FITC alone (Figure 7B).

In Vitro Interaction of mTh-LAAO with Cell Wall Proteins of *R. solani*. We have demonstrated an interaction between Th-LAAO and the hyphal surfaces of *R. solani*. Our binding assays with FITC−mTh-LAAO also suggested that Th-LAAO physically interacts with cell wall components of *R. solani*. To formally test this hypothesis, we investigated whether Th-LAAO, linked to a ConA column, could associate with *R. solani* cell wall proteins. The total cell wall protein extracts of *R. solani* are shown in Figure 8, lane a. Four components of *R. solani* cell wall protein were isolated; these formed a complex with mTh-LAAO in vitro, viz., a 63-, 30-, 17-, and 9-kDa proteins (Figure 8, lane c). We termed these four proteins *R. solani* mTh-LAAO-binding cell wall proteins (LBCWPs). The protein corresponding to the top band in lane c of Figure 8 had a similar migration pattern as that of purified mTh-LAAO (lane d, 67 kDa). We also performed a control experiment using a ConA column only, without mTh-LAAO, and demonstrated that *R. solani* LBCWPs could not be isolated nonspecifically by the ConA column alone (Figure 8, lane b).

These four LBCWPs were then identified by N-terminal Edman sequencing. Although the sequence of the 9-kDa protein could not be determined, because it may be naturally N-terminally modified, the N-terminal amino acid sequences of the 63-, 30-, and 17-kDa proteins were identified as SFSASMIND, ADGIVAVELLTTP, and RHHRG, respectively; however, there were no complete matches of any of these three sequences to the N-terminus of any cell membrane- or cell wall-related protein in the National Center for Biotechnology Information (NCBI) database. Therefore, we deduce that these three LBCWPs may be new fungal cell wall proteins. Moreover, these results confirmed the presence of mTh-LAAO cell wall binding sites on the hyphal surfaces of *R. solani*.

Thus, by affinity chromatography, we isolated the proteins in the *R. solani* cell wall responsible for interaction with mTh-LAAO, showing that mTh-LAAO forms a complex with *R. solani* cell wall proteins (LBCWPs) in vitro. This result suggests that the interaction of mTh-LAAO with LBCWPs of *R. solani*

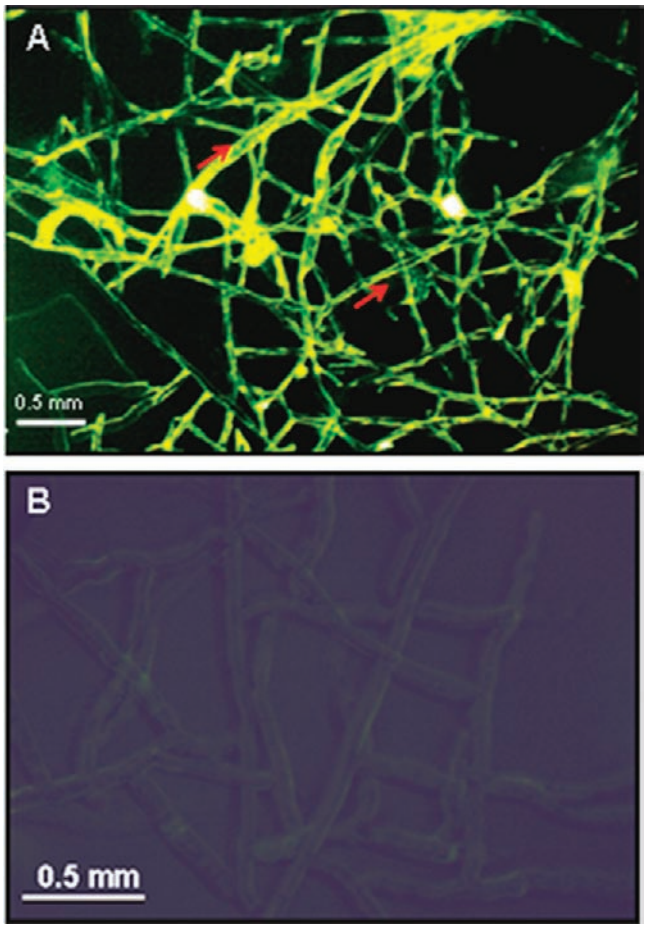

Figure 7. Interaction of mTh-LAAO with *R. solani* hyphal surfaces. (A) FITC-labeled mTh-LAAO-treated *R. solani*. Arrows indicate interaction of FITC-labeled mTh-LAAO with *R. solani* hyphal surfaces. (B) FITC-treated *R. solani* emit no appreciable hyphal surface fluorescence.

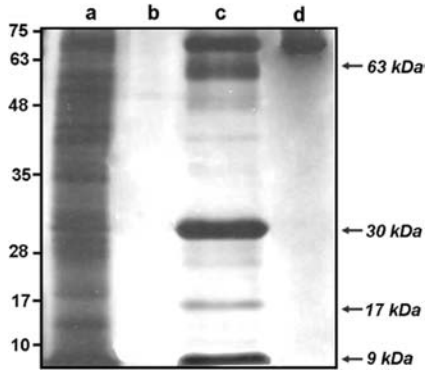

Figure 8. SDS–PAGE analysis of mTh-LAAO binding cell wall proteins (LBCWPs) of *R. solani*. Lane a, crude extracts of *R. solani* cell wall proteins; lane b, fractions of crude extracts after ConA affinity chromatography; lane c, mTh-LAAO binding cell wall proteins and mTh-LAAO; lane d, purified mTh-LAAO alone.

hyphal surfaces may be essential for the mTh-LAAO-induced hyphal lysis.

Effect of LBCWPs on mTh-LAAO-Induced Growth Inhibition in *R. solani*. In light of the mTh-LAAO-induced hyphal lysis, we further examined the significance of the interaction between mTh-LAAO and LBCWPs of *R. solani* hyphal surfaces. To this end, we investigated the effect of blocking the major interaction surfaces for binding between mTh-LAAO and LBCWPs of *R. solani* hyphal surfaces. We hypothesized that this blockage of the binding of mTh-LAAO to *R. solani* would be able to reduce growth inhibition in *R. solani*.

First, the interaction surfaces on mTh-LAAO were presaturated with a pool of *R. solani* LBCWPs. To test growth inhibition, H_2O (control treatment), mTh-LAAO, or the LBCWP–mTh-LAAO complex was incubated with *R. solani* for 5 days. As shown in Figure 9, mTh-LAAO alone induced

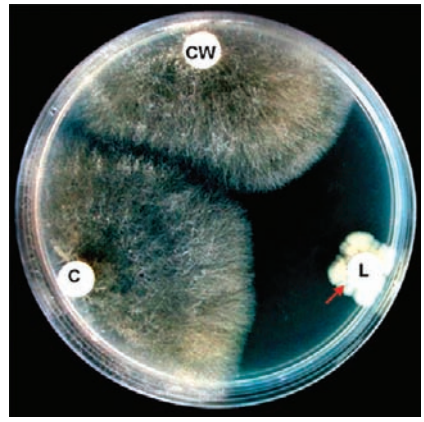

Figure 9. Effects of mTh-LAAO and isolated LBCWP–mTh-LAAO complex on *R. solani* growth. Plate assay: C, control treatment; L, Th-LAAO; CW, LBCWP–mTh-LAAO complex; arrow, hyphal lysis.

hyphal lysis in *R. solani*, but control treatment did not. As we had hypothesized, the LBCWP–mTh-LAAO complex did not inhibit *R. solani* growth. Moreover, no ROS accumulation was detectable in *R. solani* hyphae treated with LBCWP–mTh-LAAO complex (data not shown). Attenuation of the Th-LAAO-induced growth inhibition of *R. solani* by mTh-LAAO presaturated with pool of LBCWPs further supported the proposal that the interaction of Th-LAAO with LBCWPs on *R. solani* hyphal surfaces is a pivotal point in initiating the Th-LAAO-induced growth inhibition of *R. solani*.

Proposed Modes of Action of Th-LAAO in the Antagonistic Effect of *T. harzianum* ETS 323. In our previous study,[7] we identified homodimeric LAAO (Th-LAAO) among secreted proteins of *T. harzianum* ETS 323; however, monomeric Th-LAAO (mTh-LAAO) was the predominant form of the protein when *T. harzianum* was grown in the presence of deactivated *R. solani*. Thus, the deactivated *R. solani* hyphae induced an increase in the amount of Th-LAAO secreted and altered the dimerization state of Th-LAAO; this mTh-LAAO had greater hyphal lysis activity against *R. solani* than did homodimeric Th-LAAO, implicating mTh-LAAO from *T. harzianum* ETS 323 in antagonizing *R. solani*. Previously, we showed that mTh-LAAO at 3 $\mu g \cdot disk^{-1}$ induced a 50% reduction in growth of *R. solani* by the fifth day of incubation.[7] Here, we showed that this concentration of mTh-LAAO induced apoptotic features in *R. solani*, including ROS accumulation, mitochondrial dysfunction, lipid peroxidation, and DNA fragmentation. Apoptotic cell death can be initiated by different stimuli (e.g., anti-receptor antibody), which trigger cell membrane receptors; this process has been referred to as the extrinsic pathway.[23,24] Previous studies have reported that venom LAAO can interact with mammalian cell surfaces and

induce H_2O_2-mediated damage that initiates apoptosis.[2,25] However, we had directly tested the effects of H_2O_2 (10 μM) on *R. solani* but observed no ROS response or growth inhibition, implying that apoptosis of *R. solani* could not be explained fully by this mechanism.

Our results demonstrated that mTh-LAAO physically associate with LBCWPs from *R. solani* hyphal surfaces, and that such interactions induce growth inhibition in *R. solani*. However, blockage of the interaction between mTh-LAAO and *R. solani* markedly reduced mTh-LAAO-triggered hyphal lysis. One possible explanation for this observation is that these four LBCWPs on *R. solani* hyphal surfaces might have receptor-like characteristics, through which the apoptotic response can be triggered in mTh-LAAO-treated *R. solani*. Another possible explanation may be that, through binding to the LBCWPs on *R. solani* hyphal surfaces, mTH-LAAO affects the normal function of these four cell wall proteins. Indeed, in the present study, we showed that mTh-LAAO could induce growth inhibition in *R. solani* not only by an intrinsic apoptosis pathway but also by cell wall protein-mediated damage caused by interaction with mTh-LAAO. Future research is required to establish the relationship between the mTh-LAAO-induced cell wall protein-related damage and the mitochondria-mediated intrinsic apoptosis pathway.

Considered together, these findings suggest the following modes of action of Th-LAAO in the antagonistic effect of *T. harzianum* ETS 323 on *R. solani*: secretion of Th-LAAO is increased, and mTh-LAAO becomes predominant, when *T. harzianum* ETS 323 is grown in the presence of *R. solani*. Then, mTh-LAAO interacts with LBCWPs on *R. solani* hyphal surfaces; this triggers the mitochondria-mediated apoptosis pathway, including cytochrome *c* release and activation of apoptosis factors, caspases 3 and 9. Mitochondrial dysfunction leads to ROS accumulation, and apoptosis leads to DNA fragmentation in *R. solani*.

Despite strong research interest in the cytotoxic activity of LAAO,[2−4] many researchers have also paid attention to the biological purpose of LAAO in the producing host itself. Accordingly, LAAO has been identified in mouse milk (*Mus musculus*), where it is essential for defense of the mammary glands, as is shown by reduction of resistance to intramammary bacterial infection in LAAO-knockout mice.[26] Similarly, a newly identified antimicrobial LAAO has been observed to localize in the spleen, kidney, gill, and blood of the rabbitfish (*Siganus oramin*), where it may contribute to the immune defense mechanisms of the fish to microbes.[27] Interestingly, a similar defensive role of LAAO was observed in both fungus (*T. harzianum* ETS 323) and vertebrate animals (*Mus musculus* and *Siganus oramin*), although there was limited homology between Th-LAAO and other LAAO family members. This result may suggest the biological importance of LAAO for diverse organisms.

In this work, our research elucidated a novel antagonistic mechanism of *T. harzianum* ETS 323 against *R. solani*, which is mediated by mTh-LAAO. Furthermore, our study has elucidated a mechanism by which *T. harzianum* naturally exerts biocontrol over *R. solani* that is mediated by mTh-LAAO and reveals the physiological effect of this enzyme on a fungal pathogen.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional text, describing determination of ROS accumulation, and three figures, showing ROS accumulation before and after treatment with mTh-LAAO and mTh-LAAO-induced growth inhibition of *R. solani*. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

* Tel 886-8633635; fax 886-3-8633630; e-mail kcpeng@mail.ndhu.edu.tw.

Funding

The work was supported by the NSC, Taiwan, under Grant NSC 99-2313-B-212-001-MY3.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

LAAO, L-amino acid oxidase; Th-LAAO, *T. harzianum* ETS 323 L-amino acid oxidase; ROS, reactive oxygen species

■ REFERENCES

(1) Samel, M.; Vija, H. G..; Rönnholm, J. N.; Kalkkinen, S.; Siigur, E. Isolation and characterization of an apoptotic and platelet aggregation inhibiting L-amino acid oxidase from *Vipera berus berus* (common viper) venom. *Biochim. Biophys. Acta* **2006**, *1764*, 707−714.

(2) Torii, S.; Naito, M.; Tsuruo, T. Apoxin I, a novel apoptosis inducing factor with L-amino acid oxidase activity purified from western diamondback rattlesnake venom. *J. Biol. Chem.* **1997**, *272*, 9539−9542.

(3) Du, X. Y.; Clemetson, K. J. Snake L-amino acid oxidases. *Toxicon* **2000**, *40*, 659−665.

(4) Skarnes, R. C. L-Amino acid oxidase, a bactericidal system. *Nature* **1970**, *225*, 1072−1073.

(5) Bnitez, T.; Rincon, A. M.; C.Limon, M.; Codon, A. C. Biocontrol mechanisms of *Trichoderma* strains. *Int. Microbiol.* **2004**, *7*, 249−260.

(6) Tseng, S. C.; Liu, S. Y.; Yang, H. H.; Lo, C. T.; Peng, K. C. Proteomic study of biocontrol mechanisms of *Trichoderma harzianum* ETS 323 in response to *Rhizoctonia solani*. *J. Agric. Food Chem.* **2008**, *56*, 6914−6922.

(7) Yang, C. A.; Cheng, C. H.; Lee, J. W.; Lo, C. T.; Liu, S. Y.; Peng, K. C. A novel L-amino acid oxidase from *Trichoderma harzianum* ETS 323 associated with antagonism of *Rhizoctonia solani*. *J. Agric. Food Chem.* **2011**, *59*, 4519−4526.

(8) Coyle, B.; Kinsella, P.; McCann, M.; Devereux, M.; O'Connor, R.; Clynes, M.; Kavanagh, K. Induction of apoptosis in yeast and mammalian cells by exposure to 10-phenanthroline metal complexes. *Toxicol. In Vitro* **2004**, *18*, 63−70.

(9) Ohkawa, H. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **1979**, *95*, 351−358.

(10) Liu, X.; Kim, C. N.; Yang, J.; Jemmerson, R.; Wang, X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome C. *Cell* **1996**, *86*, 147−157.

(11) Suhr, S. M.; Kim, D. S. Identification of the snake venom substance that induces apoptosis. *Biochem. Biophys. Res. Commun.* **1996**, *224*, 134−139.

(12) Mahadevan, P. R.; Tatum, E. L. Relationship of the major constituents of the *Neurospora crassa* cell wall to wild-type and colonial morphology. *J. Bacteriol.* **1965**, *90*, 1073−1081.

(13) Vadassery, J.; Ranf, S.; Drzewieck, C.; Mithöfer, A.; Mazars, C.; Scheel, D.; Lee, J.; Oelmüller, R. A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of *Arabidopsis* seedlings and induces intracellular calcium elevation in roots. *Plant J.* **2009**, *59*, 193−206.

(14) Klassen, R.; Meinhardt, F. Induction of DNA damage and apoptosis in *Saccharomyces cerevisiae* by a yeast killer toxin. *Cell Microbiol.* **2005**, *7*, 393−401.
(15) Ito, S.; Ihara, T.; Tamura, H.; Tanaka, S.; Ikeda, T.; Kajihara, H.; Dissanayake, C.; Abdel-Motaal, F. F.; El-Sayed, M. A. alpha-Tomatine, the major saponin in tomato, induces programmed cell death mediated by reactive oxygen species in the fungal pathogen *Fusarium oxysporum*. *FEBS Lett.* **2007**, *581*, 3217−3222.
(16) Kobayashi, D.; Kondo, K.; Uehara, N.; Otokozawa, S.; Tsuji, N.; Yagihashi, A.; Watanabe, N. Endogenous reactive oxygen species is an important mediator of miconazole antifungal effect. *Antimicrob. Agents Chemother.* **2002**, *46*, 3113−3117.
(17) Semighini, C. P.; Hornby, J. M.; Dumitru, R.; Nickerson, K. W.; Harris, S. D. Farnesol-induced apoptosis in *Aspergillus nidulans* reveals a possible mechanism for antagonistic interaction between fungi. *Mol. Microbiol.* **2006**, *59*, 753−764.
(18) Ikemoto, H.; Tani, E.; Ozaki, I.; Kitagawa, H.; Arita, N. Calphostin C-mediated translocation and integration of Bax into mitochondria induces cytochrome c release before mitochondrial dysfunction. *Cell Death Differ.* **2000**, *7*, 511−520.
(19) Matsuyama, S.; Xu, Q.; Velours, J.; Reed, J. C. The mitochondrial F0F1-ATPase proton pump is required for function of the proapoptotic protein Bax in yeast and mammalian cells. *Mol. Cell* **1998**, *1*, 327−336.
(20) Li, P.; Nijhawan, D.; Budihardjo, I.; Srinivasula, S. M.; Ahmad, M.; Alnemri, E. S. Cytochrome C and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **1997**, *91*, 479−489.
(21) Zou, H.; Henzel, W. J.; Liu, X.; Lutschg, A.; Wang, X. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome C-dependent activation of caspase-3. *Cell* **1997**, *90*, 405−413.
(22) Mousavi, S. A. A.; Robson, G. D. Entry into the stationary phase is associated with a rapid loss of viability and an apoptotic-like phenotype in the opportunistic pathogen *Aspergillus fumigatus*. *Fungal Genet. Biol.* **2003**, *39*, 221−229.
(23) Wallach, D.; Boldin, M. P.; Goncharov, T. M.; Goltsev, Y. V.; Mett, I.; Malinin, N.; Adar, R.; Kovalenko, A.; Varfolomeev, E. Exploring cell death mechanisms by analyzing signaling cascades of the TNF/NGF receptor family. *Behring Inst. Mitt. Rev.* **1996**, *9*, 144−155.
(24) Liu, B.; Fang, M.; Schmidt, M.; Lu, Y.; Mendelsohn, J.; Fan, Z. Induction of apoptosis and activation of the caspase cascade by anti-EGF receptor monoclonal antibodies in DiFi human colon cancer cells do not involve the c-jun N-terminal kinase activity. *Br. J. Cancer* **2000**, *82*, 1991−1999.
(25) Suhr, S. M.; Kim, D. S. Identification of the snake venom substance that induces apoptosis. *Biochem. Biophys. Res. Commun.* **1996**, *224*, 134−139.
(26) Nagaoka, K.; Aoki, F.; Hayashi, M.; Muroi, Y.; Sakurai, T.; Itoh, K.; Ikawa, M.; Okabe, M.; Imakawa, K.; Sakai, S. L-Amino acid oxidase plays a crucial role in host defense in the mammary glands. *FASEB J.* **2009**, *23*, 2514−2520.
(27) Wang, F.; Li, R.; Xie, M.; Li, A. The serum of rabbitfish (*Siganus oramin*) has antimicrobial activity to some pathogenic organisms and a novel serum L-amino acid oxidase is isolated. *Fish Shellfish Immunol.* **2011**, *30*, 1095−1108.