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α-Tomatine, the major saponin in tomato, induces programmed cell death mediated by reactive oxygen species in the fungal pathogen *Fusarium oxysporum*

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Abstract **The tomato saponin α-tomatine has been proposed to kill sensitive cells by binding to cell membranes followed by leakage of cell components. However, details of the modes of action of the compound on fungal cells are poorly understood. In the present study, mechanisms involved in α-tomatine-induced cell death of fungi were examined using a filamentous pathogenic fungus *Fusarium oxysporum*. α-Tomatine-induced cell death of *F. oxysporum* (TICDF) occurred only under aerobic conditions and was blocked by the mitochondrial F_0F_1-ATPase inhibitor oligomycin, the caspase inhibitor D-VAD-fmk, and protein synthesis inhibitor cycloheximide. Fungal cells exposed to α-tomatine showed TUNEL-positive nuclei, depolarization of transmembrane potential of mitochondria, and reactive oxygen species (ROS) accumulation. These results suggest that TICDF occurs through a programmed cell death process in which mitochondria play a pivotal role. Pharmacological studies using inhibitors suggest that α-tomatine activates phosphotyrosine kinase and monomeric G-protein signaling pathways leading to Ca^{2+} elevation and ROS burst in *F. oxysporum* cells.**

Keywords: Glycoalkaloid; Apoptosis; *Fusarium oxysporum*

1. Introduction

α-Tomatine, a glycoside lycotetraose is attached to the 3-OH group of the aglycon tomatidine, is the major saponin in tomato (*Lycopersicon esculentum*). Tomato plants contain α-tomatine at 10–30 mg/kg, which is enough to kill microbes [1]. Indeed, α-tomatine has been shown to kill a broad range of fungi in vitro and functions as a resistance substance (phytoanticipin) against phytopathogens in tomato [2]. The fungicidal mode of action of α-tomatine proposed is that α-tomatine forms a complex with fungal membrane sterols with free 3β-hydroxyl groups, resulting in pore formation and loss of membrane integrity followed by leakage of cell components and cell death [3,4], references therein]. However, α-tomatine has been reported to inhibit the growth of some fungi lacking sterols in their cell membranes, suggesting that α-tomatine possesses an unknown fungicidal action [4].

Fusarium oxysporum is a phytopathogen causing a vascular wilt disease in many important crops and can be hard to be controlled [5]. *F. oxysporum* is also known as a serious emerging pathogen of humans due to the increasing number of severe cases reported and to its broad resistance to the available antifungal drugs [6,7]. Recently we found *F. oxysporum* strains that are highly sensitive to α-tomatine [8]. Understanding the mechanisms involved in the sensitivity of the strains may provide new insight into the fungicidal action of this compound and lead to the development of novel antifungal agents active against *F. oxysporum*.

In the present study, we demonstrate that α-tomatine-induced cell death of *F. oxysporum* (TICDF) occurs through a programmed cell death accompanying a rapid generation of reactive oxygen species (ROS) in the cells, which is a novel fungicidal action of α-tomatine. We also demonstrate that α-tomatine appears to activate tyrosine kinase and monomeric GTP-binding protein (G-protein) signaling pathways leading to Ca^{2+} elevation and ROS burst in *F. oxysporum* cells.

2. Materials and methods

2.1. Strains and culture conditions

F. oxysporum strains (MAFF103057, 103058, 727516, and 744001) were obtained from the National Institute of Agricultural Sciences, Tsukuba, Japan. Fungal strains were maintained on PDA medium (Daigo, Tokyo, Japan) at 25 °C. For liquid cultures, fungal strains were grown in Potato Dextrose medium (PD medium; Daigo, Tokyo, Japan) at 25 °C with shaking at 100 rpm. Spore suspensions of *F. oxysporum* were prepared from 4-day-old liquid cultures, harvested by filtration through three layers of gauze cloth, centrifuged at $3000 \times g$ for 20 min and suspended in distilled water.

2.2. Chemicals

α-Tomatine, A23187, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*″-tetraacetic acid (BAPTA), cycloheximide, dimethylthiourea, neomycin, ryanodine, and 8-(diethylamino) octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) were purchased from Sigma–Aldrich

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(St. Louis, MO, USA). Ascorbic acid, lanthanum chloride ($LaCl_3$), nifedipine, oligomycin, Ruthenium Red, and standard oxalate solution were obtained from Wako (Osaka, Japan). α-Cyano-(3,4-dihydroxy)-cinnamonitrile (AG18) and *N*-[2(*S*)-[2(*R*)-Amino-3-mercaptopropylamino]-3-methylbutyl]-Phe-Met-OH (B581) were from Calbiochem (San Diego, CA, USA). 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*″-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) was from Nakarai tesque (Kyoto, Japan). D-Erythro-sphingosine was from Alexis (San Diego, CA, USA). Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*″-tetraacetic acid (EGTA) was from Kanto Chemical (Tokyo, Japan). Wartmannin was from Alomone Labs (Jerusalem, Israel). Z-VAD-fmk was from Promega (Tokyo, Japan). α-Tomatine (4 mM) was dissolved in 50 mM sodium citrate buffer (pH 4.0) or in dimethylsulfoxide (DMSO). A23187, B581, BAPTA, BAPTA-AM, neomycin, nifedipine, ryanodine, tyrphostin A23, and wartmannin were dissolved in DMSO (final concentration: 10 mM). Solutions of cycloheximide (50 mM), EGTA (100 mM), $LaCl_3$(100 mM), and TMB-8 (10 mM) were made up in water. D-Erythro-sphingosine (10 mM), oligomycin (50 μM), and Ruthenium Red (10 mM) were dissolved in dichloromethane, methanol, and 50% DMSO, respectively. For each treatment, control experiments were performed using the appropriate volume of solvent.

2.3. Cell death assay

F. oxysporum spores were suspended in CA medium [1% (w/v) casamino acids, 10 mM ammonium sulfate, and 0.05% (w/v) yeast nitrogen base, pH 5.0] at 1×10^6 spores/ml and incubated with shaking (100 rpm) at 25 °C for 16 h. α-Tomatine was added to the vegetative mycelia and the culture was incubated at 25 °C with shaking. After varying times of incubation, an aliquot (50 μl) of the culture was taken and mixed with 5 μl of Evans blue dye (10 mg/ml). After 5 min of incubation, fungal cells were transferred onto glass slides and observed by light microscopy. In experiments to the determine inhibitory effects of chemicals on TICDF, chemicals, made up as stock solutions, were added to the fungal culture to give the appropriate concentration either 30 min before, after, or simultaneously with α-tomatine exposure. After 1 h incubation with α-tomatine, dead cells were determined with Evans blue dye staining described above. Each experiment was performed in triplicate. At the concentrations used in this study, the chemicals alone had no effect on the viability of *F. oxysporum* cells in the absence of α-tomatine.

2.4. Electrolytes leakage assay

Electrolyte loss studies were performed according to the method of Steel et al. [9]. Electrical conductivity of the fungal suspension was measured using a conductivity meter (ES-12, Horiba, Tokyo, Japan). Maximum conductivity was determined by adding chloroform at the end of each experiment.

2.5. Analysis of programmed cell death markers

For terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), *F. oxysporum* cells exposed to α-tomatine (40 μM) were fixed with 3.7% formaldehyde for 30 min, washed with PBS, and the fungal suspension was transferred onto polylysine-coated slides (Matsunami, Tokyo, Japan) and air-dried to fix fungal cells on the slides. After removing excess PBS with a filter paper, 100 μl of PBS containing Kitarase (0.4 mg/ml; Yakult, Tokyo, Japan) and Novozyme (1 mg/ml; Novo Nordisk, Bagsvaerd, Denmark) was added to the fungal cells on the slides and incubated in a humid chamber for 3 h for removing cell walls from the fungal cells. Cell permeabilization and the TUNEL reactions were carried out by using an In situ Cell Death Detection kit (Roche diagnostics, Tokyo, Japan) according to the manufacturer's instructions. TUNEL-positive cells were detected using an epifluorescence microscope (BH2-RFK, Olympus, Tokyo, Japan). Depolarization of mitochondrial membrane potential of *F. oxysporum* cells was determined by incubation of fungal cells with MitoCapture cation dye (Calbiochem) according to the manufacturer's instructions.

2.6. Detection of ROS in fungal cells

Generation of ROS by *F. oxysporum* cells was detected by epifluorescence microscopy by monitoring the conversion of non-fluorescent dihydrorhodamine 123 (DHR 123; Sigma–Aldrich) to fluorescent rhodamine 123. In time course experiments, production of ROS (H_2O_2) was measured with a luminometer BLR-201 (Aloka, Tokyo, Japan) by peroxide-dependent chemiluminescence of luminol. Fungal mycelial mat (100 mg) was suspended in 30 ml of CA medium. α-Tomatine was added to the mycelial suspension at the final concentration of 40 μM. At varying time, the mycelial suspension was filtrated with a membrane filter (pore size 0.45 μm), and the filtrate (1 ml) was mixed with 3.5 ml of 50 mM potassium phosphate buffer (pH 7.8) and 500 μl of 1.2 mM luminol in 50 mM potassium phosphate buffer in a vial set in the luminometer. The reaction was started by adding 500 μl of 10 mM potassium ferricyanide.

2.7. 2D gel electrophoresis, immunostaing, and proteomic analysis

F. oxysporum cultures were filtrated with a membrane filter and resultant fungal mat (1 g) was resuspended in 100 ml of CA medium. α-Tomatine (40 μM) was added aseptically to the culture, and then the culture was incubated with shaking. Proteins were prepared and analyzed according to the methods of Ito et al. [8]. For detection of tyrosine-phosphorylated proteins, proteins after 2D PAGE were blotted onto a polyvinylidene difluoride membrane (immobilom-P, Millipore, Bedford, MA), and the membrane was stained with anti-phosphotyrosine antibody (Amersham, Piscataway, NJ). The blots were developed using the ECL phosphorylation detection system (Amersham) and exposed on an X-ray film. For proteomic analysis, in-gel digestion with trypsin was performed according to the method reported by Kikuchi et al. [10].

3. Results

F. oxysporum cells were exposed to α-tomatine in a 1 ml culture either in a 2-ml eppendorf tube with no shaking (low-aerobic condition) or in a 15 ml tube with a sponge plug with shaking at 100 rpm (aerobic condition). Fungicidal action of α-tomatine was observed only under the aerobic condition (Fig. 1A), suggesting that the major oxygen-consuming organelle, mitochondria, would be involved in TICDF. Since all four strains of *F. oxysporum* examined showed similar responses against α-tomatine, we performed subsequent experiments using one of the strains, *F. oxysporum* f. sp. *raphani* MAFF103058.

We examined whether a specific inhibitor of mitochondrial F_0F_1-ATP synthase, oligomycin, would block the fungicidal action of α-tomatine. Oligomycin markedly inhibited TICDF, suggesting that ATP is necessary for TICDF (Fig. 1B). This result raised the possibility that α-tomatine may induce programmed cell death (PCD), since ATP is required for PCD in eukaryotic cells [11].

In PCD, caspases are activated and novel proteins are synthesized. Thus, we examined whether the broad-spectrum caspase inhibitor Z-VAD-fmk and the protein synthesis inhibitor cycloheximide would block TICDF. Both Z-VAD-fmk and cycloheximide reduced TICDF in a dose-dependent manner (Fig. 1B). PCD is characterized by a set of distinct morphological changes including DNA fragmentation and depolarization of the transmembrane potential of mitochondria. DNA fragmentation is detected in situ by the TUNEL assay, as 3′-OH DNA ends labeled with FITC-conjugated dUTP via terminal deoxynucleotidyltransferase can be visualized by fluorescence microscopy [12]. The TUNEL assay of *F. oxysporum* cells exposed to α-tomatine showed high percentage (>80%) of positive cells (Fig. 1C). Depolarization of the transmembrane potential of mitochondria, which is early event in the PCD [13], is detected by a cationic dye (Mitocapture) that fluoresces differently in healthy and PCD cells. Healthy cells show accumulation of Mitocapture in the mitochondria, producing a red fluorescence, whereas in PCD cells the dye remains in the cyto-