Antimicrobial peptaibols from *Trichoderma pseudokoningii* induce programmed cell death in plant fungal pathogens

Mei Shi,[†] Lei Chen,[†] Xiao-Wei Wang, Tian Zhang, Pei-Bao Zhao, Xiao-Yan Song, Cai-Yun Sun, Xiu-Lan Chen, Bai-Cheng Zhou and Yu-Zhong Zhang

Correspondence

Yu-Zhong Zhang
zhangyz@sdu.edu.cn
Xiao-Yan Song
xysong1209@sohu.com

State Key Laboratory of Microbial Technology, Marine Biotechnology Research Center, Shandong University, Jinan 250100, PR China

Antibiosis is one of the widespread strategies used by *Trichoderma* spp. against plant fungal pathogens, the mechanism of which, however, remains poorly understood. Peptaibols are a large family of antimicrobial peptides produced by *Trichoderma* spp. Our previous study showed that trichokonins, a type of peptaibol from *Trichoderma pseudokoningii* SMF2, exhibited antibiotic activities against plant fungal pathogens. In this study, we first demonstrated that trichokonin VI (TK VI) induced extensive apoptotic programmed cell death in plant fungal pathogens. For a deeper insight into the apoptotic mechanism involved in the action of TK VI, *Fusarium oxysporum* was used as a model. Cells of *F. oxysporum* treated with TK VI showed apoptotic hallmarks, such as exposure of phosphatidylserine, the appearance of reactive oxygen species and fragmentation of nuclear DNA. Moreover, TK VI-treated cells exhibited an accumulation of cytoplasmic vacuoles with loss of the mitochondrial transmembrane potential, and this process was independent of metacaspases. Therefore, TK VI induces metacaspase-independent apoptotic cell death in *F. oxysporum*. This represents what is believed to be the first report to reveal the antibiotic mechanism of peptaibols against plant fungal pathogens.

Received 29 June 2011

Revised 15 October 2011

Accepted 2 November 2011

INTRODUCTION

Some *Trichoderma* species, such as *Trichoderma harzianum*, *Trichoderma virens* and *Trichoderma viride*, are commercial biological control agents (BCAs) against plant fungal pathogens (Chet & Inbar, 1994; Howell, 2003; Benítez *et al.*, 2004). *Trichoderma* spp. act as BCAs through mycoparasitism, antibiosis, substrate competition, and production of antibiotics and cell wall-degrading enzymes (CWDEs) to inhibit the growth of fungal pathogens, promote plant growth and induce resistance in plants (Yedidia *et al.*, 1999; Viterbo *et al.*, 2005). Antibiosis is a widespread strategy used for defence by *Trichoderma* spp. (Pyke & Dietz, 1966; Whitmore & Wallace, 2004). *Trichoderma* spp. produce a variety of antibiotics, such as gliovirin, gliotoxin, viridin, pyrones and peptaibols, against

fungal phytopathogens (Howell, 2003; Harman *et al.*, 2004). However, among the reported biocontrol mechanisms of *Trichoderma*, very few studies have been done on their antibiotic mechanism.

Peptaibols are a large family of antibiotic peptides from soil fungi, including *Trichoderma* and related genera such as *Emerizelopsis* and *Gliocladium* (Daniel & Filho, 2007). To date, 317 peptaibols have been reported, and among them more than 190 are synthesized by *Trichoderma*. Details are compiled in the Peptaibol Database (<http://www.cryst.bbk.ac.uk/peptaibol>) (Whitmore & Wallace, 2004). Peptaibols are characterized by the presence of an unusual amino acid, α -aminoisobutyric acid (Aib), a C-terminal-hydroxylated and N-terminal-acetylated amino acid, and they are able to form voltage-dependent ion channels in lipid bilayer membranes because of their linear and amphipathic nature (Chugh & Wallace, 2001). Most research on peptaibols has focused on their biosynthetic pathways, conformational properties and amino acid sequences (Benedetti *et al.*, 1982; Wiest *et al.*, 2002). It has also been reported that peptaibols promote the inhibition of mitochondrial ATPase, uncoupling of oxidative phosphorylation, immunosuppression, inhibition of platelet aggregation and induction of fungal morphogenesis (Shima *et al.*, 1990; Matsuzaki *et al.*, 1991;

[†]These authors contributed equally to this work.

Abbreviations: BAPTA, 1,2-bis(O-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid; PCD, programmed cell death; PI, propidium iodide; PS, phosphatidylserine; ROS, reactive oxygen species; TK VI, VII and VIII, trichokonin VI, VII and VIII; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labelling.

Three supplementary figures are available with the online version of this paper.

Csermely *et al.*, 1994; Katayama *et al.*, 2001; Peltola *et al.*, 2004). However, the antimicrobial mechanisms of peptaibols are largely unknown.

Trichoderma pseudokoningii SMF2, which was described as *Trichoderma koningii* SMF2 in our previous studies (Chen *et al.*, 2009), produces three major peptaibols, trichokonin VI (TK VI), trichokonin VII and trichokonin VIII. These peptaibols were first identified by Qing Huang (Huang *et al.*, 1994, 1995). The peptaibols from *T. pseudokoningii* SMF2 have been shown to exhibit broad-spectrum antimicrobial activity against Gram-positive bacteria and filamentous fungi, including plant fungal pathogens. The aim of this study was to reveal the antibiotic mechanism of trichokonins against plant fungal pathogens. We first demonstrated that TK VI showed antibiotic activities against some plant fungal pathogens by inducing an apoptotic programmed cell death (PCD) in the fungal cells. Then, we chose *Fusarium oxysporum* as a model system to investigate the apoptotic mechanism involved in the action of TK VI. *F. oxysporum* is a harmful and ubiquitous soil-borne ascomycete, and causes vascular wilt disease in more than 100 different plant species, which is difficult to control (Mes *et al.*, 1999; Berrocal-Lobo & Molina, 2008). Our results show that TK VI induces a metacaspase-independent apoptotic PCD in parallel with the accumulation of cytoplasmic vacuoles in *F. oxysporum*, which reveals the antibiotic mechanism of peptaibol against plant fungal pathogens.

METHODS

Reagents. D-Sorbitol was obtained from Amresco. A Mitochondrial Apoptosis Detection kit (MitoCapture) was obtained from BioVision. A Fluorescein FragEL DNA Fragmentation Detection kit was purchased from Calbiochem. SYTOX Green nucleic acid stain (M_r : 600, 504/523 nm) was purchased from Sigma. Annexin-V-FLUOS, propidium iodide (PI) and a Homogeneous Caspases Assay were purchased from Roche Diagnostics. An Oxygen Species Detection kit and 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) acetoxy-methyl ester were obtained from Molecular Probes.

Strains and culture conditions. Plant fungal pathogens, *Ascochyta citrullina*, *Botrytis cinerea*, *F. oxysporum*, *Phytophthora parasitica* and *Verticillium dahliae*, were obtained from the Agricultural Culture Collection of China (ACCC) and maintained on potato dextrose agar (PDA) at 28 °C. For liquid culture, the fungal pathogens were incubated at 28 °C in potato dextrose broth (PDB) in an orbital shaker (100 r.p.m.).

Purification of trichokonins. *T. pseudokoningii* SMF2 was fermented by solid-state fermentation and TK VI was purified as previously described (Song *et al.*, 2007). The purified TK VI was directly dissolved in phosphate buffer solution.

Determination of antifungal activity. The antifungal activities of TK VI against *A. citrullina*, *B. cinerea*, *F. oxysporum*, *P. parasitica* and *V. dahliae* were investigated using the agar disk diffusion assay, as described elsewhere (Lehtopolku *et al.*, 2010). To test the effect of TK VI on the hyphal elongation of the fungal pathogens, isolates were applied to PDA plates containing different concentrations (0, 25,

50 µM) of TK VI and cultured at 28 °C for 168 h. Each test was carried out in triplicate.

Confirmation of cell death and apoptosis. To identify cells undergoing DNA fragmentation, hyphae treated with 25 µM TK VI for 12 h were subjected to terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labelling (TUNEL) analysis, as described by Cheng *et al.* (2003), using a Fluorescein FragEL DNA Fragmentation Detection kit (Calbiochem). Samples were observed under a fluorescence microscope (Nikon).

Phosphatidylserine (PS) externalization was tested on *F. oxysporum* protoplasts. To facilitate the detection of PS exposure on cell membranes, *F. oxysporum* protoplasts were used in this experiment. Protoplasts were prepared as described by Phillips *et al.* (2006), with some modifications. Glucanex (10 mg ml⁻¹; Novozyme) was used to digest the cell wall of *F. oxysporum* hyphae for 1 h. A total of 4×10^6 protoplasts were resuspended in 100 ml isotonic solution (1 M sorbitol, 10 mM Tris/HCl, pH 7.5) and treated with 10 and 25 µM TK VI. PS exposure on the protoplast surface was determined after 4 h of TK VI treatment by Annexin V assay using Annexin V-FITC and PI according to the manufacturer's instructions. Samples were observed under a fluorescence microscope (Nikon) and analysed by flow cytometer (Epics XL-MCL, Coulter).

Morphological changes of cells treated with TK VI. Membrane permeabilization was measured by the uptake of SYTOX Green dye (M_r : 600, 504/523 nm). The *F. oxysporum* hyphae were analysed using a fluorescence microscope (Nikon). For the detection of SYTOX Green uptake, an excitation wavelength of 488 nm and an emission wavelength of 500–554 nm were used.

For transmission electron microscopy, *F. oxysporum* hyphae were initially fixed in 0.1 M sodium phosphate buffer containing 2.5% (v/v) glutaraldehyde (pH 7.4). Next, the hyphae were fixed in 0.1 M sodium phosphate buffer containing 1% (v/v) OsO₄ (pH 7.2). Then the hyphae were embedded in Ultracut (Leica) and sliced into 60 nm sections. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a JEM-1230 transmission electron microscope (JEOL).

Analysis of mitochondrial membrane potential and production of reactive oxygen species (ROS). After the fungal hyphae had been treated with 10 or 25 µM TK VI, changes in mitochondrial membrane potential ($\Delta\psi_m$) were evaluated using MitoCapture according to the manufacturer's instructions. Hyphae were incubated at 37 °C for 15 min in 1 ml diluted MitoCapture reagent and subsequently analysed by fluorescence microscopy with a fluorescence microscope (Nikon).

To facilitate the detection of ROS formation using flow cytometry, protoplasts were used to analyse ROS production. Protoplasts were incubated in 10 or 25 µM TK VI for 6 h. Intracellular ROS production was determined using an Image-iT LIVE Green Reactive Oxygen Species Detection kit following the manufacturer's instructions. Samples were observed under a fluorescence microscope (Nikon) and analysed by EPICS XL-MCL flow cytometer.

Metacaspase activity analysis. In order to lyse cells efficiently, protoplasts of *F. oxysporum* were prepared and treated with different concentrations (0, 5, 10, 25 and 50 µM) of TK VI and then subjected to a metacaspase activity assay using the Homogeneous Caspases Assay (fluorimetric) kit according to the manufacturer's instructions (Roche Diagnostics).

Statistical analysis. All results are expressed as mean $\pm$ SD. $P < 0.01$ was considered statistically significant. All statistical analyses were performed using SPSS 11.5 for Windows.

Fig. 1. Detection of PS externalization in *F. oxysporum* after TK VI treatment. (a) Exposure of PS in cells treated with TK VI. Cells were treated with TK VI (10 or 25 μ M) for 4 h. Fluorescence and differential interference contrast (DIC) micrographs showing PS exposure of cells are as follows: healthy protoplasts, Annexin⁻, PI⁻; early apoptotic protoplasts, Annexin⁺, PI⁻; late apoptotic or necrotic protoplasts, Annexin⁺, PI⁺. (b) Exposure of PS analysed on a flow cytometer. Cells were treated with TK VI (10 or 25 μ M) for 4 h and analysed using flow cytometry with FITC-labelled Annexin V and PI. Bottom left, viable cells (PI⁻, Annexin V-FITC⁻); bottom right, early apoptotic cells (PI⁻, Annexin V-FITC⁺); top right, late apoptotic/necrotic cells (PI⁺, Annexin V-FITC⁺); top left, necrotic cells. Numbers in the quadrants indicate the proportions of cells in the corresponding areas. Experiments were performed in triplicate, with similar results. (c) Cells were treated in the same way as in (a). The quantitative analysis of apoptosis in cells was analysed by FACS with Annexin V/PI. Results were expressed as means $\pm$ SD of triplicate experiments (each performed in duplicate). ^a $P < 0.01$ and ^b $P < 0.01$, TK VI treatment versus control; ^c $P < 0.01$, TK VI + BAPTA versus TK VI treatment.

RESULTS

TK VI is an antimicrobial peptide with a broad spectrum of pro-apoptotic activity in plant fungal pathogens

Our previous study primarily demonstrated that trichokonnins exhibit antimicrobial activity against several fungal pathogens (Song *et al.*, 2006). The antifungal activity of TK VI was further evaluated *in vitro* against the phytopathogenic fungi *A. citrullina*, *B. cinerea*, *F. oxysporum*, *P. parasitica* and *V. dahliae*. TK VI showed strong antifungal activity against *A. citrullina*, *B. cinerea*, *F. oxysporum* and *P. parasitica*, and moderate activity against *V. dahliae* (Supplementary Fig. S1). With 25 or 50 μ M TK VI treatment, the colony diameters of the first four isolates decreased dramatically compared with that of the control. Although the diameter of the *V. dahliae* isolate displayed only a small decrease after 25 and 50 μ M TK VI treatment, the whitish *V. dahliae* colony turned brownish after TK VI treatment, and the brownish hyphae exhibited apoptosis (data not shown), indicating that TK VI caused growth retardation in *V. dahliae*. However, there was no further significant decrease in the diameter of the isolates when the TK VI concentration was increased above 50 μ M (data not shown).

Then, we tried to find the mechanism by which TK VI causes cell suicide of plant fungal pathogens. Since apoptosis is a common pattern of cell death caused by many stresses (Liang *et al.*, 2008), we first tested whether the antifungal activity of TK VI acted through the rapid induction of apoptosis in plant fungal pathogens. The fragmentation of nuclear DNA is one of the most common cytological markers of PCD in yeast and mammalian cells (Madeo *et al.*, 2002). Therefore, we investigated whether TK VI induced PCD in the tested plant fungal pathogens by detecting DNA fragmentation using TUNEL assays. Exposure of the hyphae of the above five fungal phytopathogens to 25 μ M TK VI resulted in a dramatic increase in the proportion of TUNEL-positive nuclei compared with the control population (Supplementary Fig. S2), showing that DNA fragmentation occurred in TK VI-treated cells. After treatment with TK VI for 12 h, the percentages of TUNEL-positive cells in *F. oxysporum*, *B. cinerea*, *A. citrullina*, *P. parasitica* and *V. dahliae* reached approximately 80, 70, 60, 45 and 40 %, respectively. Taken together, the results strongly suggested that TK VI plays a pro-apoptotic role via its potent antifungal activity. Accordingly, *F. oxysporum* was used as a model genetic system to elucidate the apoptotic process triggered by TK VI.

TK VI treatment induces exposure of PS in *F. oxysporum* cells

In addition to fragmentation of nuclear DNA, another biochemical hallmark of apoptotic cell death is the translocation of PS from the cytoplasmic surface to the

external surface of the cell membrane (Häcker, 2000). Exposure of PS at the surface of apoptotic cells is readily detected using fluorescently labelled Annexin V, which specifically binds PS. To further characterize TK VI-induced apoptotic cell death in *F. oxysporum*, we detected PS surface exposure by Annexin V staining of the TK VI-treated protoplasts. Plasma membrane integrity was simultaneously assessed by PI dye exclusion. In 10 μ M TK VI-treated protoplasts, the proportion of Annexin V-positive/PI-negative cells (early apoptotic cells) increased with time (Fig. 1). Simultaneously, the late-apoptotic cells

Fig. 2. Membrane integrity of TK VI-treated cells. The permeabilization of membranes was monitored using SYTOX Green. Cells were treated with 25 and 50 μ M TK VI for 12 h (a) or 24 h (b). SYTOX Green staining of nuclei was analysed using a fluorescence microscope. DIC, differential interference contrast micrographs.

increased in the protoplasts treated with 25 μM TK VI, based on the increase of the Annexin V- and PI-positive population. Therefore, TK VI was able to cause PS exposure and induce apoptotic death in *F. oxysporum* cells. However, when the protoplasts were pretreated with BAPTA (a calcium chelator), the onset of apoptosis induced by TK VI dramatically diminished (Fig. 1c), indicating that Ca^{2+} is involved in TK VI-mediated apoptosis in *F. oxysporum*.

TK VI triggers a change of fungal membrane permeability and disintegration of subcellular structures

Membrane damage of *F. oxysporum* hyphae exposed to TK VI was evaluated with the SYTOX Green uptake assay. Fungal hyphae grown in the absence of TK VI displayed no fluorescence (Fig. 2a). Similar results were observed with hyphae treated with 25 μM TK VI for 12 h (Fig. 2a). However, SYTOX Green entered the nucleus and combined with the DNA in the cells treated with 25 μM TK VI for 24 h (Fig. 2b). Furthermore, when hyphae were incubated with 50 μM TK VI for 24 h, approximately 55% of hyphae stained positive for SYTOX Green (Fig. 2b). The results indicated that TK VI could disrupt the integrity of the *F. oxysporum* cell membrane either via long-term treatment at a low concentration or via high-concentration treatment.

Ultrastructural changes accompanying PCD in the hyphae of *F. oxysporum* cells were detected using transmission electron microscopy after 16 h of TK VI treatment. Most cells treated with 25 μM TK VI were drastically vacuolated, and the size of the vacuoles reached 2–3 μm , while control cells did not develop any discernible vacuoles (Fig. 3a, b). Moreover, significant mitochondrial swelling was observed in the cells treated with 50 μM TK VI for 16 h (Fig. 3c). Mitochondria were swollen, with disrupted membranes and the organization of the cristae was lost. In contrast, control cells showed intact mitochondrial morphology, with densely packed and ordered cristae (Fig. 3a). Morphologically, the vacuoles were derived from mitochondria or endoplasmic reticula. However, if cells were pre-treated with BAPTA, the number of vacuoles was decreased (Fig. 3d), suggesting that vacuolation induced by TK VI was associated with calcium.

TK VI has an effect on mitochondrial membrane permeabilization and intracellular ROS production in *F. oxysporum*

An increasing amount of evidence shows that mitochondria play central roles in the regulation of apoptotic cell death (Pereira *et al.*, 2008). In the sequence of events taking place in mitochondria during the course of apoptosis, loss of the mitochondrial transmembrane potential ($\Delta\psi_{\text{m}}$) appears to be the initial event (Eisenberg *et al.*, 2007). To study the effect of TK VI on the $\Delta\psi_{\text{m}}$ of *F. oxysporum* cells,

Fig. 3. Morphological changes in TK VI-treated cells. Cells were treated with different doses of TK VI, or pretreated with 10 mM BAPTA. Subsequently, ultrastructural morphological changes associated with cell death were detected after 16 h of treatment with TK VI. N, nucleus; M, mitochondrion; V, vacuole; ER, endoplasmic reticulum. Extensive cytoplasmic vacuolization is indicated by arrows. (a) Control, (b) 25 μM TK VI treatment, (c) 50 μM TK VI treatment, (d) BAPTA + 50 μM TK VI treatment.

F. oxysporum hyphae treated with TK VI were detected with MitoCapture reagent, which emits an orange-red fluorescence in healthy cells and emits green fluorescence in apoptotic cells. Under the fluorescence microscope, the control cells displayed bright orange-red fluorescence. After 6 h of treatment with 10 μM TK VI, 30% of treated cells displayed green fluorescence, indicating that $\Delta\psi_{\text{m}}$ loss occurred in these cells. Moreover, 25 μM TK VI treatment led to a significantly higher population of cells (>75%) with $\Delta\psi_{\text{m}}$ loss, suggesting that mitochondria are involved in the regulation of apoptotic cell death of *F. oxysporum* induced by TK VI (Fig. 4a).

Since ROS generation is mostly governed by mitochondria, loss of mitochondrial membrane potential triggers ROS generation, and increased ROS production leads to further mitochondrial disruption. In plant and fungal cells, ROS have been shown to be necessary and sufficient to induce certain forms of apoptosis (Pereira *et al.*, 2008; Perrone

Fig. 4. Mitochondrial changes and ROS production in TK VI-treated cells. (a) Loss of $\Delta\psi_m$ in TK VI-treated cells. Right panels: cells were treated with TK VI for 6 h and subjected to mitochondrial transmembrane potential assessment using a MitoCapture reagent. Loss of $\Delta\psi_m$ was visualized as a reduction in the fluorescence signal in the FL1 channel. Left panels: differential interference contrast (DIC) micrographs. (b) Detection of intracellular ROS using fluorescence microscopy. Right panels: protoplasts were treated with 10 or 25 μM TK VI for 6 h. ROS production was then detected using the Image-iT LIVE Green Reactive Oxygen Species Detection kit. Left panels: differential interference contrast (DIC) micrographs. (c) Enumeration of cells exhibiting the accumulation of ROS. Cells treated with TK VI (10 or 25 μM) for 6 h were analysed using flow cytometry. ^a $P < 0.01$ and ^b $P < 0.01$, TK VI treatment versus control.

et al., 2008). We thus examined whether TK VI treatment induced ROS production in *F. oxysporum* protoplasts. To assay ROS production, we loaded the protoplasts with 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy- H_2DCFDA) after TK VI treatment. In the presence of ROS, carboxy- H_2DCFDA is oxidized to carboxy-2',7'-dichlorofluorescein (carboxy-DCF), which can be visualized under a fluorescence microscope. As shown in Fig. 4(b), control cells showed very weak carboxy-DCF fluorescence, indicating that they produced little ROS. In contrast, the cells treated with 25 μM TK VI for 6 h showed strong fluorescence, indicating that much more ROS was produced in these cells than in control cells. Further detection using flow cytometry showed that ~70 % of cells treated with 25 μM TK VI displayed dramatically

increased levels of cellular ROS, and ~30 % of cells treated with 10 μM TK VI displayed increased ROS levels (Fig. 4c). Taken together, TK VI triggered an increase of ROS in the cells of *F. oxysporum* in a dose-dependent manner.

TK VI triggers metacaspase-independent apoptosis in *F. oxysporum*

Activation of effector caspases, such as caspases 3, 7, 8 and 9, is responsible for the proteolytic cleavage of a diverse range of structural and regulatory proteins in apoptosis (Madeo *et al.*, 2002, 2004). Several components functionally related to the mammalian caspases, named metacaspases, have been identified in fungi (Madeo *et al.*, 2002). To determine whether the apoptosis in *F. oxysporum*

triggered by TK VI was related to metacaspase-mediated proteolysis, metacaspase activity was determined. As shown in Fig. 5, metacaspase activity in the lysates of both control and TK VI-treated cells was at background levels, whereas the activity in the lysates of positive control cells (provided by the Homogeneous Caspases Assay kit) was nearly 10 times higher than that in the lysates of treated cells. This result showed that the metacaspases in the apoptotic *F. oxysporum* cells induced by TK VI were not activated, which indicates that TK VI induces metacaspase-independent apoptosis in *F. oxysporum*.

DISCUSSION

Although peptaibols are known antibiotic peptides, their antimicrobial mechanisms are largely unclear. A previous study demonstrated that trichokonins produced by *T. pseudokoningii* SMF2 inhibit several important plant-pathogenic fungi (Song *et al.*, 2006). In this study, TK VI was shown to have strong antifungal activity against *F. oxysporum*, *A. citrullina*, *B. cinerea* and *P. parasitica*. Therefore, the peptaibol TK VI might have broad-spectrum antifungal activity against plant-pathogenic fungi. More importantly, TK VI induced extensive apoptosis of mycelia of the tested plant-pathogenic fungi, suggesting that TK VI exerts antifungal activity against plant-pathogenic fungi through an apoptotic pathway. Subsequently, *F. oxysporum* was used as a model for a detailed study of the apoptotic mechanism.

Fig. 5. Metacaspase activity in TK VI-treated cells. Cells were treated with different concentrations of TK VI, as indicated, for 16 h, and the metacaspase activity of each sample and of the positive control was measured by using a Homogeneous Caspases Assay kit (fluorimetric). The positive control cells were provided in the kit. DEVDase activity=caspase-3 protease activity.

Fungi can undergo autophagic- or apoptotic-type PCD on exposure to antifungal agents, developmental signals or stress factors (Roze & Linz, 1998; Semighini *et al.*, 2006; Hamann *et al.*, 2008; Ramsdale, 2008). PCD has been described in a range of model fungi, with a particular focus on the yeasts *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Candida albicans* (Mousavi & Robson, 2004; Reiter *et al.*, 2005; Phillips *et al.*, 2006; Almeida *et al.*, 2008; Andrés *et al.*, 2008). However, an understanding of typical apoptotic mechanisms in plant-pathogenic fungi was lacking. The physiological changes due to TK VI treatment were evidence of apoptotic cell death in *F. oxysporum*, and included PS externalization, accumulation of ROS, and nuclease-mediated DNA strand breakage. Moreover, when cells were pretreated with BAPTA, the exposure of PS in plasma membranes was absent. It has been reported that long-sequence peptaibols, exemplified by alamethicins, seem to form ionic channels in chromaffin cells in the 'barrel-stave' mode which are permeable to Ca^{2+} (Béven & Wróblewski, 1997; Bonnafeous *et al.*, 1982). In the view of Qing Hua (Huang *et al.*, 1994), TK VI is a potent agonist of the L-type Ca^{2+} channel. Based on our result, it could be deduced that TK-mediated PCD is a calcium-dependent process in *F. oxysporum*, suggesting that Ca^{2+} is a major player in the interaction between peptaibols and phyto-pathogenic fungi. Interestingly, TK VI-treated cells exhibited the accumulation of perinuclear vesicular structures. The vacuoles were transformed into a few very large ones when the concentration of TK VI was increased, and morphologically these resembled autophagic vacuoles in animal and yeast cells. The vacuolation was truncated in the absence of Ca^{2+} , giving rise to the hypothesis that Ca^{2+} plays a pivotal role in autophagy-like cell death in pathogenic fungi.

Peltola *et al.* (2004) demonstrated that the crude methanolic cell extract from a *T. harzianum* strain dissipates the mitochondrial membrane potential of human lung epithelial carcinoma cells (A549 cell line). Our result is consistent with this. Mitochondria played a central role in TK VI-induced apoptosis in *F. oxysporum*. Growing evidence suggests that mitochondrial dysfunction plays a key role in oxidative stress (Perrone *et al.*, 2008; Schrauwen *et al.*, 2010). In particular, mitochondria are also the primary source of ROS, a common indicator of oxidative stress which may play a pivotal role in mitochondrial dysfunction and in the regulation of apoptotic factors. Once generated, ROS further lead to cell injury through cell membrane lipid destruction, cleavage of DNA and damage of proteins (Cheng *et al.*, 2008; Feissner *et al.*, 2009). Recent work (Cheng *et al.*, 2003; Leiter *et al.*, 2005) on *Aspergillus nidulans* indicates that intracellular ROS might be necessary for fungal apoptosis. Here we showed that the accumulation of ROS was also associated with apoptosis induced by TK VI in *F. oxysporum*. These findings are consistent with previous reports that ROS and mitochondria play important roles in the induction of PCD in lower and higher eukaryotes.

Fig. 6. Schematic diagram of the metacaspase-independent apoptosis pathway involved in the mechanism of action of TK VI in *F. oxysporum* cells. TK VI promotes the elevation of cytosolic Ca^{2+} [Ca^{2+}]_c in *F. oxysporum* cells. Cells exhibit the main hallmarks of the apoptotic process, such as PS exposure, dissipation of mitochondrial transmembrane potential, and an increase of intracellular ROS levels. The process is independent of metacaspases and is accompanied by the accumulation of cytoplasmic vacuoles.

Metacaspases are the proteases of the caspase family that are found in plants, fungi and protozoa, and they are the functional homologues of animal caspases. Some reports show that metacaspases are involved in PCD, but do not possess caspase-specific proteolytic activity (Liang *et al.*, 2008; Mazzoni & Falcone, 2008). However, until now, the role of metacaspases in cell death has remained enigmatic, and both up- and downregulation of metacaspases have yielded conflicting data (Vercammen *et al.*, 2007). Our results showed no activation of metacaspases in *F. oxysporum* in the presence of TK VI, suggesting that the metacaspases were not required for TK VI-induced apoptotic PCD in *F. oxysporum*.

Based on our results, a molecular biocontrol mechanism of TK VI against plant-pathogenic fungi is proposed (Fig. 6). TK VI efficiently induces an apoptotic phenotype in fungal cells that is characterized by PS exposure, dissipation of mitochondrial transmembrane potential, and the accumulation of intracellular ROS. The process is independent of metacaspases and is accompanied by the accumulation of cytoplasmic vacuoles. The elevation of cytosolic Ca^{2+} might play a crucial role in the apoptosis. Our previous study revealed that the peptaibol TK VI also induces a characteristic form of cell death, essentially featuring apoptosis and autophagy, in human hepatocellular carcinoma cells (Shi *et al.*, 2010). The peptaibol alamethicin induces apoptosis in *Arabidopsis thaliana* (Rippa *et al.*, 2010). Therefore, peptaibols may induce apoptosis in different cell systems via complex and diverse mechanisms.

Although the details of the signalling and effectors involved in apoptosis in plant-pathogenic fungi triggered by TK VI remain under investigation, this is believed to be the first report to demonstrate that the antibiotic mechanism of peptaibol against plant-pathogenic fungi occurs through apoptosis.

ACKNOWLEDGEMENTS

The work was supported by Hi-Tech Research and Development Program of China (2011AA090704), National Natural Science Foundation of China (30870047, 81071804), Specialized Research Fund for the Doctoral Program of Higher Education (BA2009YY009), Program of Shandong for Taishan Scholars, Natural Science Foundation of Shandong Province (JQ200910, 2008BS02019) and Independent Innovation Foundation of Shandong University (2009TS079, 2011DX002).

REFERENCES

- Almeida, B., Silva, A., Mesquita, A., Sampaio-Marques, B., Rodrigues, F. & Ludovico, P. (2008). Drug-induced apoptosis in yeast. *Biochim Biophys Acta* **1783**, 1436–1448.
- Andrés, M. T., Viejo-Díaz, M. & Fierro, J. F. (2008). Human lactoferrin induces apoptosis-like cell death in *Candida albicans*: critical role of K^+ -channel-mediated K^+ efflux. *Antimicrob Agents Chemother* **52**, 4081–4088.
- Benedetti, E., Bavoso, A., Di Blasio, B., Pavone, V., Pedone, C., Toniolo, C. & Bonora, G. M. (1982). Peptaibol antibiotics: a study on the helical structure of the 2-9 sequence of emerimicins III and IV. *Proc Natl Acad Sci U S A* **79**, 7951–7954.
- Benítez, T., Rincón, A. M., Limón, M. C. & Codón, A. C. (2004). Biocontrol mechanisms of *Trichoderma* strains. *Int Microbiol* **7**, 249–260.
- Berrocal-Lobo, M. & Molina, A. (2008). *Arabidopsis* defense response against *Fusarium oxysporum*. *Trends Plant Sci* **13**, 145–150.
- Béven, L. & Wróblewski, H. (1997). Effect of natural amphipathic peptides on viability, membrane potential, cell shape and motility of mollicutes. *Res Microbiol* **148**, 163–175.
- Bonnafous, J. C., Dornand, J. & Mani, J. C. (1982). Alamethicin or detergent permeabilization of the cell membrane as a tool for adenylate cyclase determination. *Biochim Biophys Acta* **720**, 235–241.
- Chen, L. L., Liu, L. J., Shi, M., Song, X. Y., Zheng, C. Y., Chen, X. L. & Zhang, Y. Z. (2009). Characterization and gene cloning of a novel

- serine protease with nematocidal activity from *Trichoderma pseudo-koningii* SMF2. *FEMS Microbiol Lett* **299**, 135–142.
- Cheng, J., Park, T. S., Chio, L. C., Fischl, A. S. & Ye, X. S. (2003). Induction of apoptosis by sphingoid long-chain bases in *Aspergillus nidulans*. *Mol Cell Biol* **23**, 163–177.
- Cheng, W. C., Leach, K. M. & Hardwick, J. M. (2008). Mitochondrial death pathways in yeast and mammalian cells. *Biochim Biophys Acta* **1783**, 1272–1279.
- Chet, I. & Inbar, J. (1994). Biological control of fungal pathogens. *Appl Biochem Biotechnol* **48**, 37–43.
- Chugh, J. K. & Wallace, B. A. (2001). Peptaibols: models for ion channels. *Biochem Soc Trans* **29**, 565–570.
- Csermely, P., Radics, L., Rossi, C., Szamel, M., Ricci, M., Mihály, K. & Somogyi, J. (1994). The nonapeptide leucinostatin A acts as a weak ionophore and as an immunosuppressant on T lymphocytes. *Biochim Biophys Acta* **1221**, 125–132.
- Daniel, J. F. S. & Filho, E. R. (2007). Peptaibols of *Trichoderma*. *Nat Prod Rep* **24**, 1128–1141.
- Eisenberg, T., Büttner, S., Kroemer, G. & Madeo, F. (2007). The mitochondrial pathway in yeast apoptosis. *Apoptosis* **12**, 1011–1023.
- Feissner, R. F., Skalska, J., Gaum, W. E. & Sheu, S. S. (2009). Crosstalk signaling between mitochondrial Ca^{2+} and ROS. *Front Biosci* **14**, 1197–1218.
- Häcker, G. (2000). The morphology of apoptosis. *Cell Tissue Res* **301**, 5–17.
- Hamann, A., Brust, D. & Osiewacz, H. D. (2008). Apoptosis pathways in fungal growth, development and ageing. *Trends Microbiol* **16**, 276–283.
- Harman, G. E., Howell, C. R., Viterbo, A., Chet, I. & Lorito, M. (2004). *Trichoderma* species – opportunistic, avirulent plant symbionts. *Nat Rev Microbiol* **2**, 43–56.
- Howell, C. R. (2003). Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis* **87**, 4–10.
- Huang, Q., Tezuka, Y., Kikuchi, T. & Momose, Y. (1994). Trichokonin VI, a new Ca^{2+} channel agonist in bullfrog cardiac myocytes. *Eur J Pharmacol* **271**, R5–R6.
- Huang, Q., Tezuka, Y., Hatanaka, Y., Kikuchi, T., Nishi, A. & Tubaki, K. (1995). Studies on metabolites of mycoparasitic fungi. IV. Minor peptaibols of *Trichoderma koningii*. *Chem Pharm Bull (Tokyo)* **43**, 1663–1667.
- Katayama, T., Miyagawa, K., Kodama, T. & Oikawa, S. (2001). Trichorzin HA V, a member of the peptaibol family, stimulates intracellular cAMP formation in cells expressing the calcitonin receptor. *Biol Pharm Bull* **24**, 1420–1422.
- Lehtopolku, M., Nakari, U. M., Kotilainen, P., Huovinen, P., Siitonen, A. & Hakanen, A. J. (2010). Antimicrobial susceptibilities of multidrug-resistant *Campylobacter jejuni* and *C. coli* strains: *in vitro* activities of 20 antimicrobial agents. *Antimicrob Agents Chemother* **54**, 1232–1236.
- Leiter, E., Szappanos, H., Oberparleiter, C., Kaiserer, L., Csernoch, L., Pusztahelyi, T., Emri, T., Pócsi, I., Salvenmoser, W. & Marx, F. (2005). Antifungal protein PAF severely affects the integrity of the plasma membrane of *Aspergillus nidulans* and induces an apoptosis-like phenotype. *Antimicrob Agents Chemother* **49**, 2445–2453.
- Liang, Q. L., Li, W. & Zhou, B. (2008). Caspase-independent apoptosis in yeast. *Biochim Biophys Acta* **1783**, 1311–1319.
- Madeo, F., Herker, E., Maldener, C., Wissing, S., Lächelt, S., Herlan, M., Fehr, M., Lauber, K., Sigrist, S. J. & other authors (2002). A caspase-related protease regulates apoptosis in yeast. *Mol Cell* **9**, 911–917.
- Madeo, F., Herker, E., Wissing, S., Jungwirth, H., Eisenberg, T. & Fröhlich, K. U. (2004). Apoptosis in yeast. *Curr Opin Microbiol* **7**, 655–660.
- Matsuzaki, K., Shioyama, T., Okamura, E., Umemura, J., Takenaka, T., Takaishi, Y., Fujita, T. & Miyajima, K. (1991). A comparative study on interactions of α -aminoisobutyric acid containing antibiotic peptides, trichopolyn I and hypelcin A with phosphatidylcholine bilayers. *Biochim Biophys Acta* **1070**, 419–428.
- Mazzoni, C. & Falcone, C. (2008). Caspase-dependent apoptosis in yeast. *Biochim Biophys Acta* **1783**, 1320–1327.
- Mes, J. J., Wit, R., Testerink, C. S., de Groot, F., Haring, M. A. & Cornelissen, B. J. (1999). Loss of avirulence and reduced pathogenicity of a gamma-irradiated mutant of *Fusarium oxysporum* f. sp. *lycopersici*. *Phytopathology* **89**, 1131–1137.
- Mousavi, S. A. & Robson, G. D. (2004). Oxidative and amphotericin B-mediated cell death in the opportunistic pathogen *Aspergillus fumigatus* is associated with an apoptotic-like phenotype. *Microbiology* **150**, 1937–1945.
- Peltola, J., Ritieni, A., Mikkola, R., Grigoriev, P. A., Pócsfalvi, G., Andersson, M. A. & Salkinoja-Salonen, M. S. (2004). Biological effects of *Trichoderma harzianum* peptaibols on mammalian cells. *Appl Environ Microbiol* **70**, 4996–5004.
- Pereira, C., Silva, R. D., Saraiva, L., Johansson, B., Sousa, M. J. & Côte-Real, M. (2008). Mitochondria-dependent apoptosis in yeast. *Biochim Biophys Acta* **1783**, 1286–1302.
- Perrone, G. G., Tan, S. X. & Dawes, I. W. (2008). Reactive oxygen species and yeast apoptosis. *Biochim Biophys Acta* **1783**, 1354–1368.
- Phillips, A. J., Crowe, J. D. & Ramsdale, M. (2006). Ras pathway signaling accelerates programmed cell death in the pathogenic fungus *Candida albicans*. *Proc Natl Acad Sci U S A* **103**, 726–731.
- Pyke, T. R. & Dietz, A. (1966). U-21,963, a new antibiotic. I. Discovery and biological activity. *Appl Microbiol* **14**, 506–510.
- Ramsdale, M. (2008). Programmed cell death in pathogenic fungi. *Biochim Biophys Acta* **1783**, 1369–1380.
- Reiter, J., Herker, E., Madeo, F. & Schmitt, M. J. (2005). Viral killer toxins induce caspase-mediated apoptosis in yeast. *J Cell Biol* **168**, 353–358.
- Rippa, S., Eid, M., Formaggio, F., Toniolo, C. & Béven, L. (2010). Hypersensitive-like response to the pore-former peptaibol alamethicin in *Arabidopsis thaliana*. *Chem BioChem* **11**, 2042–2049.
- Roze, L. V. & Linz, J. E. (1998). Lovastatin triggers an apoptosis-like cell death process in the fungus *Mucor racemosus*. *Fungal Genet Biol* **25**, 119–133.
- Schrauwen, P., Schrauwen-Hinderling, V., Hoeks, J. & Hesselink, M. K. (2010). Mitochondrial dysfunction and lipotoxicity. *Biochim Biophys Acta* **1801**, 266–271.
- Semighini, C. P., Hornby, J. M., Dumitru, R., Nickerson, K. W. & Harris, S. D. (2006). Farnesol-induced apoptosis in *Aspergillus nidulans* reveals a possible mechanism for antagonistic interactions between fungi. *Mol Microbiol* **59**, 753–764.
- Shi, M., Wang, H. N., Xie, S. T., Luo, Y., Sun, C. Y., Chen, X. L. & Zhang, Y. Z. (2010). Antimicrobial peptaibols, novel suppressors of tumor cells, targeted calcium-mediated apoptosis and autophagy in human hepatocellular carcinoma cells. *Mol Cancer* **9**, 26.
- Shima, A., Fukushima, K., Arai, T. & Terada, H. (1990). Dual inhibitory effects of the peptide antibiotics leucinostatins on oxidative phosphorylation in mitochondria. *Cell Struct Funct* **15**, 53–58.
- Song, X. Y., Shen, Q. T., Xie, S. T., Chen, X. L., Sun, C. Y. & Zhang, Y. Z. (2006). Broad-spectrum antimicrobial activity and high stability

of trichokonins from *Trichoderma koningii* SMF2 against plant pathogens. *FEMS Microbiol Lett* **260**, 119–125.

Song, X. Y., Xie, S. T., Chen, X. L., Sun, C. Y., Shi, M. & Zhang, Y. Z. (2007). Solid-state fermentation for trichokonins production from *Trichoderma koningii* SMF2 and preparative purification of trichokonin VI by a simple protocol. *J Biotechnol* **131**, 209–215.

Vercammen, D., Declercq, W., Vandenabeele, P. & Van Breusegem, F. (2007). Are metacaspases caspases? *J Cell Biol* **179**, 375–380.

Viterbo, A., Harel, M., Horwitz, B. A., Chet, I. & Mukherjee, P. K. (2005). *Trichoderma* mitogen-activated protein kinase signaling is involved in induction of plant systemic resistance. *Appl Environ Microbiol* **71**, 6241–6246.

Whitmore, L. & Wallace, B. A. (2004). The Peptaibol Database: a database for sequences and structures of naturally occurring peptaibols. *Nucleic Acids Res* **32** (Database issue), D593–D594.

Wiest, A., Grzegorski, D., Xu, B. W., Goulard, C., Rebuffat, S., Ebbole, D. J., Bodo, B. & Kenerley, C. (2002). Identification of peptaibols from *Trichoderma virens* and cloning of a peptaibol synthetase. *J Biol Chem* **277**, 20862–20868.

Yedidia, I., Benhamou, N. & Chet, I. (1999). Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. *Appl Environ Microbiol* **65**, 1061–1070.

Edited by: R. P. Oliver