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

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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 trichokinin 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.

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; Benitez 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 *Emerichellopsis* 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, \(\alpha\)-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;...
Peptaibols induce apoptosis in fungal pathogens

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 (Molecular Probes) was 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, C. 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 × 10⁶ 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 cytometry (Epic XL–MCL, Coulter).

Morphological changes of cells treated with TK VI. Membrane permeabilization was measured by the uptake of SYTOX Green dye (M₅₆; 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 (ΔΨₘ) 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 ± SD. P<0.01 was considered statistically significant. All statistical analyses were performed using SPSS 11.5 for Windows.

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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 μM) 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 μM) 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 ± SD of triplicate experiments (each performed in duplicate). *P<0.01 and **P<0.01, TK VI treatment versus control; ***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 trichokonins 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 μM 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 μM 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 μM (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 μM 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 μM 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 μM 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.](http://mic.sgmjournals.org)
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 Ca2+ 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 (∆Ψ_m) appears to be the initial event (Eisenberg et al., 2007). To study the effect of TK VI on the ∆Ψ_m of *F. oxysporum* cells, *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 ∆Ψ_m loss occurred in these cells. Moreover, 25 μM TK VI treatment led to a significantly higher population of cells (>75%) with ∆Ψ_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
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$_2$DCFDA) after TK VI treatment. In the presence of ROS, carboxy-H$_2$DCFDA 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 \textit{F. oxysporum} cells induced by TK VI were not activated, which indicates that TK VI induces metacaspase-independent apoptosis in \textit{F. oxysporum}.

**DISCUSSION**

Although peptaibols are known antibiotic peptides, their antimicrobial mechanisms are largely unclear. A previous study demonstrated that trichokonins produced by \textit{T. pseudokoningii} SMF2 inhibit several important plant-pathogenic fungi (Song \textit{et al.}, 2006). In this study, TK VI was shown to have strong antifungal activity against \textit{F. oxysporum}, \textit{A. citrullina}, \textit{B. cinerea}, and \textit{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, \textit{F. oxysporum} was used as a model for a detailed study of the apoptotic mechanism.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig_5.png}
\caption{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.}
\end{figure}

Fungi can undergo autophagic- or apoptotic-type PCD on exposure to antifungal agents, developmental signals or stress factors (Roze & Linz, 1998; Semighini \textit{et al.}, 2006; Hamann \textit{et al.}, 2008; Ramsdale, 2008). PCD has been described in a range of model fungi, with a particular focus on the yeasts \textit{Saccharomyces cerevisiae}, \textit{Schizosaccharomyces pombe} and \textit{Candida albicans} (Mousavi & Robson, 2004; Reiter \textit{et al.}, 2005; Phillips \textit{et al.}, 2006; Almeida \textit{et al.}, 2008; Andrè\textit{s} \textit{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 \textit{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\textsuperscript{2+} (Bèven & Wróblewski, 1997; Bonnafous \textit{et al.}, 1982). In the view of Qing Hua (Huang \textit{et al.}, 1994), TK VI is a potent agonist of the L-type Ca\textsuperscript{2+} channel. Based on our result, it could be deduced that TK-mediated PCD is a calcium-dependent process in \textit{F. oxysporum}, suggesting that Ca\textsuperscript{2+} is a major player in the interaction between peptaibols and phytopathogenic 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\textsuperscript{2+}, giving rise to the hypothesis that Ca\textsuperscript{2+} plays a pivotal role in autophagy-like cell death in pathogenic fungi.

Peltola \textit{et al.} (2004) demonstrated that the crude methanolic cell extract from a \textit{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 \textit{F. oxysporum}. Growing evidence suggests that mitochondrial dysfunction plays a key role in oxidative stress (Perrone \textit{et al.}, 2008; Schrauwen \textit{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 \textit{et al.}, 2008; Fei\textit{s}ner \textit{et al.}, 2009). Recent work (Cheng \textit{et al.}, 2003; Leiter \textit{et al.}, 2005) on \textit{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 \textit{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.
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\textsuperscript{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.

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**REFERENCES**


![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\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{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.](http://mic.sgmjournals.org)
serine protease with nematicidal activity from Trichoderma pseudo-


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