TOXIN- AND CADMIUM-INDUCED CELL DEATH EVENTS IN TOMATO SUSPENSION CELLS RESEMBLE FEATURES OF HYPERSENSITIVE RESPONSE

Elena T. Iakimova[1,2*+], Ernst J. Woltering[2,+] and Zhenia P. Yordanova[3]

[1]Regional Research Center and Extension Service of Floriculture and Agriculture (RCNPO), 1222 Negovan, Sofia, BULGARIA
[2]Wageningen University & Research Centre, Agrotechnology and Food Science Group (AFSG), P.O. Box 17, 6700 AA Wageningen, THE NETHERLANDS
[3]Department of Plant Physiology, Faculty of Biology, Sofia University "St. Kliment Ohridski", 8 Dragan Tzankov Blvd, Sofia, BULGARIA

*Correspondence to: Elena T. Iakimova, e-mail: Elena.Iakimova@wur.nl
Present address: Wageningen University & Research Centre, Agrotechnology and Food Science Group (AFSG), P.O. Box 17, 6700 AA Wageningen, THE NETHERLANDS; Tel: +31 317 475002; Fax: +31 317 475347

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ABSTRACT

Elicitors of different origin (fumonisin B1, fungal toxin), camptothecin (alkaloid from *Camptotheca acuminata*), mastoparan (wasp venom) and the heavy metal (cadmium) were tested for their ability to induce programmed cell death (PCD) in a model system of tomato cell culture, line MsK8. By employing a pharmacological approach the involvement of proteolysis, oxidative stress and ethylene in the suicidal cascade is shown. The caspase-specific peptide inhibitors: irreversible caspase-1 (ICE)-inhibitor acyl-Tyr-Val-Ala-Asp-chloromethylketone and the broad range caspase inhibitor benzyoxycarbonyl-Asp-2,6-dichlorobenzoyloxymethylketone effectively reduced cell lethality providing a sound indication that in tomato suspension cells the applied inducers promote cell death that resembles features typical for PCD. A lack of

[+]The authors Dr. E.T. Iakimova and Prof. Dr. E.J. Woltering have equally contributed to the paper.

inhibition occurred at mastoparan-induced cell death in response to the caspase inhibitors, while the serine protease inhibitor aminoethylbenzenesulphonyl fluoride caused substantial reduction of cell mortality. Significant inhibition was detected after administration of ethylene inhibitor, aminoethoxyvinyl glicine and the antioxidants L-galactonic acid-ă-galactone and catalase. The results indicate that the cell death response at the exposure to biotic and abiotic stressors may employ an activation of similar cell death pathways and that caspase-like- and non-caspase-like-dependent biochemical processes may be operative. In addition, the presented comparative study suggests that the reaction of tomato suspension cells to diverse cell death stimulating compounds at least partially coincides with the cell death machinery involved in the plant hypersensitive response and during PCD in animal cells.

Key words: cadmium, camptothecin, calcium, caspase-like proteases, ethylene, fumonisin B1, hypersensitive response, mastoparan, oxidative stress, tomato cell culture, programmed cell death

INTRODUCTION

Programmed cell death (PCD) is an active process of cell suicide that is an important mechanism of development and survival present in all eukaryotes. The PCD machinery is activated developmentally and in response to diverse biotic and abiotic insults. It involves a sequence of biochemical and molecular events leading to controlled disassembly of the cells. Specific morphological features of PCD are cell shrinkage, blebbing of the plasma membrane, condensation and fragmentation of the nucleus, internucleosomal cleavage of DNA and formation of DNA-containing (apoptotic-like) bodies. Morphological similarities have been detected between animal cells undergoing apoptosis and dying plant cells, including hallmarks such as cell and cytoplasm shrinkage and DNA laddering (Wang et al., 1996a, 1996b; de Jong et al., 2000). During plant development, processes that conform to the general definition of PCD are cell death during xylogenesis, aerenchyma formation, plant reproductive processes, leaf and petal senescence and endosperm development (Lam, 2004). Furthermore, cell death in response to pathogen attack and to a variety of abiotic factors such as ozone, UV radiation and heavy metals also falls within the definition of PCD. A form of programmed cell death associated with plant resistance to pathogens is the hypersensitive response (HR), an elaborate mechanism to counteract the spread of pathogens which is characterized by rapid, localized death of cells (lesion formation) at the site of infection (Gilchrist, 1998; Heath, 2000; Greenberg and Yao, 2004). HR can be triggered by pathogens, by their toxins or by purified elicitors such as harpins from *Pseudomonas syringae* and *Erwinia amylovora*, the fungal toxins victorin from *Trichoderma viridae* (He, 1996), cryptogein from

Phythophthora cryptogea (Hirasawa et al., 2005), AAL toxin from *Alternaria alternata* and mycotoxin fumonizin B1 (FumB1) from *Fusarium moniliforme* (Wang et al., 1996a, 1996b). Also, plant viruses such as *tobacco mosaic virus* (TMV) have been reported to elicit HR (del Pozo et al., 2004).

Generally, apoptotic cell death engages a sequence of cysteinyl aspartate-specific proteases (caspases) activation events in which initiator caspases activate downstream executioner caspases that process a variety of target proteins eventually leading to the apoptotic phenotype (Hengartner, 2000). No structural homologues of animal caspases have been identified in plants, although other enzymes, such as metacaspases, vacuolar processing enzymes (VPEs) and saspases (subtilisin-like serine proteases) have been reported to functionally mimic the caspase-like effects (Coffeen and Wolpert, 2004; Woltering, 2004).

Caspases can selectively be inhibited by small peptides, mimicking the substrate recognition site, carrying electrophiles such as aldehydes, nitriles or ketones at their C terminal that react with the active site cystein. Evidence exists that caspase-like proteases, as in animal systems, participate in the programmed cell death in plants (del Pozo and Lam, 1998; de Jong et al., 2000; Richael et al., 2001; Woltering et al., 2002; Chichkova et al., 2004). Apart from the possible involvement of caspase-like proteases in plant cell death, other key components of the animal apoptotic pathway such as increased production of reactive oxygen species (ROS) (Neill et al., 2002; Laloi et al., 2004), proteolysis (de Jong et al. 2000) and ethylene production (He et al., 1996; Woltering et al., 2003) have also been found to actively participate in the signal transduction, indicating that PCD in plants may proceed through a similar mechanism as in animal cells (Hoeberichts and Woltering, 2003; Iakimova et al., 2005).

The aim of present work was to study the effect of chemicals and toxins of different origin on the induction of cell death in tomato suspension cells and, by using a pharmacological approach, to elucidate the involvement of caspase-like and serine proteases, ROS and ethylene in the cell death signaling. For cell death induction, compounds known to induce HR in animal and plant systems (the fungal toxin fumonisin B1 (Fum B1) and the wasp venom mastoparan (MP) were introduced and their potency to stimulate cell death pathways was compared to the effects of the alkaloid camptothecin (CPT), and the heavy metal cadmium, applied as $CdSO_4$. Together with the inducers, we have administrated specific inhibitors that proved to be effective in reducing cell mortality thus indicating that the studied biochemical events participate in cell death in tomato suspension cells. Possible caspase- and non-caspase dependent cell death pathways are discussed in connection with the HR and animal apoptotic cell death.

MATERIAL AND METHODS

Chemicals

Acyl-Tyr-Val-Ala-Asp-chloro-methylketone (Ac-YVAD-CMK) and benzyoxycarbonyl-Asp-2,6-dichloro-benzoyloxymethylketone (Z-Asp-CH2-DCB) were purchased from Bachem AG, Bubendorf, Switzerland; Murashige-Skoog + vitamins from Duchefa, Haarlem, The Netherlands and all other chemicals from Sigma-Aldrich, Zwijndrecht, The Netherlands. Camptothecin (CPT), mastoparan (MP), Ac-YVAD-CMK, Z-Asp-CH2-DCB and AEBSF were dissolved in dimethylsulfoxide (DMSO) at final concentration 0.1% v/v.

Plant material

Tomato (*Lycopersicon esculentum* Mill.) cell suspension culture, line Msk8 (Koorneef et al., 1987) was grown on a liquid medium supplemented with 5 µM naphthylacetic acid, 1 µM N^6-benzyledenine and vitamins as described by Adams and Townsend (1983) and kept on a rotary shaker (80 rpm). Cells were subcultured every 7 d by making a 1:4 dilution in 20 ml of fresh medium in 100-ml flasks with aluminum screw caps.

Cell death induction and inhibition

The tomato suspension cells were used for experiments 5 d after subculture. Cell death inducers (Fum B1, CPT, $CdSO_4$ or MP) were added either alone or simultaneously with inhibitors to 5 ml of suspension culture in 30-ml flasks with screw caps. Cell death was calculated 24 h after the chemical treatments as a percentage of dead cells to the total number of cells after staining the living cells with 0.002% fluorescein diacetate (FDA) and counting the cells in three non-overlapping microscope fields at inverted fluore-scent microscope (Axiovert, Carl Zeiss, Darmstadt, Germany). The effect of cell death inhibitors is shown as percentage inhibition with a reference to the control, non-treated cells. DMSO was tested alone and, in the indicated final concentration, had no effect on cell mortality. The other chemicals were dissolved in water. All compounds (alone and in combinations) were tested in at least three concentrations and in at least three independent sets of experiments. The shown concentrations are the lowest that either inducing or inhibiting cell death.

Imaging

Bright field images of tomato suspension cells were obtained using the transmission channel and the 488 nm excitation line of the argon laser of a TCS SP2 AOBS confocal laser scanning microscopy system (Leica-Microsystems GmbH, Mannheim, Germany) mounted on inverted DM IRE2 microscope.

Statistical analysis

Data were processed by standard Excel Office software and the statistical significance of presented values (average of at least three independent experiments) was compared by standard error of the means (SEM_{n-1}).

RESULTS

Induction of cell death in suspension cultured tomato cells

For cell death induction, tomato suspension cells were exposed to compounds, known as PCD inducers in other plant and animal model systems: Fumonisin B1 (FumB1), CPT, MP and $CdSO_4$. Reproducible cell death induction (determined by FDA staining of living cells) occurred in the presence of 5 µM MP, 100 µM $CdSO_4$, 5 µM CPT, and 20 µM FumB1. After 24 h the control cells did not show cell death rate higher than 2.5% (Fig. 1). The trend of cell death induction and inhibition in all cases was sustained until 48 h (data not included).

At all reported treatments, similar morphological pattern of cytoplasm shrinkage and compaction of the nuclei that are typical for programmed cell death was observed (Fig. 2).

Inhibition of cell death induced by FumB1, CPT, $CdSO_4$ and MP

Activation of caspase-like proteases is a key event in apoptotic cell death in animal systems. To determine the involvement of caspase-like proteases in cell death in tomato suspension cells, we have administrated the irreversible caspase-1 (ICE)-inhibitor acyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-CMK) and the broad spectrum caspase inhibitor benzyoxycarbonyl-Asp-2,6-dichlorobenzoyloxymethylketone (Z-Asp-CH2-DCB). The caspase inhibitors were tested in a range of concentrations between 1 nM and 1 µM and most pronounced cell death inhibition occurred at concentrations of 100 nM. The application of Z-Asp-CH2-DCB and Ac-YVAD-CMK to CPT, FumB1 and $CdSO_4$-treated cells, led to a significant inhibition of cell death ranging from 80 to 99% (Fig. 3). Although FumB1 showed lower cell death inductive effect in comparison to CPT, $CdSO_4$ and MP, the response was effectively quenched by Ac-YVAD-CMK and Z-Asp-CH2-DCB (Fig. 1, 3). Remarkable inhibition (3.5 – 4 times) of cell death occurred when the peptide inhibitors were introduced simultaneously with $CdSO_4$ (82-95% inhibition) or CPT (80-86% inhibition). Interestingly, no effect of the caspase inhibitors was detected at their administration with MP (Fig. 3). Together, the results indicate that cell death induced by FumB1, $CdSO_4$ and CPT is a type of programmed cell death where caspase-like proteases operate in the signal transduction cascade. The lack of inhibition of MP-induced cell death by the caspase inhibitors may point to a different mechanism, involving caspase-independent proteolytic cascade. To test whether MP-induced cell death could be suppressed by protease inhibitors, we have administrated the serine protease inhibitor aminoethyl-benzenesulphonyl fluoride (AEBSF). The effect of AEBSF on MP-stimulated and on the cell lethality in response to the other elicitors

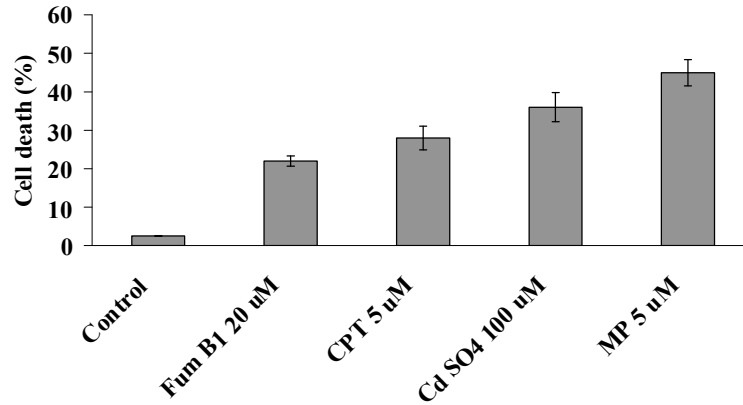

Figure 1. Effect of 20 µM Fumonisin B1 (FumB1), 5 µM camptothecin (CPT), 100 µM $CdSO_4$ or 5 µM mastoparan (MP) on cell death induction in tomato suspension cells. The cells were left untreated (control) or were treated with chemicals for 24 h and cell death was calculated after FDA staining of the living cells. Error bars indicate SEM (n-1).

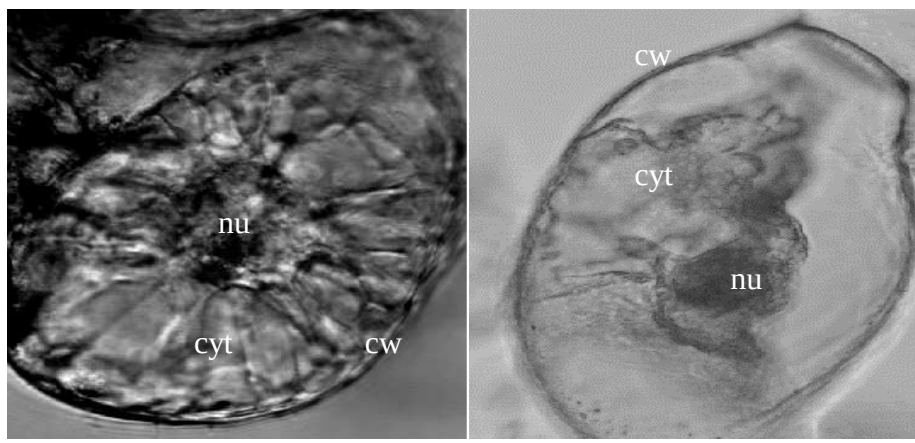

Figure 2. Bright field images of tomato suspension cells representing typical cell morphology at any of the treatments with CPT, FumB1, MP or $CdSO_4$. The images were obtained using the transmission channel and the 488 nm excitation line of the argon laser of a TCS SP2 AOBS confocal laser scanning microscopy system (Leica-Microsystems GmbH, Mannheim, Germany) mounted an inverted DM IRE2 microscope. The image on the left: living cell – diffused nuclei and no cytoplasm shrinkage; the image on the right: dead cell showing the characteristic for PCD compacted nuclei and shrunken cytoplasm separated from the cell wall. Cw – cell wall; nu – nuclei; cyt – cytoplasm.

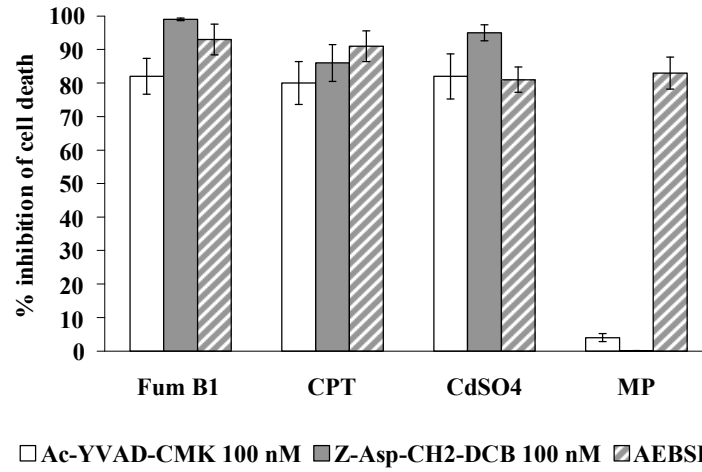

Figure 3. Effect of 100 nM irreversible caspase-1 (ICE)-inhibitor acyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-CMK), 100 nM broad range caspase inhibitor benzyl-oxycarbonyl-Asp-2,6-dichlorobenzoyloxymethylketone (Z-Asp-CH2-DCB) and 1 mM serine protease inhibitor aminoethylbenzenesulphonyl fluoride (AEBSF) on cell death in tomato suspension cells induced by 20 µM Fumonisin B1 (FumB1), 5 µM camptothecin (CPT), 100 µM $CdSO_4$ or 5 µM mastoparan (MP). Cell death was calculated after 24 h of treatment followed by FDA staining of the living cells and is expressed as % of inhibition. Error bars indicate ±SEM.

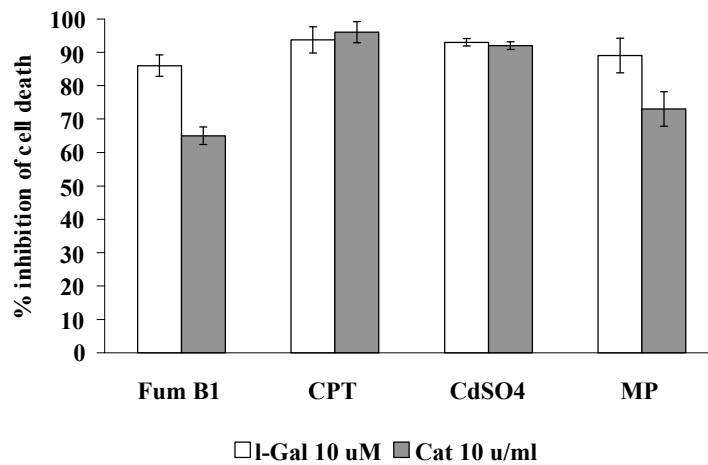

Figure 4. Effect of antioxidants 10 µM L-galactonic acid-ă-galactone (L-Gal) and 10 U/ml catalase (Cat) on cell death in tomato suspension cells induced by 20 µM Fumonisin B1 (FumB1), 5 µM camptothecin (CPT), 20 µM 100 µM $CdSO_4$ or 5 µM mastoparan (MP). Cell death was calculated after 24 h of treatment followed by FDA staining of the living cells and is expressed as % of inhibition. Error bars indicate ±SEM.

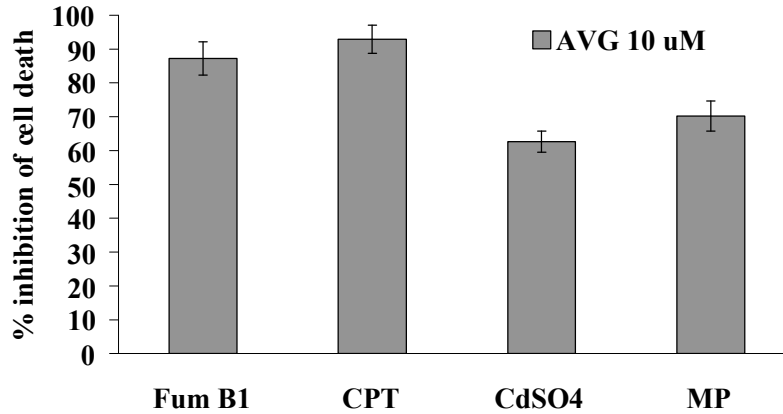

Figure 5. Effect of 10 µM ethylene synthesis inhibitor aminoethoxyvinyl glicine (AVG) on cell death in tomato suspension cells induced by 20 µM Fumonisin B1 (FumB1), 5 µM camptothecin (CPT), 100 µM $CdSO_4$ or 5 µM mastoparan (MP). Cell death was calculated after 24 h of treatment followed by FDA staining of the living cells and is expressed as % of inhibition. Error bars indicate ±SEM.

appeared to reduce strikingly cell death, which demonstrates that serine proteases are employed in the activation of cell death machinery in tomato suspension cells, irrespective of the nature of the applied inducers.

To elucidate the involvement of oxidative stress in cell death signalling in tomato suspension cells when challenged with different inducers, the cells were treated with antioxidants, such as 10 µM L-galactonic acid-ă-galactone (L-Gal) and 10 U/ml catalase. Treatment with 10 ěM L-Gal (an immediate precursor of ascorbic acid) remarkably blocked the cell death that occurred in response to all applied stressors showing 85-96% inhibition (Fig. 4). Hydrogen peroxide (H_2O_2) scavenging enzyme catalase also significantly decreased cell lethality. These results point to the participation of ROS formation in cell death machinery that is causally related to cadmium, CPT, FumB1, and MP-induced cell death.

The plant hormone ethylene is an important molecule in plant PCD signal transduction. We have tested the effect of the ethylene inhibitor AVG on chemical-induced cell death (Fig. 5). When applied alone, ethylene and AVG did not exert any changes of cell mortality in comparison to non-treated control cells (results not shown). The administration of AVG remarkably reduced cell death induced by all compounds used, which is a sound evidence that ethylene plays a role in the transmission of cell death signals.

DISCUSSION

The compounds tested for cell death inducing activity in tomato suspension cells were chosen due to their documented effect on cell death in several other systems. Here we confirm that CPT, FumB1, $CdSO_4$, and MP are potent inducers of cell death also in tomato suspension cells (Fig. 1). The observed cell shrinkage and compaction of the nucleus that occurred in response to applied stress stimuli is an additional prove that cell death in tomato culture is a kind of PCD (Fig. 2).

CPT is an alkaloid isolated from *Camptotheca acuminata* (inhibitor of topisomerase 1, used as anticancer drug) and has earlier been shown to induce cell death in tomato suspension cells (de Jong et al., 2000). The cell death induced by CPT resembles several features of PCD and is inhibited by caspase inhibitors (see also Fig. 3). This indicates that CPT-induced cell death is apoptotic-like in nature.

Fumonisin B1 is a sphinganine analogue, a host-selective mycotoxin isolated from necrotrophic fungal pathogen *Fusarium moniliforme* and is found to induce plant cell death (Wang et al., 1996a; Stone et al., 2000; Spassieva et al., 2002). Infiltration with FumB1 is shown to trigger a characteristic HR in *Arabidopsis* leaves and protoplast culture (Stone et al., 2000; Watanabe and Lam, 2006), implicating signaling pathways that require salicylate-, jasmonate- and ethylene-mediation (Asai et al., 2000). The observed inhibition of FumB1-induced cell death in the tomato culture treated with Ac-YVAD-CMK (Fig. 3) suggests that caspase-1-like proteases might be active in cell death signaling cascade. Such a suggestion is in accordance with the findings of Kuroyanagi et al. (2005) who have shown that VPE, which exhibits caspase-1 activity, is involved in a fungal toxin-induced cell death in *Arabidopsis* leaves. In addition, other caspases may also been activated, since the broad range caspase inhibitor Z-Asp-CH-DCB also showed a high cell death inhibiting potency.

In animal cells, cadmium toxicity is associated with PCD exhibiting the characteristic features of apoptosis including cell shrinkage, activation of endonucleases, nuclear condensation and DNA fragmentation (Hamada et al., 1997). It has been reported that in tobacco suspension cells $CdSO_4$ induces cell death associated with typical apoptosis-like morphological changes (Fojtová et al., 2002). These observations suggest that, as in animal cells, cadmium toxicity in plants may also be associated with PCD. Our results indicate cadmium ability to cause apoptotic-like cell death in tomato cells and show that caspase-like proteases (evidenced by cell death inhibition in the presence of caspase inhibitors) are among the players at cell death execution (Fig. 3, see also Yakimova et al., 2006).

MP is venom isolated from wasp, and it has been reported that responses to MP treatment in plants

include induction of an oxidative burst (Takahashi et al., 1998; Legendre et al., 1992). In our experiments, MP-induced cell death was not reversed by the caspase inhibitors (Fig. 3) and may therefore proceed through a mechanism that does not employ caspase-like proteases. It has recently been found that not all programmed cell death events are inhibited by the same caspase inhibitors. For instance, cell death during the HR induced by TMV is inhibited by Ac-YVAD-CMK, which is an inhibitor of caspase 1 but not caspase 3 (Hatsugai et al., 2004). Similarly, cell death in *Picea abies* embryogenic lines was inhibited by caspase 6 inhibitor (z-VEID-fmk) but not by caspase 9 inhibitor (z-LEHD-fmk) (Bozhkov et al., 2004). In addition, MP-stimulated cell death in tomato cells might belong to another kind of PCD, which may exhibit morphological and biochemical features that do not entirely copy the apoptotic characteristics (van Doorn and Woltering, 2005), and where caspase-like proteases might not be operative.

In animal cell lines serine proteases have been shown to mediate cell death at conditions of caspase inhibition and are suggested to operate apart of the caspase pathway (Egger et al., 2003). In plants, AEBSF has been demonstrated to be a potent inhibitor of cell death in soybean suspension (Levine et al., 1996) and Arabidopsis cultured cells (Tiwari et al., 2002). Lack of inhibition of MP-induced cell death (Fig. 3) in the presence of caspase inhibitors and the remarkable decrease of MP-induced cell death upon administration of serine protease inhibitor observed in our experiments suggest that non-caspase dependent biochemical routes might participate in MP-induced cell death.

To investigate the participation of oxidative stress in cell death cascade, we applied the ROS quenching compounds L-Gal and catalase. The inhibition of chemical-induced cell death by antioxidants shows that oxidative stress is instrumental in cell death in tomato cells (Fig. 4). This is in line with the finding that the prevention of ROS accumulation may modulate the cell death response (Neill et al., 2002) and that ROS are at least partially involved in PCD activity of different cell death inducers. The results on the effect of tested antioxidants on MP and FumB1 have not been reported before and are additional prove for the role of oxidative stress in the cell suicidal cascade.

It has been demonstrated on various experimental plant systems that ethylene plays an intrinsic role in programmed cell death and senescence (Hoeberichts and Woltering, 2003). It has been shown (in *Arabidopsis* and tomato) that cell death induced by the mycotoxin FumB1 involves ethylene signaling (Wang et al., 1996a, 1996b; Asai et al., 2000) and treatments with ethylene antagonists aminooxyacetic acid and silver thiosulphate in oat mesophyll cells have effectively inhibited victorin-induced PCD (Curtis and Wolpert, 2004). Over-produced ethylene is also reported

to closely correlate with expression of hypersensitive cell death symptoms in hybrid tobacco seedlings (Yamada and Murabashi, 2003). The role of ethylene in signal transduction events in cell death-stimulated tomato cells was studied by simultaneous application of the inducers and ACC synthase inhibitor AVG. AVG was very effective in reducing cell death in FumB1- and MP-stimulated cells (Fig. 5), in compliance with its cell-death reducing effect observed in CPT- or Cd-treated tomato cells (see also de Jong et al., 2000; Yakimova et al., 2006). These results indicate that ethylene participate in the transmission of signals for cell death in response to inducers of different origin.

Taken together, the presented data show that the administrated compounds can activate PCD in tomato suspension cells in a manner similar to that in animal model systems and are an additional indication that complicated mechanism operates in plant PCD in which caspase-like proteases, oxidative stress and the plant hormone ethylene are among the major factors involved in the signal transduction cascade. In the search for counterpart in cell death machinery, here we demonstrate that cell death inducers of biotic and abiotic origin can cause biochemical changes in tomato suspension cells similar to those found at plant hypersensitive response. The reported comparative analysis of cell death response to chemicals of various nature suggest that diverse elicitors may employ the activation of similar cell death pathways and that caspase-like- and non-caspase-like-dependent biochemical cascade may be operative. Since the cell death signaling in response to pathogen attacks and abiotic stresses share common mechanisms, the understanding of underlying biochemical and molecular events may shed more light into disease resistance processes in plants.

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ŚMIERĆ KOMÓRKI INDUKOWANA PRZEZ TOKSYNY POCHODZENIA BIOLOGICZNEGO I KADM W ZAWIESINIE KOMÓREK POMIDORA MA PODOBNY MECHANIZM JAK REAKCJA NADWRAŻLIWOŚCI

Elena W. Iakimova, Krnst H. Woltering
i Zhenia B. Yordanova

S T R E S Z C Z E N I E

Badano zdolność elicytorów różnego pochodzenia: (fumonizyna B1, toksyna grzybowa), kamptothecyna (alkaloid z *Camtotheca acuminata*), mastoparan (jad osy) oraz ciężki metal (kadm) do indukowania programowanej śmierci komórki (programmed cell death – PCD) w modelowym systemie kultury zawiesinowej komórek pomidora linii (klonu) MsK8. Wykazano, że procesy proteolizy, stres oksydacyjny oraz etylen biorą udział w kaskadzie sygnału samobójczej śmierci komórki. Specyficzny dla kaspazy-1 inhibitor acylo-Tyr-Val-Ala-Asp-chloro-methylketon oraz niespecyficzny inhibitor kaspaz benzyoxycarbonylo-Asp-2,6-dichlorobenzoyloxymethylketon znacząco zmniejszały śmiertelność komórek, co stanowiło dowód, że w kulturze zawiesinowej komórek pomidora zastosowane elicitory indukują śmierć komórki zgodnie z mechanizmem PCD. W przypadku śmierci komórek indukowanej przez jad osy inhibitory kaspaz nie powodowały znaczącej reakcji, natomiast inhibitor proteazy serynowej, fluorek amino-etylobenzenosulfonylowy spowodował istotny spadek śmiertelności. Znaczące obniżenie śmiertelności zanotowano także po zastosowaniu inhibitora etylenu, amino-ethoxyvinyloglicyny oraz antyoksydantów ă-galactonu kwasu L-galaktonowego i katalazy. Wyniki te wskazują, że śmierć komórek w rekcji na traktowanie biotycznymi i abiotycznymi stymulatorami może przebiegać za pośrednictwem podobnych szlaków metabolicznych i może być związana z aktywacją zarówno zależnych od kaspaz, jak i od nich niezależnych procesów. Ponadto zaprezentowane wyniki wskazują, że rekcja komórek pomidora na traktowanie różnymi induktorami programowanej śmierci przypomina mechanizm śmierci komórek w reakcji nadwrażliwości u roślin oraz w reakcji programowanej śmierci komórek u zwierząt.

Słowa kluczowe: kadm, kamptothecyna, wapń, podobne do kaspaz proteazy, etylen, fumonizyna B1, reakcja nadwrażliwości, mastoparan, stres oksydacyjny, kultura zawiesinowa komórek pomidora, programowana śmierć komórki