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Role of ethylene and phospholipid-mediated signalling in mycotoxin-induced programmed cell death in the apical part of maize roots

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Abstract: Maize (*Zea mays* L. cv. Thermo) root segments were treated for 24 h with 100 μg mL^{-1} of zearalenone and its derivatives α- and β-zearalenol. The mycotoxin treatment resulted in cell death which was evident by Evans blue staining and was accompanied by DNA release/fragmentation. Mycotoxin-induced programmed cell death (MPCD) was abolished by sub-micromolar concentrations of caspase-specific peptide inhibitors pointing to a MPCD mechanism similar to animal apoptosis. Here we demostrate that exogenous ethylene and ethylene precursor (aminocyclopropane-1-carboxylic acid; ACC) substantially blocked MPCD while the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) did not markedly reduce cell death rate. In addition, Western blot analyses revealed that MPCD was induced via ethylene-regulated expression of DAD1 protein. Pre-treatment of root segments with inhibitors of phospholipase C and D signalling pathway intermediates significantly reduced the rate of MPCD. Treatment with mastoparan and lyso-phosphatidic acid (L-PA), G protein activator and analogue of the lipid second messenger phosphatidic acid (PA), respectively, stimulated cell death. Furthermore, application of lipid and protein kinase inhibitors (wortmannin, Go 6983, staurosporine) also reduced cell death, indicating that various kinases are a part of signalling cascade involved in MPCD. Taken together, the results presented in this paper provide direct evidence that MPCD exhibits formal apoptotic-like features, involves caspase-mediated pathway and is regulated via ethylene and phospholipid signal transduction pathways.

Key words: *Zea mays* L.; ethylene; phospholipids; programmed cell death; zearalenone; zearalenol.

Introduction

Fungi are ubiquitous in the biosphere and represent a significant problem throughout the world due to their capability to produce a plethora of mycotoxins that can be poisonous to animal and human cells. Zearalenone (ZEN, [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone]) is one of the most significant and worldwide distributed mycotoxins (Schollenderger et al. 2007; Zinedine et al. 2007). The initial evidence for ZEN toxicity was derived predominantly from studies on humans and animals. Unfortunately, limited information about the toxic effects of ZEN in plants is available. For example, it was demonstrated that ZEN can be toxic to plant cells causing a leakage of electrolytes and various organic compounds, inhibition of plant cell membrane transport (Vianello & Macri 1978), inhibition of the oxidative phosphorylation (Vianello & Macri 1981), chromosome damages (Kumar & Sinha 1995) and disorders in photosynthesis and growth processes (Kosćielniak et al. 2009). In the apical part of maize root application of ZEN and its derivates α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) caused a rapid depolarization of plasma membrane potential (E_M) and inhibited a strong superoxide dismutase insensitive nitro blue tetrazolium (NBT) reduction activity (Repka et al. 2014). Interestingly, ZEN, but not its derivatives α-ZEL and β-ZEL, significantly decreased respiration of maize root cells (Repka et al. 2014).

In contrast, several experimental studies clearly proved that ZEN is a key substance controlling plant development (Meng et al. 1992) and regulating flowering process perhaps via activity similar to plant hormones (Biesaga-Kosćielniak & Filek 2010). Moreover, a ZEN-stimulated course of photochemical reactions in PSII under salt stress and protection of the photosynthetic apparatus against the consequences of strong illumination were identified in wheat and soybean seedlings (Kosćielniak et al. 2009).

The exact assessment of the biological role of ZEN is further complicated by the fact that it can undergo a set of biotransformations in metabolically active plant cells. It was experimentally proved that ZEN can be transformed to α-zearalenol (α-ZEL), β-zearalenol (β-ZEL) and ZEN-4-glusocside (ZEN-4-

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Glc) by plant enzymes as was shown by Engelhardt et al. (1988) in maize suspension cells. Moreover, α-zearalenol-4-glusocside (α-ZEL-4-Glc) and also β-zearalenol-4-glusocside (β-ZEL-4-Glc) were detected in these cultures (Engelhardt et al. 1999). More recently, an array of 17 different metabolites, most prominently glucosides, malonylglucosides, di-hexose- and hexose-pentose disaccharides of ZEN, and α-ZEL and β-ZEL, were detected in mycotoxin treated seedlings of *Arabidopsis thaliana* (Berthiller et al. 2006). The picture further complicates when formed substances, often referred to as "masked mycotoxins" (Gareis et al. 1990), are reactivated in metabolically active cells to the parent toxin.

Programmed cell death (PCD) represents an inherent cellular physiological process that controls and regulates normal plant organ development, morphogenesis and overall cellular homeostasis (Pennel & Lamb 1997). Previous studies have demonstrated that PCD can be induced by a plethora of biotic stresses including plant pathogen attacks (Richberg et al. 1998) and/or plant pathogen-derived elicitors (e.g. harpins, Choi et al. 2013). Furthermore, a few studies have indicated that also various fungus-secreted toxin molecules have profound effect on PCD induction. However, the mechanism underlying mycotoxin-triggered PCD is currently poorly understood. PCD-eliciting mycotoxin, fumonisin B1 (FB1), induced apoptosis-like PCD in wild-type *Arabidopsis* protoplast (Asai et al. 2000) and the execution of PCD required functional salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) mediated signalling pathways. Recently, two ubiquitin ligases, RING DOMAIN LIGASE3 (RGLG3) and RGLG4, were instrumental in control of FB1-triggered PCD by modulating the JA signalling pathway in *A. thaliana* (Zhang et al. 2015). Fusaric acid (FA) treatment of tobacco suspension cells resulted in the production of several hallmarks of PCD, and activation of caspase-3-like protease modulated by nitric oxide (NO) signaling molecule was responsible for the FA-induced PCD (Jiao et al. 2013). Moreover, there is evidence that ROS production, down regulation of antioxidative enzymes activities, upregulation of lipid peroxidation, and mitochondrial dysfunction were crucial for the onset of PCD (Singh & Upadhyay 2014; Jiao et al. 2014). Treatment of maize roots with ZEN and its derivates (α-ZEL and β-ZEL) resulted in the onset and progression of the cell death which was dependent on both, the type and concentration of mycotoxin (Repka et al. 2014).

The present paper reports on the use of a pharmacological approach to elucidate whether ZEN- and its derivates-induced cell death exhibits features of PCD, and to investigate the involvement of ethylene and phospholipid signalling pathways.

Material and methods

Plant material and growing conditions

The experiments were conducted on hybrid maize cultivar NK Thermo produced by Syngenta Seeds (Basel, Switzerland) and provided by Interagros (Bratislava, Slovakia). Seeds were surface-sterilized with sodium hypochlorite (1% available chlorine) for 2 min and rinsed three times in sterile distilled water for 4 min. The seeds were germinated on a moistened filter paper in square Petri dishes in the dark at 21 °C for 3 days.

Chemicals

ZEN, α-ZEL and β-ZEL were obtained from Sigma-Aldrich (Deisenhofen, Germany). The specific and non-specific caspase inhibitors were purchased from Bachem AG (Budendorf, Switzerland) and BioVision, Inc., (San Francisco, USA), Nemadipine A, mastoparan and mastoparan inactive analog M-17 from Enzo Life Sciences (Farmingdale, USA), Edelfosine from EMD Chemicals (Gibbstown, USA) and FIPI (N-[2-[4-(2,3-Dihydro-2-oxo-1H-benzimidazol-1-yl)-1-piperidinyl]ethy]-5-fluoro-1H-indole-2-carboxamide hydrochloride) from R&D Systems (Minneapolis, USA). Broad spectrum PKC inhibitor – Go 6983 (3-[1-[3-(Dimethylamino) propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione) was purchased from Tocris Bioscience (Bristol, UK), lysophosphatidylethanolamine (L-PEA, 18:0), lysophosphatidylcholine, L-α-lysophosphatidic acid (L-PA) and phosphatidic acid (PA) from Avanti Polar Lipids Inc., (Alabaster, USA). Ethylene was provided by Messer Griesheim GmbH (Griesheim, Germany). All other chemicals were from Sigma-Aldrich (Deisenhofen, Germany).

Root treatments and pharmacological assays

For treatments, apical root segments (10 mm) were cut from 5 days old maize seedlings and put into liquid 0.5 × MS (Murashige & Skoog, 1962) medium (control) supplemented with cell death inducers and inhibitors. For pharmacological experiments, apical root segments were pre-treated for 1 h in 0.5 × MS liquid medium with a broad range of concentrations (from nM to mM) of inhibitors dissolved etiher in DMSO (concentration 0.1% v/v) or in water with the same amount of DMSO and/or water as a control. Alternatively, mycotoxins and cell death inhibitors were added to apical root segments simultaneously. In general, pharmacological substances were assayed with and without the addition of mycotoxins (100 μg mL^{-1}). Lowest concentrations of inhibitors giving significant reduction of mycotoxin-induced cell death are presented. After different incubation times, seedlings were collected, immediately frozen in liquid nitrogen and stored at –70 °C prior further analysis.

Cell death quantification

Dead cells were determined and quantified using Evans blue according to Repka (2006). The stained root segments were observed under a Leica DMIRB microscope equipped with an Leica DC450 colour CCD camera (1.2 Mpixel) and digital images were processed with Leica LAS software (Leica Microsystems AG, Heerbrugg, Switzerland).

In situ detection of DNA fragmentation (TUNEL assay)

Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling was performed using an ApopTag Fluorescein *In Situ* Apoptosis Detection kit for indirect immunofluorescence staining (Merck/Millipore AG, Darmstadt, Germany) with a few minor modifications of the manufacturer's instructions. Briefly, root sections (5 mm long) were treated with proteinase K (250 μg mL^{-1}) for 15 min, with TdT enzyme for 1.5 h at 37 °C, and with anti-digoxigenin-fluorescein isothiocyanate (FITC) conjugate for 1 h at 25 °C. Either mycotoxin- or DNAse I-treated root explants were used as a positive control for the TUNEL assay. For the mycotoxin

treatment, maize root explants (10 per experiment) were transferred to the medium supplemented with different concentrations of mycotoxins and cultivated for another 10 h. For DNAse I treatment, the sections were preincubated in DN buffer (30 mM Tris-HCl pH 7.2, 4 mM $MgCl_2$, 0.1 mM DTT; Shishkova & Dubrovsky 2005) for 5 min and incubated with DNAse I (500 μg cmL^{-1} in DN) for 15 min at 25 °C. Omission of TdT enzyme in the reaction served as a negative control. After TUNEL staining, sections were mounted in 50% glycerol, 0.15% n-propylgallate, containing 0.5 μg mL^{-1} DAPI (4'6-diamidino-2-phenylindole) and observed under an epifluorescence inverted microscope equiped with a fluorescence led illumination module (DM IL LED) and CCD (2.8 Mpix, DFC 7000T) camera (Leica Microsystems AG, Heerbrugg, Switzerland). Digital images were processed by Leica LAS software. In all experimental groups, the number of TUNEL-positive cells in 10 root explants was counted and five samples from each group were evaluated. Differences between the number of TUNEL-positive cells in the control and experimental samples were statistically analyzed using the ANOVA; post hoc analysis was performed using Dunn's test.

Ethylene emission measurements

For determination of endogenous ethylene production fresh excised apical 10-mm long root segments (100 mg FW) were placed in 50 mL hermetically capped vials, flushed with ethylene-free air and incubated at room temperature for 1 h. At time zero respective mycotoxins were injected into the vial (100 μg mL^{-1} final concentration) and from this period ethylene was allowed to accumulate for 2 h intervals. One mL of headspace air was sampled and injected into a gas chromatograph (Model 2000, Perkin-Elmer, Norwalk, USA) packed with 0.32 cm × 1.2 m Poropak-QS 80/100 column and equiped with a flame ionization detector (FID). Helium was the carrier gas used at 8 psi. The injector, owen and detector temperatures were 175 °C, 75 °C and 175 °C, respectively. The total amount of ethylene produced during a period was calculated from the ethylene production rates at different times. Ethylene production was expressed as nL h^{-1}. Three determinations were performed for each point. In a separate experiment stress ethylene was allowed to accumulate for 24 h and was analyzed as described above. This experiment was repeated at least twice.

Western blotting

Root extracts containing 100 μg of total protein were separated by 12.5% SDS-PAGE, blotted onto WESTRAN BA-85 (0.45 μm) nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany), and probed with the specific DAD1 and tubulin antibody at a dilution of 1 : 1000 in TEN buffer (Repka 2006). Horseradish peroxidase (PRX)-conjugated goat antirabbit IgG (Kirkegaard & Perry Labs., Gaithersburg, USA) was used as a secondary antibody, and the reaction was visualized by enhanced chemiluminescence using SuperSignal West Pico (Pierce, Rockford, USA) as a substrate.

Photography and statistics

At least 5 pictures from randomly selected roots were taken in all experiments. Each experiment was repeated at least twice for each condition. Digital pictures were directly obtained with a *Coolsnap* CCD camera (RS Photometrics, USA). Only representative pictures are shown. All data were analyzed using a one-way analysis of variance (ANOVA) with $P < 0.05$ or 0.01. Means and standard deviations were calculated from three independent experiments (n = 10 apical root segments); post hoc analysis was performed using Dunn's test.

Results

Effect of ZEN and its derivatives on programmed cell death

At a macroscopic level, a considerable increase in Evans blue uptake was detected in the 10 mm long apical root segments treated for 24 h with 100 μg mL^{-1} of ZEN and its derivatives α-ZEL and β-ZEL (Fig. 1A). The Evans blue uptake was observed also in the control roots, but to a much lower extent in comparison with the mycotoxin-treated root segements (Fig. 1A, Control). As a result of cell death, the accumulation of the dye revealed a strict mycotoxin-specific pattern. After 24 h, maximum cell death was induced by β-ZEL, followed by ZEN and then α-ZEL (Fig. 1B).

To explore whether the observed Evans blue uptake activity actually mimics the PCD, specific test (*in situ* TUNEL assay) was used to detect a major hallmark of apoptosis. DNA fragmentation analysis of control (untreated) apical root segments revealed that < 10% of cells had TUNEL-positive nuclei (Fig. 1C). Compared with the control roots, the mycotoxin-treated root segments contained nuclei with stronger green fluorescence. The percentage of the TUNEL-positive nuclei in the mycotoxin-treated root segments differed and revealed the toxin specific patterns. When data for all root segments were analyzed, the TUNEL-positive nuclei in ZEN-, α-ZEL- and β-ZEL-treated roots segments reached the approximate values 73%, 48% and 92% of total nuclei, respectively (Fig. 1C). Collectively, these results indicate that mycotoxin treatment can cause DNA fragmentation.

Caspase-specific inhibitors block mycotoxin-induced cell death

To evaluate if mycotoxin-induced cell death in maize apical root segments is mediated by some caspase-like proteases, caspase-specific inhibitors at a concentration of 0.1 μM were applied simultaneously with ZEN, α-ZEL and β-ZEL. The human caspase-1 inhibitor YVAD-CMK (Z-Tyr-Val-ala-Asp-chloromethylketone) and the broad range caspase inhibitor ZASP-DCB (Z-Asp-CH2-DCB, benzyoxycarbonyl-asp-2,6-dichlorobenzoyloxymethyl-ketone) or BOC-FMK (BOC-Asp (OMe)-fluoromethylketone) significantly reduced the rate of mycotoxin-induced cell death (Fig. 2). Also the application of the human caspase-3 inhibitor DEVD-CMK (Z-Asp-Glu-Val-Asp-chloromethylketone) and caspase-9 inhibitor LEDH-CMK (Z-Leu-Glu-His-Asp-fluoromethylketone) to maize root segments incubated in 100 μg mL^{-1} of respective mycotoxins, effectively blocked the cell death (Fig. 2). As a negative control, apical root segments were treated with two caspase-unrelated peptide inhibitors MEO-CMK (MeOSuc-AAPV-CMK, methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl- ketone) or ZFA-FMK (Z-Phe-Ala-

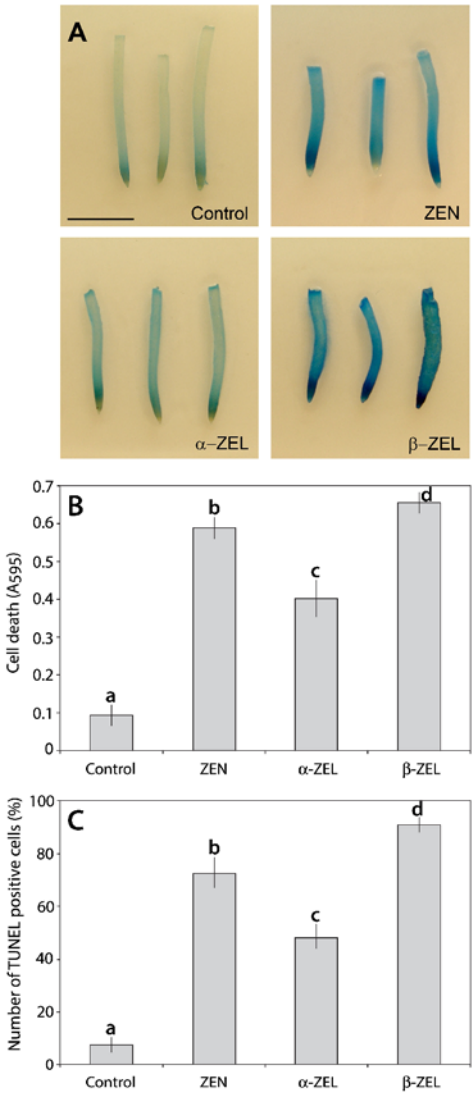

Fig. 1. ZEN and its derivatives induce cell death. A) – Effect of mycotoxins on Evans blue uptake at 24 h post-mycotoxin treatment. Representative pictures of three roots per treatment are shown. Bar = 500 μm; B) – Quantification (A_{595}) of cell death in Evans blue stained maize apical root segments 24 h post-mycotoxin treatment; C) – Percentage of TUNEL positive cells determined by TUNEL assay 24 h post-mycotoxin treatment. Mean values ± SD ($n = 10$). Different letters indicate statistical significance according to Dunn's test ($P < 0.05$).

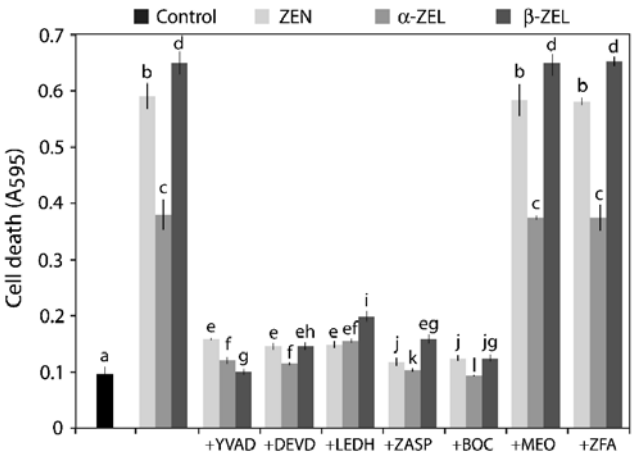

Fig. 2. Dependence of mycotoxin-induced cell death on caspase-like proteases. Root segments were treated with respective mycotoxins (100 μg mL^{-1}) alone or together with caspase inhibitors (0.1 μM). Cell death was determined and quantified by Evans blue staining 24 h after treatment. Mean values ± SD ($n = 10$). Different letters indicate statistical significance according to Dunn's test ($P < 0.05$). YVAD (Z-Tyr-Val-ala-Asp-chloromethylketone), ZASP (Z-Asp-CH2-DCB, benzyoxycarbonyl-asp-2,6-dichlorobenzoyloxymethylketo-ne), BOC (BOC-Asp(OMe)-fluoromethylketone), DEVD (Z-Asp-Glu-Val-Asp-chloromethylketone), LEDH (Z-Leu-Glu-His-Asp-fluoromethylketone), MEO (MeOSuc-AAPV-CMK, methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone), ZFA (Z-Phe-Ala-FMK, benzyloxycarbonyl-Phe-Ala-fluoromethylketone).

FMK, benzyloxycarbonyl-Phe-Ala-fluoromethylketone). Tested peptide inhibitors did not substantially affect mycotoxin-induced cell death (Fig. 2). These results indicate that the mycotoxin-induced cell death pathway employs caspase-like proteases and argue for the fact that mycotoxin-induced cell death reflects some features of animal apoptosis.

Mycotoxin-induced cell death is alleviated with exogenous ethylene

To test if ethylene mediates mycotoxin-induced cell death, a time course analysis of ethylene production in control and mycotoxin-treated apical root segments was performed. Ethylene was allowed to accumulate for successive periods of 2 h in the absence (control) or presence of ZEN, α-ZEL or β-ZEL (100 μg mL^{-1}). The treatment of root segments with respective mycotoxins reduced the basal ethylene production rate gradually with apparent mycotoxin-specific kinetics (Fig. 3A). The most effective in the lowering of ethylene production was ZEN which almost completely abolished ethylene production within 10 h (Fig. 3A, ZEN). α-ZEL and β-ZEL completely inhibited ethylene production within 22 and 18 h, respectively (Fig. 3A, α-ZEL and β-ZEL). As a positive control, root segments were treated with 100 μM $CdCl_2$, which induced significant rise in transient ethylene production (Fig. 3A, Cadmium).

In addition, 24 h accumulation experiments were also performed. When the root segments were treated with ethylene biosynthesis inhibitor, aminoethoxyvinylglycine (AVG, 1 μM), endogenous ethylene production was slightly inhibited (Fig. 3B, AVG) and this inhibition was more efficient with higher concentrations of AVG (data not shown). In contrast, treatment of root segments with the precursor of ethylene aminocyclopropane-1-carboxylic acid (ACC, 1 μM) induced a significant rise in ethylene production (Fig. 3B, ACC). In this experiment, all three tested mycotoxins almost completely blocked endogenous ethylene production and this mycotoxin-induced effect can be partially alleviated by simultaneous addition of 1 μM ACC (Fig. 3B).

To further elucidate the role of ethylene signalling in mycotoxin-induced cell death in maize apical root segments, inhibitor (AVG) and stimulator (ACC) spe-

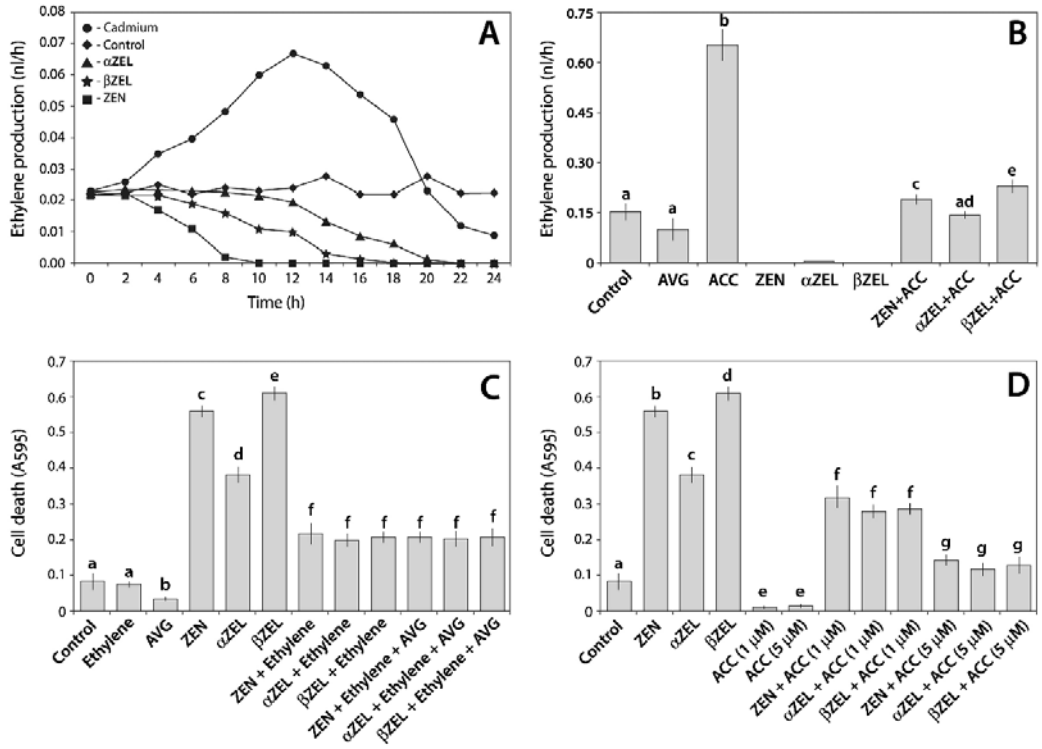

Fig. 3. Effect of ZEN and its derivatives on ethylene production and cell death progression. A) – Time course of ethylene production in control, cadmium-stressed and mycotoxin-treated apical root segments; B) – Effect of mycotoxins, ethylene inhibitor (AVG) and ethylene stimulator (ACC) on ethylene production during a 24 h treatment; C) – Effect of exogenous ethylene and AVG on cell death. Root segments were simultaneously treated with respective mycotoxins and either ethylene or AVG and cell death was determined and quantified by Evans blue staining 24 h after treatment; D) – Concentration-dependent effect of ACC (ethylene precursor) on mycotoxin-induced cell death. Root segments were simultaneously treated with respective mycotoxins and different concentrations of ACC and cell death was determined and quantified by Evans blue staining 24 h after treatment. Mean values ± SD ($n = 3$). Different letters indicate statistical significance according to Dunn's test ($P < 0.05$).

cific to ethylene biosynthesis pathway were applied. When applied alone (100 μL L^{-1}), the exogenous ethylene did not affect the rate of cell death in root explants (Fig. 3C). Treatment with 1 μM AVG for 24 h resulted in a marked decrease of cell death when compared to both control and ethylene. When ZEN, α-ZEL or β-ZEL (100 μg mL^{-1}) were combined with exogenous ethylene (100 μL L^{-1}), the cell death rates after 24 h were reduced to ∼ 50–60%. Furthermore, the observed effect of ethylene on mycotoxin-induced rates of cell death could not be reversed by additional treatment with AVG (1 μM). Addition of exogenous ACC (1 or 5 μM) had an apparent effect on cell death, but simultaneous application of ACC with ZEN, α-ZEL or β-ZEL also effectively blocked mycotoxin-induced cell death by a concentration dependent manner (Fig. 3D).

Mycotoxin-induced cell death requires inhibition of DAD1 protein expression

Preliminary antibody microarray protein expression profiling experiments have revealed that challenge of maize roots with ZEN and its derivatives stimulated accumulation of distinct apoptosis-related proteins and repression of anti-apoptotic genes (unpublished data). Among them, defender against apoptotic death (DAD1) protein displayed an interesting expression pattern. As revealed by subsequent Western blot analysis, expression of maize DAD1 was constitutive in control (untreated) root segments (Fig. 4). In contrast, the expression of DAD1 was profoundly repressed upon mycotoxin treatment. While ZEN and β-ZEL at 100 μg mL^{-1} blocked the expression of DAD1 completely, the percentage of α-ZEL-induced suppression reached 88% when compared to control. The reason for this variability is not clear but may be related to differences in perception of cells. The effect of mycotoxins on repression of DAD1 accumulation in apical root segments was fully (ZEN and β-ZEL) or partially (α-ZEL) reversed by additional treatment with exogenous ethylene (100 μL mL^{-1}), although application of ethylene alone did not affect the DAD1 expression level (Fig. 4). Moreover, application of ethylene biosynthesis inhibitor AVG (1 μM) to root segments did not substantially lower the expression of DAD1 protein. These results collectivelly indicate that restoration of mycotoxin-induced repression of DAD1 was not related to ethylene synthesis and possibly requires other cellular components that determine cell death fate.

Lipid and calcium signalling is instrumental in mycotoxin-induced cell death

To further explore the signal transduction pathways involved in mycotoxin-induced cell death in apical root segments, pharmacological inhibitors and stimulators

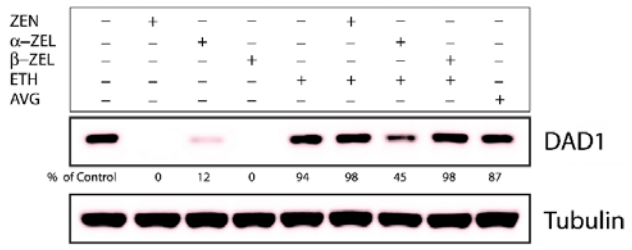

Fig. 4. Western blot analysis of the effects of respective mycotoxins, exogenous ethylene and AVG on expression of DAD1 protein in maize apical root segments. Each lane was loaded with 100 μg of total protein. Tubulin expression confirmed the equal loading in each lane and the immunospecific signal was developed using a chemiluminiscent substrate.

related to various intermediates and enzymes in phospholipid signalling were tested. Activation of phospholipase C (PLC) in all living cells has a strict calcium requirement. As shown in Fig. 5A, treatment of apical root segments with the calcium channel blocker $LaCL_3$ (150 μM), cell permeable L-type calcium channel protein inhibitor Nemadipine A (50 μM) and the calmodulin inhibitor W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; 15 μM) significantly reduced the rate of cell death induced by tested mycotoxins. Also the modulator of phosphoinositol metabolism, LiCl (100 μM), which inhibits inositol monophosphatase (IMP) activity, markedly reduced mycotoxin-induced cell death.

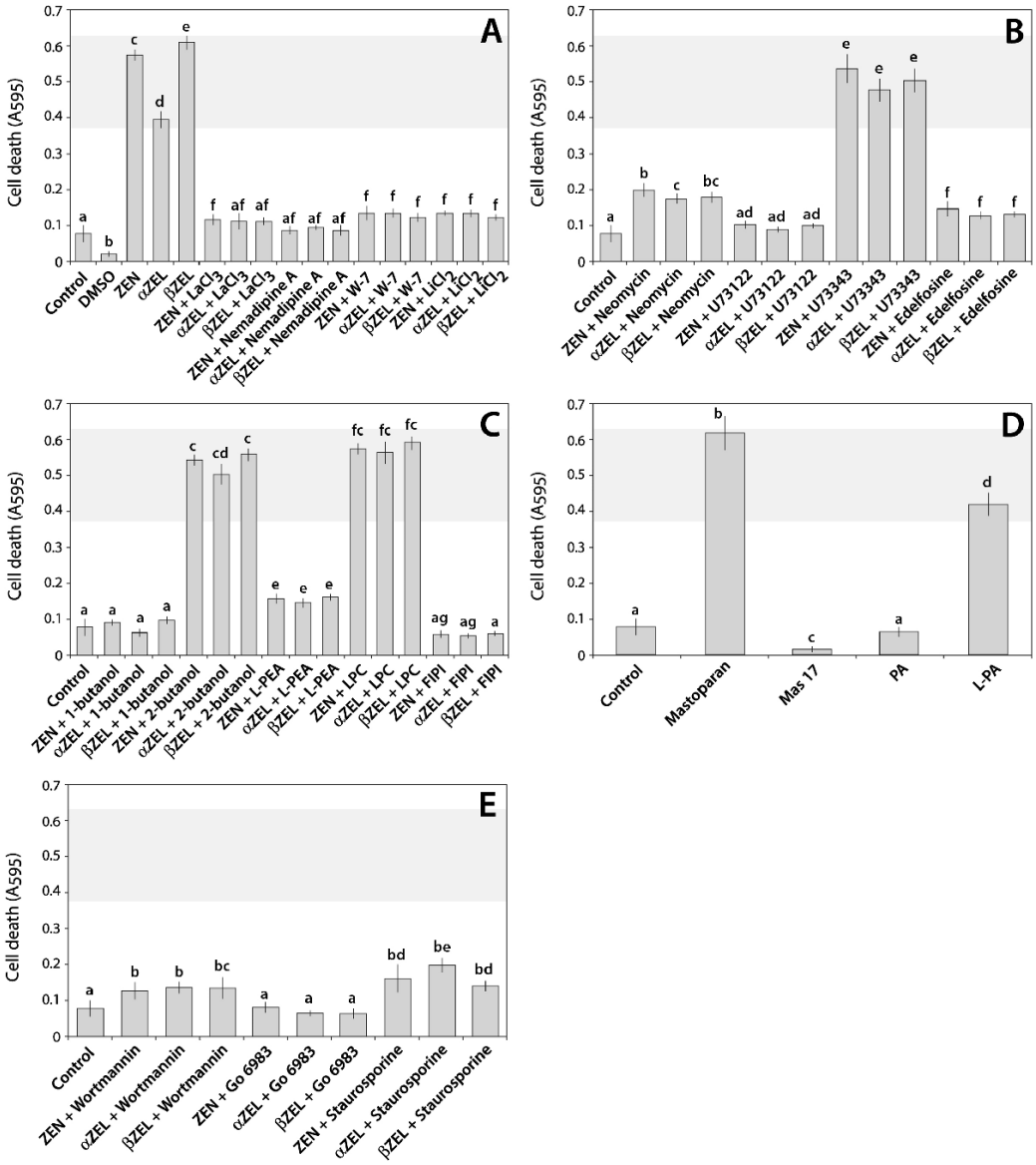

Fig. 5. Phospholipid signalling is instrumental in mycotoxin-induced cell death. A) – Effect of inhibitors of calcium and lipid signalling on mycotoxin-induced cell death; B) – Effect of Phospholipase C (PLC) signalling inhibitors on mycotoxin-induced cell death; C) – Effect of Phospholipase D (PLD) signalling inhibitors on mycotoxin-induced cell death; D) – Effect of phospholipid signalling stimulators and their inactive analogues on cell death; E) – Effect of different lipid and protein kinase inhibitors on cell death. Cell death was determined and quantified by Evans blue staining 24 h after treatment. Horizontal gray bars indicate the cell death range induced by ZEN and its derivatives when applied to the root segments alone. Mean values ± SD ($n = 3$). Different letters indicate statistical significance according to Dunn's test ($P < 0.05$).

Next, we examined the effects of various PLC inhibitors on the extent of mycotoxin-induced cell death (Fig. 5B). Application of neomycin (5 μM) significantly decreased mycotoxin-induced cell death. The rate of cell death was substantially reduced by incubation of root segments in medium containing 15 μM U73122, an inhibitor of phosphoinositide-specific PLCs (PI-PCLs), whereas application of 15 μM U73343, an inactive analog of U73122, had no effect on cell death. Edelfosine (100 μM), another PI-PCLs inhibitor, was also significantly effective in reducing mycotoxin-induced cell death. There was an apparent inhibitor-specific capacity to reduce the rate of ZEN-, α-ZEL- and β-ZEL-induced cell death.

Phospholipase D (PLD) transfers the phosphatidyl group of structural phospholipids to a primary alcohol rather than water, producing phosphatidylalcohol and inhibiting both, the PLD-mediated production of phosphatidic acid (PA) and PA-dependent cellular responses. To investigate whether PA could be involved in mycotoxin-induced cell death, root explants were treated with 0.5% 1-butanol or 2-butanol. In 1-butanol-treated roots the rate of cell death was significantly reduced. The secondary alcohol, 2-butanol, which cannot serve as PLD substrate was used as a negative control. As expected, 2-butanol did not affect the mycotoxin-induced cell death rate (Fig. 5C). Lysophosphatidylethanolamine (L-PEA) is a naturally occuring lipid produced by phospholipase A_2 (PLA_2)-mediated hydrolysis of phosphatidylethanolamine and is one of the few known inhibitors of PLD. Addition of 50 μM L-PEA significantly reduced cell death. In comparison, the related phospholipid such as lysophosphatidylcholine (LPC; 50 μM) had no significant effect on mycotoxin-induced cell death. The inhibition of mycotoxin-induced cell death was further increased by application of 1 μM 5-fluoro-2-indolyl deschlorohalopemide (FIPI), a PLD1/2 dual pharmacological inhibitor (Fig. 5C).

Both PLC and PLD may be activated via G protein-coupled signalling. As shown in Fig. 5D, G protein activator mastoparan (5 μM), but not its inactive analog Mas 17 (5 μM), significantly increased cell death indicating that phospholipid signalling cascade is involved in the process. Addition of native PA (50 μM) to maize root explants did not substantially affect cell death, probably due to its poor uptake by the cells. On the other hand, the marked stimulatory effect on cell death was observed after application of lyso-phosphatidic acid (L-PA; 15 μM), an analogue of PA derived from either activity of PLC or PLD.

Downstream molecular targets of PLC- and PLD-derived second messengers may be a wide spectrum of protein and/or lipid kinases, e.g. MAPKs, PA-kinase (PAK) and phosphatidylinositol 3-kinase (PI-3-kinases). Application of 5 μM staurosporine, a broad spectrum protein kinase inhibitor, significantly inhibited mycotoxin-induced cell death indicating that some classes of kinases are involved in the cell death signalling cascade. The PI-3-kinase inhibitor wortmannin (10 μM), as well as protein kinase C inhibitor Go 6983 (15 μM) decreased mycotoxin-induced cell death. These results show that PI-3-kinases and protein kinase C (probably phospholipid-dependent) are a part of the signalling pathway instrumental in mycotoxin-induced cell death (Fig. 5E).

Discussion

In our previous study, we demonstrated that ZEN and its derivatives α- and β-zearalenole, collectively termed here as ZENs, could induce a sequence of physiological and biochemical events resulting in the onset and the progression of root cell death (Repka et al. 2014). However, the implication of putative signalling pathways and the roles of relevant signalling molecules in ZENs-induced cell death remained unknown.

The above data indicate that the changes observed in the ZENs-treated root explants resemble the PCD hallmarks reported previously for plant cells challenged with toxins produced by other *Fusarium* species (Jiao et al. 2013). Moreover, as revealed in this work by independent cell death assays, the pattern of ZENs-induced PCD in maize root explants is shown to be mycotoxin-specific.

Earlier reports have indicated that caspase-like proteases play a critical role during PCD in plants (Jiao et al. 2013). Results presented in this paper further demonstrate that the administration of the synthetic peptide inhibitor YVAD-CMK, DEVD-CMK or LEDH-CMK specific to human caspases 1, 3 and 9, respectively, effectively blocked the ZENs-induced cell death. True caspases have not been descibed in plants yet and thus caspase specific inhibitors may exert their PCD inhibitory effects via other molecular targets. In various plant-pathogen systems vacuolar processing enzymes (VPEs), a group of cysteine endopeptidases, or subtilisin-like serine proteases were found to exhibit caspase-like activity and could be effectively blocked by inhibitors specific to human caspases (Coffeen & Wolpert 2004; Krzymowska et al. 2007).

Numerous studies have shown that ethylene plays an important role in regulating and modulating plant responses, including PCD, to biotic stresses (Plett et al. 2009; Singh & Upadhyay 2014). In the present study, ethylene production was significantly inhibited by ZENs treatment and this response can be partially reversed by simultaneous addition of 1 μM ACC (Fig. 3B). Unexpectedly, suppressing ZENs-induced ethylene production was accompanied with significant rise in PCD rate. Interestingly, this rise in cell death was at least partially prevented by simultaneous addition of ethylene precursor ACC or exogenous ethylene suggesting that this molecule is an important player to bring out the process of PCD in maize roots. The effect of ethylene on ZENs-induced cell death was not reversed by simultaneous addition of AVG indicating that the observed decrease in the cell death rate was related rather to exogenously applied, than the biosynthetically-produced ethylene (Fig. 3C). There are conflicting results with

regard to the exact role of ethylene in mediating plant cell death. Previously it has been demonstrated that treatment with exogenous ethylene can increase disease symptoms such as necrosis and spread of pathogens (Hoffman et al. 1999). On the other hand, it has also been shown that presence of ethylene is essential for resistance to some pathogens, e.g. *Erwinia carotovora* or *Botrytis cinerea* (Thomma et al. 1999; Norman-Setterblad et al. 2000). The possible explanations for the observed effect of ethylene on ZENs-induced progression of PCD may come from the study of the role of ethylene receptors in Fumonisin B1 (FB1)-induced cell death in *Arabidopsis* (Plett et al. 2009). The evidence points to the key roles of ethylene receptors EIN4 and ETR1 which have distinct functions in ethylene signalling. When ethylene binds at EIN4, cell death is accelerated through repression of transcription factor *ERF1* which is known to be essential for pathogen resistance. Ethylene signalling through ETR1 inhibits cell death most likely through positive regulation of *PDF1.2* and *ERF1* genes. It is important to note, the EIN4 and ETR1 regulate the expression of other ethylene receptors and collectively impact the expression of downstream defence genes. Taken together, fine-tuning the balance between the two receptors may affect the outcome of mycotoxin-induced cell death.

Preliminary antibody microarray expression profiling of a variety of positive and negative regulator proteins involved in the events of PCD have been performed in ZENs-treated maize root explants. The most intriguing expression profile has been observed for DAD1 protein, a product of putative PCD supressor gene in both animals and plants (Apte et al. 1995; Dong et al. 1998). We demonstrated that the DAD1 expression declined dramatically upon mycotoxin treatment, when the PCD program proceeded, and its disappearing is concomitant with the blocking of ethylene production. The observation that a strong inhibitory effect of ZENs on PCD and DAD1 expression may be reversed by exogenous ethylene argues for the vital role of this hormone in maintaining cellular homeostasis. The participation of ethylene in the regulation of PCD and DAD1 expression has been independently reported from the study of plant senescence, flower pollination and fruit ripening (Yamada et al. 2004), all representing a modified plant form of PCD (Jones & Dangl 1996). At present, the exact role of ethylene-regulated expression of DAD1 protein in ZENs-induced PCD is not clear. The DAD1 protein is a subunit of a membrane-embedded oligomeric complex, the OST (Kelleher & Gilmore 2006), involved in post-translational modification (glycosylation) of many proteins in endoplasmic reticulum (ER). On the basis of the above data, we hypothesize that the role DAD1 in response of cells to ZENs may be 2-fold. Permanent loss of DAD1 in ZENs-treated root cells would initiate and/or contribute to the amplification of ER stress. An alternative is that ZENs-induced degradation of DAD1 induces persistent accumulation of either misglycosylated or unglycosylated proteins resulting in the activation of pro-death unfolded protein response (UPR) pathways. Regardless of the type of mechanism both cases lead to cell death. Finally, this conclusion needs to be verified experimentally and alternative mechanisms cannot, however, be ruled out.

The increase in cytosolic Ca^{2+} levels in plant cells appears to be an important component of plant responses to abiotic and biotic stress. In line with this statement we show that several calcium channel blockers ($LaCL_3$, Nemadipine A, W-7) inhibit the effect of ZENs on cell death. One of the best-described calcium releasing second messengers is phosphatidylinositol-triphosphate (IP_3), which is released from the membrane phospholipids by the activity of phosphoinositide-specific PLC (PI-PLC; Mueller-Roeber & Pical 2002). Recent data, however, indicate that IP_6 is more potent in releasing Ca^{2+} (Zonia & Munnik 2006) and this molecule has been implicated pathogen resistance in *Aarabidopsis* (Murphy et al.2008). In plants, different IMPs have been found sensitive to LiCl inhibition (Gillaspy et al.1995). The observation that LiCl inhibits ZENs-induced cell death suggests that fluctuations in the level of free inositol may affect the processes in inositide cascade and support the idea that ZENs may stimulate PLC signalling.

Likewise in animals, also in plants the G-protein-activated signalling pathways can be artificially activated by primary alcohols and the G-protein stimulator mastoparan (Munnik et al. 1995). Both, mastoparan and L-PA induced cell death in maize roots indicating that lipid signalling is involved in this process and that PA may be an important intermediate generated either through PLD or PLC activity (Testerink & Munnik 2005).

Several inhibitors of either PLC (neomycin, U-73122, Edelfosine) or PLD (1-butanol, L-PEA, FIPI) inhibited the effect of ZENs on cell death. The involvement of PLC, PLD and their products, namely PA, in the response to various kinds of stress has been experimentally demonstrated in plants (Ruelland et al. 2015).

Evidence has shown that downstream targets of PLC- and PLD-derived second messengers may be a plethora of protein and lipid kinases (Munnik & Meijer 2001). Pharmacological assays with specific inhibitors of PI-3 kinase and protein kinase C, as well as with a broad spectrum protein kinase inhibitors, significantly inhibited cell death rate in response to ZENs treatment. This observation indicates the involvement of PI-3 kinases and eventually other kinases in the signal transduction cascade leading to ZENs-induced PCD. PI-3 kinase prevents the spread of PCD during plant antiviral hypersensitive response (Liu et al. 2005).

The plant responses to ethylene are repressed by constitutive triple response 1 (CTR1) protein kinase which is a homolog of the mammalian Raf-1 kinase (MAPKKK). Testerink et al. (2008) have demonstrated that CTR1 function is negatively regulated by PA and proposed a model on the role of PA in activation of downstream ethylene signalling pathway by inhibition of CTR1 kinase activity. Based on the presented data

and in line with this model we hypothesize that ZENs secreted to the plant cells may exert their functions as molecular switches that turn off the downstream ethylene regulated defence pathways by inhibition of ethylene biosynthesis. ZENs-induced toxicity expressed on a background of already compromised cells may be sufficient to manifestation of cell death. Finally, a challenge for future research is the elucidation of the involvement of CTR1 and the specific role of lipid second messengers like PA in ZENs-induced PCD.

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