MODULATION OF PROGRAMMED CELL DEATH IN A MODEL SYSTEM OF XYLOGENIC ZINNIA (*ZINNIA ELEGANS*) CELL CULTURE

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ABSTRACT

Programmed cell death is an integral part of the latest stage of differentiation of the tracheary elements of plant xylem vascular system. In this study, by applying a pharmacological approach with specific peptide inhibitors, we have elucidated the involvement of plant caspase-like proteases in cell death signaling during vessel elements formation in xylogenic zinnia (Zinnia elegans) cell culture. Cell culture was isolated from zinnia leaves and exposed to chemical treatments. Broad spectrum caspase inhibitor, caspase 1 and caspase-3 inhibitors were administrated to the cell culture where the cells were induced to differentiate with the addition of 1 mg/L benzylaminopurine and 0.1 mg/L α-naphthalene-acetic acid. The differentiation was substantially suppressed in presence of 100 nM concentration of the inhibitors. This indicates that the re-differentiation in cultured mesophyll zinnia cells is a PCD event that most probably occurs through signaling involving caspase-like proteases. The reported results are the first that provide evidence for participation of plant caspase-related enzymes in xylogenesis in zinnia. Laser scanning confocal microscopy revealed differentiation associated structural cellular changes, including formation of large vacuole, secondary wall thickenings, tonoplast rupture and cell autolysis to form hollow dead tracheary elements..

Keywords: proteolysis, programmed cell death, tracheary elements, xylogenesis

Introduction

Programmed cell death (PCD) is a cell suicidal genetically programmed developmentally and environmentally stimulated mechanism (9). An example of developmental PCD in vascular plants is the PCD that occurs at the final stage of cell differentiation during the formation of xylem vascular system (6). Xylem vessels (water conducting tubes) are composed of a number of fused vessel or tracheary elements (TEs) that are dead, hollow cells with patterned lignified cellulose secondary walls. TEs originate through re-differentiation of root and shoot pro-cambium and cambium cells and undergo autolysis as they differentiate and mature (3). In mammalian cells, a type of PCD commonly referred to as apoptosis, is mediated by cysteinyl-aspartic proteases (caspases)-activation events involving initiator caspases and downstream execution caspases resulting in a typical phenotype of cell shrinkage, chromatin condensation, nuclear DNA cleavage and, cellular disintegration into apoptotic bodies, that are digested by macrophages (7, 14). No structural homologues of animal caspases exist in plants, but caspase peptide inhibitors known to inhibit caspases in animal models have been shown as potent abolishers of PCD in plant systems indicating the existence of plant proteases with substrate specificity and functional similarity to animal caspases (1, 2, 13, 16).

Xylogenic zinnia (*Zinnia elegans)* cell culture derived from leaf mesophyll cells has been established as a unique model system to study PCD mechanisms during xylogenesis (5). Cytokinins and auxins are both required for re-differentiation process *in vitro* and *in planta*. Plant hormones (4) and other signaling substances and metabolic pathways (brassinosteroids, calcium, ROS, abscisic and jasmonic acids, nitric oxide, ethylene, MAP-kinases, pH, osmotic values, etc.) are described to be involved in the integration and control of TE formation (3, 4). No evidence has earlier been shown about the involvement of plant caspase-like proteases in PCD during TE formation in zinnia cell culture (1, 3, 15).

The involvement of PCD in the process of TE differentiation suggests that xylogenesis could be manipulated by controlling the PCD signaling. In turn, modulations of the initiation and the progress of PCD may alter the anatomy of the TEs. Hence, the use of appropriate PCD signaling modulators may lead to improved properties of the xylem vessels, finally influencing the quality and shelf life of the plants.

In order to better understanding of the PCD signaling involved in the process of xylogenesis, the present work addressed a possible participation of plant proteases with functional similarity to animal caspases in PCD events during tracheary elements (TEs) formation in a model system of *Zinnia elegans* cell culture. We also aimed to elucidate the potential of pharmaceutical treatments for manipulation of TE generation and to study the structural cellular changes associated with TE differentiation.

Materials and methods

Cell culture isolation and chemical treatments

Seedlings of zinnia (*Zinnia elegans)* cv. Envy, were raised in peat-based commercial potting compost (85% peat, 15% clay) at growing conditions of 16 h day photoperiod, day/night temperature 25/20^{o}C and lower than 70% RH. Mesophyll cells were isolated in sterile conditions from the first true leaves (3rd leaf pair just emerging) of 14-day old seedlings, by gentle mechanical homogenization of surface-sterilized leaves in a culture medium following, the protocol of Fukuda and Komamine (5) with slight modifications. Briefly, the leaves were sterilized in 0.15% sodium hypochlorite, leaf homogenate was filtered through 50 μm nylon mesh and the filtrate was centrifuged at 150 g for 90 sec. The pellet was re-suspended with the medium to achieve cell density of 2.10^{5} cell/ml and, the obtained suspension was cultured in dark at 26^{o}C in orbital shaker at 80 rpm. For induction of TE differentiation, 24 h after the isolation, 3 ml portions of the cell culture were treated with 0.1 mg/l α-naphthalene-acetic acid (NAA) and 1mg/l benzylaminopurine (BA) and cultured in 6-well culture plates at the above conditions. Non-hormone induced culture served as a negative control. A range of concentrations of irreversible broad-ranged human caspase-3 inhibitor benzyoxycarbonyl-Asp-2,6-dichlorobenzoyloxymethylketone (Z-Asp-CH_2-DCB), irreversible caspase-1 inhibitor Tyr-Val-ala-Asp-chloromethylketone (Ac-YVAD-CMK), and the reversible caspase-3 inhibitor Acyl-Asp-Glu-Val-l-aspartic acid aldehyde (Ac-DEVD-CHO), as well as cysteine protease inhibitors L- transepoxysuccinyl-leucylamido-(4-guanidino)butane) (E64), iodoacetamide (IA) and N-ethylmaleimide (NEM)) were tested alone and in combination with the hormones for its potency to modulate the process of differentiation. Lowest concentrations of the inhibitors that exerted most pronounced effects are presented. Controls with hormones and without caspase inhibitors were set up. Cell counting of living, dead and differentiated cells was executed by using Axiovert Carl Zeiss microscope at 100 x magnification.

Microscopic observations of cell morphology and imaging

The living cells were detected by FDA staining (green fluorescence visible in the living cells only) and the dead cells were distinguished by propidium iodide (PI), permeable in the dead nuclei. Calculfuor White (0.0005%) was used to stain the patterns of cellulose bands profiling the secondary cell wall thickenings in the differentiated TEs. Images were collected by 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.

Data analysis

Cell counting was performed in three non-overlapping randomly chosen microscopic fields per each sample. Presented data are averaged of at least three independent experiments after counting of approximately 80 cells per microscopic field. Values are compared by standard error of the means (SEM $_{(n-1)}$).

Results and Discussion

Freshly isolated zinnia mesophyll cell culture contained more than 80% viable cells. Treatments of the cells with 1 mg/L BA+0.1 mg/L NAA induced approximately 40% of the cells to trans-differentiate in TEs (**Fig. 1**), the first TEs generated the earliest 48 h after administration of the hormones and the process was terminated 120 h after hormone addition.

We have tested other hormone types (e.g. 2,4D), and cytokinin/auxin ratios but only the indicated 10/1 ratio of BA to NAA proved to be efficient for achieving a maximum of 40% differentiation. In addition, when the culture was treated with various hormone combinations and cell death inhibitors the TE yield varied, suggesting that the ratio and type of auxin and cytokinin might affect the process of re-differentiation by interfering with the cell death inhibiting chemicals (data not shown).

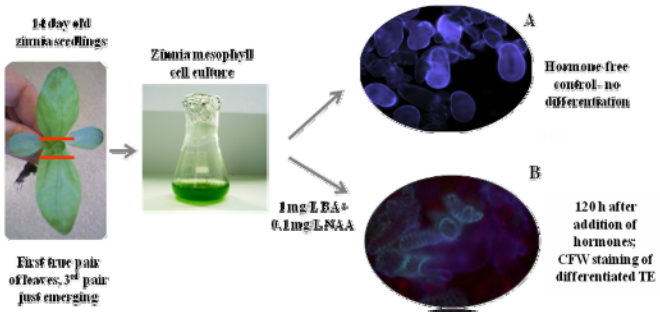

Fig. 1. Schematic presentation of isolation and induction of zinnia cell culture. For details on culture isolation see Materials and Methods. Confocal field images show CFW stained: A. non-differentiated non-hormone treated cells; B. differentiated cells 120 h after administration of 1 mg/L BA+0.1 mg/L NAA.

The established generation of TEs indicates that the studied zinnia cell culture indeed has a potential to re-differentiate when exposed to induction with proper ratio of phytohormones.

Three consecutive stages, each associated with specific physiological states of the cells, typical morphological features and expression of specific set of genes are described to occur during the process of trans-differentiation in zinnia cells (3, 15). Stage I includes de-differentiation of mesophyll cells and acquisition of competence for re-differentiation, during stage II synthesis and deposition of secondary wall material occurs and the final stage III involves a progression of PCD process associated with (in this order) formation of large vacuole, rupture of the tonoplast, DNA fragmentation, disappearing of the nucleus, autolysis of cell content and formation of hollow dead TEs. In our experiments, the three stages were monitored and, in addition, formation of vessel-like elements of fused TEs was observed (**Fig. 2**).

To examine the morphological changes in the cellular constituents that occur during TE differentiation, we have performed observations by laser-scanning confocal microscopy. Bright green fluorescence of FDA in the viable cells showed intact cytoplasm and intact vacuole, non permeable for the dye (**Fig. 3A**).

Fully differentiated TEs showed auto fluorescence from the secondary cell walls (**Fig. 3A**). Red coloration of the living cells was due to chlorophyll auto-fluorescence (**Fig. 3B**). CFW staining of the cellulose fibers in the cell walls allowed qualitative analysis of the TE differentiation including distinguishing of the different stages of the formation of cell wall thickenings as represented in fully differentiated (completed secondary wall patterning) TEs (**Fig. 3B)** and in differentiating (incomplete secondary cell walls) (**Fig. 3C**) cells.

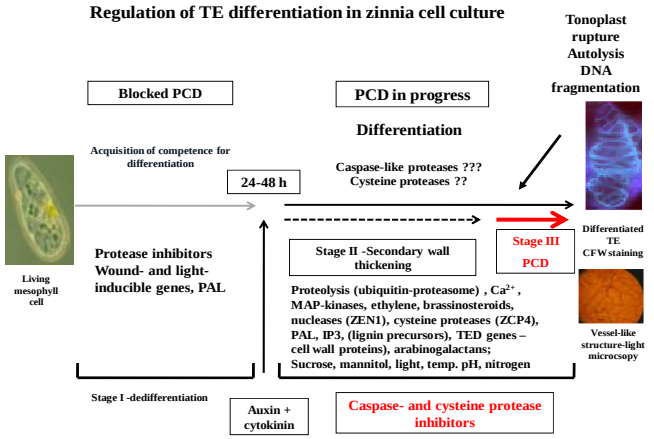

Fig. 2. Schematic presentation of the regulation of TE differentiation in Zinnia cell culture.

The late stage of TE formation was preceded by vacuole rupture while the dead compacted nucleus was still preserved, as clearly visible by in debt color imaging of PI stained TE (**Fig. 3D**). TEs maturation was completed by autolysis of the cell content followed by degradation of the dead nucleus, to form hollow differentiated cells (**Fig. 3B**). This data demonstrate additional details of the progressive structural changes involved in autolytic formation of TEs and is in line with the cellular features of TEs described by other authors (4, 12)

It has been documented that hydrolases, nucleases, cysteine and serine proteases, participate in the final stage of PCD during TE differentiation (**Fig. 2**) of zinnia cultured cells (8, 11, 13, 15, 17). No information is as yet available about the involvement of caspase-like plant proteases.

To elucidate the possible involvement of plant caspase-like proteases in the PCD signalling during the process of TE differentiation, the zinnia cells were induced to differentiate with 0.1 mg/L NAA and 1 mg/L BA and treated with 100 nM concentrations of human caspase inhibitors Z-Asp-CH2-DCB, Ac-YVAD-CMK and Ac-DEVD-CHO known to interfere with PCD events in plant cells (2, 16). Remarkable inhibition of the TEs differentiation in comparison to hormone treatment alone occurred 120 h after administration of 100 nM Z-Asp-CH2-DCB.

Lower but substantial inhibition was detected also at application of Ac-DEVD-CHO and Ac-YVAD-CMK. In favor of the amount of produced TEs, the cell viability in

hormone-induced culture was about 40% lower than in the

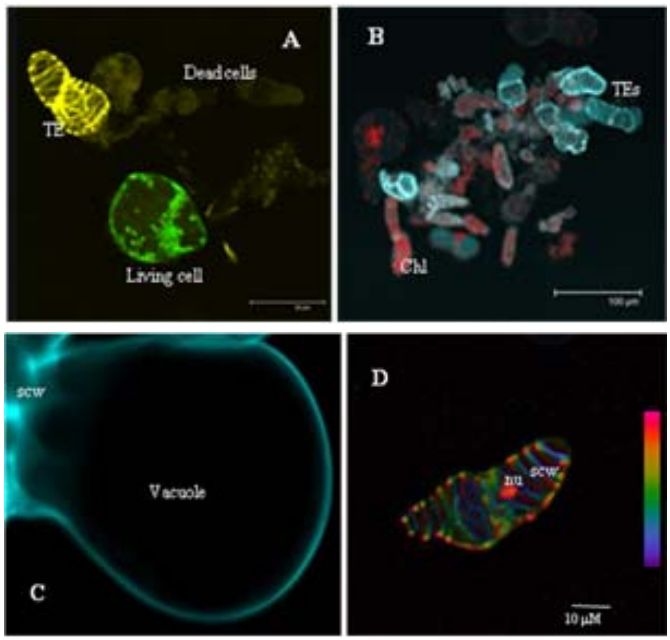

Figure 3. Confocal images of zinnia suspension cultured cells, representing the morphology of TEs differentiation. **A**. Green fluorescence of FDA stained living cell – visible are intact cytoplasm and intact vacuole; yellow auto fluorescence is visible from the secondary cell walls of fully differentiated TE; non-differentiated dead cells do not show fluorescence. Scale bars = 50 µm; **B**. Field image of living and differentiated cells after specific CFW staining of the cell walls (blue fluorescence); red fluorescence is emitted from the chlorophyll of the living cells. Scale bar = 100 µm; **C**. Large vacuole in a differentiating cells, blue fluorescence indicates the incomplete CFW stained secondary cell walls; **D**. In debt color image of hollow differentiating cell with preserved nuclei (PI positive) and lysed cell content; color code: purple – front, red - back. Scale bars = 10 µm. The images were taken 120 h after administration of 0.1 mg/L NAA and 1 mg/L BA. Nu, nucleus; scw, secondary cell wall; TEs, tracheary elements; v, vacuole.

negative control. Interestingly, at the addition of Z-Asp-CH2-DCB the cell death decreased compared to the hormone treated cells, but no change in the number of dead cells occurred in presence of caspase-1 and caspase 3 inhibitors. This might be due to either eventual interaction between the chemicals in the culture medium or due to still unknown reason. To the best of our knowledge, these results are the first evidence that plant proteases with possible substrate specificity similar to animal caspases are involved in the xylem cell death signaling in zinnia cultured cells.

The cell culture was also exposed to treatments with cysteine protease inhibitors E 64, IA and NEM. TE generation was inhibited by 84 % at the addition of E64 and no differentiation was detected in presence of IA and NEM. This shows that cysteine proteases are intimately involved in the mechanisms of TE differentiation. The administration of protease inhibitors IA and NEM suppressed the cell death in the culture and completely abolished the process of differentiation. This might be due to inhibition of broad range of cysteine proteases that are involved in processes supporting the differentiation, such as cell-to-cell communication. The inhibiting effect of E64 on TE generation is additional evidence that cysteine proteases are involved in cell death at TE differentiation. This is in line with reports from other laboratories (11).

The established suppression of TE formation at the administration of the human caspase inhibitors provide the first evidence that re-differentiation of cultured mesophyll zinnia cells is a PCD event that, in addition to cysteine protease signaling, most probably involves activation of caspase-like protease cascade. Enclosed high resolution confocal images show important details of cellular structural changes associated with re-differentiation. Further studies are necessary to elucidate the expression of genes encoding for the relevant caspase-like enzymes.

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