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Calcium-Calmodulin complex mediates the trans-differentiation of *Zinnia elegans* leaf mesophyll cells in to tracheary elements in cell suspension culture

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ABSTRACT

Leaf mesophyll cells of *Zinnia elegans* cv Envy could be induced to be trans differentiated in to tracheary elements by auxin and cytokinin in cell suspension culture, with peak differentiation at 96 hours, which has been used as a model system in studying the physiology and biochemistry of cellular differentiation. In the present study, the involvement of calcium ions and calmodulin (CaM) in the process of tracheary element (TE) differentiation was examined. Calcium chelator EGTA was used to deplete the Ca^{2+} concentration in the medium while calmodulin inhibitors trifluoferazin (TFP) and chlorpromazine (CPZ) were used to inhibit the calmodulin activity. Blocking the Ca^{2+} sequestration with 0.1mM EGTA and the inhibition of calmodulin action by TFP and CPZ at 10 and 20 µM concentrations significantly inhibited the TE differentiation process. It implies that, Ca^{2+} itself and Ca/CaM complex has a specific role to play in the trans-differentiation process of *Zinnia* mesophyll cells in to tracheary elements. Further increase in EGTA upto 0.5mM, TFP and CPZ upto 40 µM concentrations resulted a high rate of cell death probably due to non-specific toxic effects, which may lead to membrane damage.

Keywords: *Zinnia elegans*, xylem differentiation, calcium, calmodulin.

INTRODUCTION

Cyto-differentiation has been an important area of research for a long period because it leads to an understanding of the way that plant cells design and adopt their structure to specialized functions without changing the genome but merely by selective gene expression. Tracheary element (TE) differentiation is an excellent example of cyto-differentiation in higher plants (Torrey *et al.* 1971). When mesophyll cells of *Zinnia elegans* cv Envy (a member of the composite family) are cultured in a medium containing auxin and cytokinin, the mesophyll cells re-differentiate directly into TE without major cell expansion (Fukuda 1992, 1996). This model system is found unique in plant *in vitro* systems because of the readily inducible, synchronous differentiation of up to 60% of the cell population (Domingo *et al.* 1998).

Importance of calcium as a plant macro nutrient has been known for decades though its mode of action was obscure. Recently a considerable progress has been made in understanding the role of calcium ion as a second messenger in plants. It was revealed that Ca^{2+} ions play a vital role in mediating plant responses to external stimuli of both abiotic origin i.e. light, cold, heat, movement, hypoxia and drought and, biotic origin i.e. phyto-hormones, pathogens and interactions with symbionts (Snedden and Fromm 1998). Thus, it is clear that, calcium triggers a myriad of cellular processes that influence growth, development and physiology, which allow plants to adopt to the changing environment. Calcium dependent modulations of cellular processes occur *via* intracellular calcium binding proteins. Of these, calmodulin is one of the best characterized. It has no catalytic activity of its own, but calcium bound calmodulin activates numerous target proteins involved in a variety of cellular processes (Poovaih and Reddy 1987; Poovaih *et al.* 1996). A similar behavior has been reported in animal cells also where calmodulin has been implicated in many fundamental signaling pathways, activating the functions of various kinases, phosphatases, ion channels and other cytosolic enzymes (Crivici and Ikura 1995; Gnegy 1995). Thus, it is very likely that calcium ion is associated with cell wall metabolism and it has been

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Abbreviations: CaM - calmodulin, CPZ - chlorpromazin, EGTA - ethylene glycol-bis N, N' tetra acetic acid, PCD - Programmed cell death, TE - tracheary elements, TFP - trifluoperazin

shown that, disturbing the normal calcium relations with a calcium specific ionophore A-23187 results in the irregular deposition of wall constituents and inhibition of growth (Reiss and Herth 1977). Auxin induced cell elongation probably requires calmodulin action, since calmodulin binding compounds were highly effective in controlling auxin dependent elongation growth of oat coleoptile segments and wheat coleoptiles (Elliot *et al.* 1983). Induction of xylogenesis in cultured explants of lettuce claimed to require calmodulin action and the TE differentiation in suspension cultured cells of *Zinnia elegans* also required the uptake of extra cellular calcium, mediated by calmodulin (Roberts and Baba 1987). Thus, it is highly probable that, calcium sequestration accompany the onset of cell wall thickening during TE formation in *Zinnia* mesophyll cells. In furthering the understanding of the involvement of Ca^{2+} and calmodulin in TE differentiation process, the present study was conducted using a calcium chelator and two calmodulin inhibitors

MATERIALS AND METHODS

Plant material

Seeds of *Zinnia elegans* (L.) cv Envy were planted in plastic pots filled with 1:1 mixture of vermiculite and compost after surface sterilization with 0.5% NaOCl for 10 minutes. Upon germination, seedlings were thinned down to 2 vigorous plants per pot and grown in climate chambers (MB-Teknik climate chambers) under 14/10 hours light/dark cycle at 25°C and 75% RH. Light was provided with cool, fluorescent tubes (40W, Phillips) at photon flux density of 136 $\mu I\ M^{-2}\ S^{-1}$ of photosynthetically active radiation.

Isolation and culture of mesophyll cells

The first pair of true leaves was collected after 14 days at the end of the dark period, the distal 2/3 of leaf blades were separated, surface sterilized with 0.3% NaOCl mixed with a few drops of Tween 20 for 10 minutes. Then rinsed 5 times with sterilized distilled water and once with hormone free culture medium (Fukuda and Komamine 1980). Then, leaf pieces were transferred to a beaker containing ca. 100 ml of culture medium and cut in to small pieces and gently macerated using a glass pestle on a sterile, stainless steel mesh (400 μm), while washing down with the medium to separate the cells from the debris. The cell suspension so obtained was filtered through 85 μm mesh under vacuum to separate small cell clumps, centrifuged twice at 150g for 3 minutes to wash the cell suspension free of small cell particles, finally re-suspended in the culture medium with hormones. The resulting cell suspension mostly consists of intact, single mesophyll cells. Cell counts were taken under differential interference contrast microscopy (Leitz Biomed) on a haemocytometer to adjust the cell density to $1.5x10^5$ cells ml^{-1}. Fukuda & Komamini (1980) medium supplemented with 0.5μM naphthaleneacetic acid and 0.5μM benzyl adenine as specified by Church & Galston (1988) was used to culture the cells. Hormones were added after autoclaving the medium through sterile filtration with 0.22 μm filters (Millipore). The calcium chelator EGTA and two calmodulin inhibitors; CPZ and TFP (Sigma Chemical Co Ltd., USA) were used to deprive the Ca^{2+} ion availability and to block the calmodulin action. Calcium chelator and calmodulin inhibitors were used at following concentrations; 0.1 and 0.5mM EGTA, 10, 20 and 40μM TFP and 10μM CPZ in the experiment 1 while 10, 20 and 40 μM TFP and CPZ were used in the experiment 2. Chemicals were added at the beginning of the culture and pH was adjusted to 5.6 afterwards. Then, cell suspension was transferred to 50ml Erlenmeyer flasks at a rate of 5ml/ flask and incubated on an orbital shaker at 65 rpm in the dark at 27°C and sampling was done under green light (445nm).

Cell counts were taken after 72 and 96 hours of culturing after staining the samples with Evans blue. Dead cells were quickly stained with Evans blue, facilitating fast cell identification and counting. It was important to strain the cell suspension to break the small cell clumps before staining and counting. Cells in the suspension ccould be divided into three groups; live cells (L, not stained with Evans blue), tracheary elements (T, can be easily identified by wall banding patterns and also stained with Evans blue) and dead cells (D, stained with Evans blue). The % of live cells represents [(T+L)/(T+L+D)]x100 and the % of tracheary elements (TE) represents [T/(T+L)]x100. Three replicates were used for each treatment and three counts were taken from each flask. Analysis of variance and Duncan's Multiple Range Test were used to test the significance of the results and to compare the treatment means. Experiments were repeated at least twice and only reproducible results are presented.

RESULTS AND DISCUSSION

Effect of Ca chealator (EGTA) and calmodulin inhibitors (TFP and CPZ) on TE differentiation process, estimated as the % of TEs/ live cells, is presented in Figures 1 and 2. Effect of 0.1 and 0.5 mM EGTA, 10, 20, 40 μM TFP and 10 μM CPZ on

the % of tracheary elements is shown in Figure 1. The highest % of TEs were found after 96 hours of incubation irrespective of treatments. There was a rapid increase in the % of TEs from 72-96 hours in the control where the highest % of TEs has been observed, compared to the marginal increase seen in the rest of the treatments. It was evident from the results, that calcium chealator and both of the calmodulin inhibitors strongly inhibited the TE differentiation process. A significant, partial inhibition, compared to the control could be observed with EGTA 0.1mM (by 43.4%), CPZ 10 µM (by 43.9%) and TFP 10- µM (by 47.7%) treatments compared to the control. TFP 20-µM treatment has completely inhibited the TE formation compared to the partial inhibition observed with 10 µM concentration. However, the mean number of live cells/ml has not changed significantly in the control, EGTA 0.1 mM, CPZ 10 µM, TFP 10 and 20 µM treatments (values are not indicated). This behavior probably showed a selective inhibition of differentiation process through the deprivation of the availability of calcium ion and blocking the mediation of calmodulin action in the TE differentiation process. Further increase in the concentration of above chemicals (0.5mM EGTA and 40 µM TFP) caused complete inhibition of TE differentiation and inflicted an extremely high rate of cell death, probably due to non-specific toxic effects of these chemicals at above concentrations.

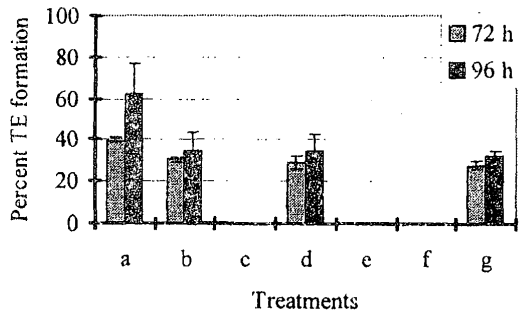

Fig. 1. Effect of calcium chelator EGTA and calmodulin antagonists; TFP and CPZ on TE differentiation of *Zinnia elegans* mesophyll cells in cell suspension culture.
Cell counts were taken at 72 and 96 hours after the incubation and the % of tracheary elements is expressed in proportion to the total number of live cells at the time of cell counts.
Legend:
a-Control* (61.84% TE formation at 96 hours of incubation)
b-EGTA, 0.1 mM (34.94% TE formation, 43.4% inhibition compared to the control)
c-EGTA, 0.5 mM (very high rate of cell death, no TEs were found)
d-TFP, 10 µM (34.68% TE formation, 47.7% inhibition compared to the control)
e-TFP, 20 µM (complete inhibition of TE formation without affecting the number of live cells, compared to the control, b, d and g)
f-TFP, 40 µM (very high rate of cell death, no TEs were found)
g-CPZ, 10 µM (27.74% TE formation, 43.9% inhibition compared to the control)
*The values in parenthesis are referred to 96 hours of culture

Effect of calmodulin inhibitors; CPZ and TFP at different concentrations on TE differentiation

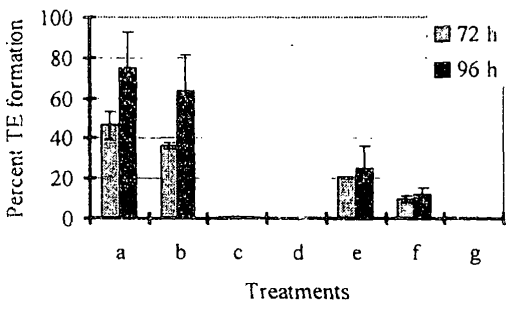

Fig. 2. Effect of calmodulin inhibitors; trifluoferazin and chlorpromazine on tracheary element differentiation of *Zinnia elegans* leaf mesophyll cells in cell suspension culture.
Cell counts were taken at 72 and 96 hours after incubation and the % of tracheary elements was expressed in proportion to the number of live cells at the time of counts.
Legend:
a-Control (74.84% TE formation)
b-TFP 10 µM (53.56% TE formation, only 15% inhibition compared to the control, the difference is not significant)
c-TFP 20 µM (only 0.85% TE formation, 98.8% inhibition compared to the control)
d-TFP 40 µM (very high rate of cell death, no TEs were found)
e-CPZ 10 µM (24.71% TE formation, 67% inhibition compared to the control)
f-CPZ 20 µM (12.34% TE formation, 83.5% inhibition compared to the control)
g-CPZ 40 µM (very high rate of cell death, no TEs were found)
*Values in parenthesis refer to 96 hours of culture

process is presented in Figure 2. Results obtained were complementary and confirmatory in nature to the findings of the previous experiment. The highest % of TEs could be observed in the control (74.84%) as expected. There is a sharp increase in the % of TE formation between 72-96 hours of culture in control as observed in experiment 1 and in 10-µM TFP treatment also. However, in the rest of the treatments, the increase was marginal indicating that the inhibitory action has already taken place. TFP 10 µM treatment resulted in a 15% inhibition even though, it is not significantly lower compared to the control. This is contrary to the previous results, where the same treatment resulted in a significant inhibition while TFP 20 µM treatment almost completely inhibited the TE formation (by 98.8%), confirming the previous results. CPZ also has significantly inhibited TE formation with an increasing tendency with increasing concentrations; by 67% at 10 µM concentration where the degree of inhibition was quite high compared to the experiment, and by 83.5% at 20µM levels. When the concentration increases further to 40-µM level, both calmodulin inhibitors resulted in an extremely high rate of cell death, confirming the non-specific toxic effect of these chemicals as observed in the previous experiment.

TE differentiation has been identified as a programmed cell death (PCD) where the formation of mature TEs were completed by the loss of cell contents including the nucleus, plastids,

mitochondria, golgi apparatus and the endoplasmic reticulum and, by the partial digestion of primary walls forming a functional cell corpse that makes a strand of water conducting vessels of the xylem tissue in intact plants (Roberts *et al.*1988). Isolated mesophyll cells in cell suspension culture system could completely differentiated into TEs in the absence of the direct contact with other cells, demonstrating the autonomous nature of the TE differentiation process that characterizes morphological changes, which takes place during PCD (Groover and Jones 1993). The sequence of events that occured during the TE differentiation process was closely co-ordinated and regulated by a group of differentially regulated genes, which were expressed at different stages of the process (Ye and Verner 1993). Antosiewicz and Polisenseky (1995) suggested that, the dynamic modulation of CaM gene expression in plants is also reflected in gene specific, developmentally regulated, organ, tissue and cell specific expression patterns, which conveniently fitted into a process like cell autonomous trans-differentiation of *Zinnia* mesophyll cells into TEs. Kobayashi and Fukuda (1994) reported that the involvement of Ca^{2+} and Ca/CaM complex in TE differentiation process correlated with the expression of CaM related genes, which are expressed at early and late stages of the differentiation process, as it was confirmed by the stage specific inhibition of TE differentiation by the use of CaM antagonists. It has been further reported that, at later stages of the TE differentiation process CaM levels increased transiently and subsequently a few CaM binding proteins start to be expressed in a differentiation specific manner. Possibility that Ca/CaM system was involved in the progression of TE differentiation is also supported by higher levels of membrane associated Ca^{2+} ions observed in TE precursor cells compared to the control *Zinnia* cells, as observed by Roberts and Haigler (1989, 1990). This has been further confirmed by the findings of Kobayashi and Fukuda (1996). Observations of Polisensky and Braam (1996) that, induction of the expression of at least some of these CaM related genes was mediated by a rise in cytosolic Ca^{2+} in response to the external stimuli in general, fitted well with the previous observations which were specific to the *Zinnia* system.

In the present study it was observed that the depletion of Ca^{2+} concentration in the medium by the use of Ca^{2+} chelator EGTA (0.1mM) as well as the inhibition of calmodulin action by using calmodulin antagonists; CPZ and TFP (20 μM) has drastically reduced the TE differentiation process i.e.; by 43.4%, 83.5% and 98.8% respectively (Figs 1 &2), once again confirming the essential role played by Ca/CaM complex in TE differentiation process. Since the calcium chelator and CaM inhibitors were in the medium from the initiation of culture, present results do not provide evidence for the time sequence of the mediation of the above on TE differentiation process. Regulation of the inhibition of TE differentiation process by chelating Ca^{2+} and blocking CaM activity may occur at different stages through different pathways. It can be any of the following processes like; prevention of the transduction of initiation signal of hormones (at early stages), blocking the expression of the differentiation specific calmodulin related genes and inhibition of the execution of PCD by preventing the autolysis of internal cellular organelles etc (at later stages), which may ultimately contribute to the inhibition process in varying magnitudes, in a mutually inclusive or exclusive manner. Yet, our results provide distinct evidence for the necessity of intracellular Ca^{2+} sequestration and CaM mediation for the completion of TE differentiation process in *Zinnia* mesophyll cell suspension culture system, in an agreeable manner with contemporary findings. However, it was also found that, higher concentrations of EGTA (0.5mM), TFP and CPZ (40 μM) has resulted in a high rate of cell death probably due to non-specific toxic effects, which may be attributed to the changes in membrane properties causing leakage and premature cell death prior to the onset of differentiation process.

CONCLUSION

This study provideed conclusive evidence for necessity of intra-cellular Ca^{2+} sequestration and calmodulin mediation in the trans-differentiation of *Zinnia* mesophyll cells into tracheary elements in cell suspension culture. Both calmodulin antagonists could almost completely inhibit TE differentiation at 20 μM concentration, while 10 mM EGTA resulted in a significant inhibition, demonstrating the distinct role played by Ca^{2+}/ calmodulin system in the process. The fact that only the TE differentiation was affected by moderate concentrations of above chemicals, without significantly changing the total number of live cells shows the selective inhibition of differentiation.

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