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Programming of Cell Death during Xylogenesis*

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Death of tracheary elements which compose vessels and tracheids is a typical example of programmed cell death in plants. An *in vitro* system using *Zinnia* mesophyll cells which differentiate directly into tracheary elements has provided various types of data on the cell death process. In this paper, we will summarize recent results obtained using the *Zinnia* system and discuss the programming of cell death during tracheary element differentiation.

Key words: Apoptosis — Cysteine protease — DNase — Programmed cell death — Tracheary element differentiation — Vacuole — *Zinnia elegans*

Introduction

In plants as well as in animals, cell death plays an essential role in the organization of plant body; the abortion of the primordia of female flowers in unisexual flowers involves programmed cell death (Dellaporta and Calderon-Urrea 1993). Death of tapetum and stomium cells is also programmed during anther development (Goldberg *et al.* 1993). Another conspicuous example is death of tracheary elements (TEs) (Fukuda 1996, 1997b). Two major components of conductive tissues in plants are vessels/tracheids and sieve tubes. Sieve tubes, responsible for the transport of nutrients such as sucrose, are formed from sieve-tube elements, which are still living but often lose their nucleus and are supported by companion cells. In contrast, vessels and tracheids, which are responsible for the transport of water and salts, are formed from dead TEs. Differentiating TEs deposit the patterned secondary wall thickenings and, at the final stage, they lose their cell contents including the nucleus to become a hollow tube.

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Abbreviations: TE, tracheary element.

In vitro Zinnia system

We have established an experimental system in which single cells isolated from *Zinnia* mesophyll differentiate into TEs (Fukuda and Komamine 1980a, b). In this system, TE differentiation occurs directly from single mesophyll cells without cell division. This system is thought to be an efficient system for the study of cell death during TE differentiation (Fukuda 1997a, b). To date, using the *Zinnia* system, various events during TE differentiation have been reported (Fukuda 1996, 1997a). Figure 1 is the summary of these events. In the differentiation, initiation occurs by wounding and by the cooperative action of auxin and cytokinin. A morphological change characteristic of TEs, localized secondary wall thickening, starts at 60 hr of culture; and a little later, autolysis starts. The process of the *in vitro* TE differentiation is found to be divided into three stages, Stage I, II and III. Stage I involves dedifferentiation during which isolated mesophyll cells lose their potential to function as photosynthetic cells and acquire the ability to differentiate in the new environment. It should be emphasized that this dedifferentiation is functional dedifferentiation and is not accompanied by cell division. In Stage II, cells that have dedifferentiated may restrict their potency of differentiation from pluripotency, whereby they differentiate into xylem and/or phloem cells, to a single potency for differentiating into TEs. Stage III involves a variety of specific events leading to secondary wall formation and cell death.

Cell Death Process

Epifluorescent microscopical observation of living *Zinnia* cells revealed that an immature TE has all its organelles including the nucleus, vacuole, many active mitochondria, and chloroplasts (Hideo Kuriyama, unpublished result). However, such a TE rapidly loses all of them. The lose of organelles occurs 6 hr after the secondary wall formation starts (Shigemi Aoyagi, unpublished result). Observation using an electron microscope indicated that vacuole collapse is a critical step in death of TEs (Fukuda 1996, Groover *et al.* 1997). It is after vacuole collapse that single membrane-organelles, ER and Golgi bodies, swell at first at their

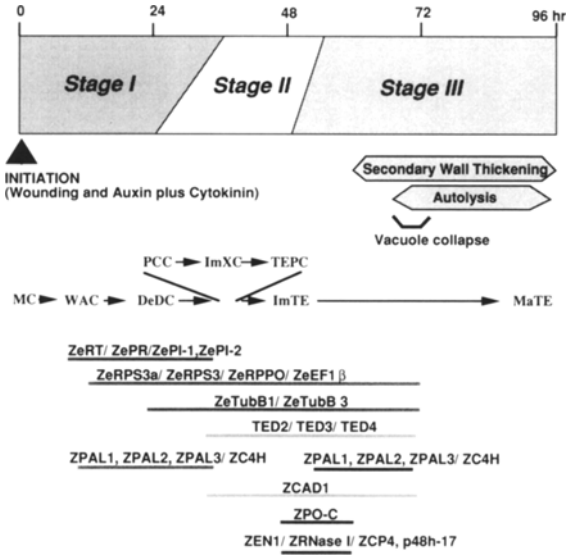

Fig. 1. Time sequences of the accumulation of various transcripts during transdifferentiation from single *Zinnia* mesophyll cells into TEs. Transdifferentiation from *Zinnia* mesophyll cells into tracheary elements is induced by wounding and a combination of auxin and cytokinin. The process of transdifferentiation is divided into three stages: Stage I, Stage II, and Stage III. Stage I corresponds to the functional dedifferentiation process. In Stage II, the developmental potential of the dedifferentiated cells becomes restricted from the pluripotent capability to differentiate into immature xylem and/or phloem cells to the single capability to differentiate into TEs. Stage III involves TE-specific events. Time sequences of the accumulation of transcripts for various genes isolated from *Zinnia elegans* are shown as bars. MC, meristematic cells; WAC, wound activated cells; DeDC, dedifferentiated cells; PCC, procambial cells; ImXC, immature xylem cells; TEPC, tracheary element precursor cells; ImTE, immature tracheary elements; MaTE, mature tracheary elements. (After Fukuda 1997a with some modifications)

ends and then over their entire length and become balloon-like structures. Finally they disappear. A little later than the degradation of single membrane-organelles, the degeneration of double membrane-organelles such as chloroplasts and mitochondria becomes visible. First, the matrix of chloroplasts is degraded and then their membranes are degraded. It is well known that chromosomal condensation and nuclear fragmentation occur during the apoptotic cell death in animals (Kerr and Harmon 1991). During autolysis of TE differentiation, however, the first sign of nuclear degradation is swelling of the nuclear membrane after the vacuole collapse; then the nuclear matrix is degraded. Thus, we think that one of the most essential irreversible points of death of TEs is the tonoplast disruption.

Cysteine Protease

To understand the autolytic step of cell death, we have examined the activities of various proteases in cultured *Zinnia* cells. Among them, one protease activity increases in close association in time with autolysis. This activity is partially purified through CM Sepharose, Phenyl superose and Superose 12 (Minami and Fukuda 1995). The purified protease is a basic protease with molecular mass of about 30 kD. Its activity is enhanced by DTT and completely inhibited by leupeptin and E-64. These and other properties including substrate specificity suggest that the protease is a cysteine-protease. The pH optimum of the enzyme is about 5.5, which is almost the same as the pH in the vacuole, suggesting that this cysteine protease works in the vacuole or in the cytoplasm after vacuole collapse.

What will happen to TE differentiation if we prevent the activity of such a cysteine protease in cells? To answer this question, a membrane permeable E-64 analogue was added to the culture medium just before, at, and just after the secondary wall thickenings start (Yoriko Watanabe, unpublished result). The addition of the inhibitor before morphological changes resulted in the inhibition of TE formation itself, but the addition at or just after the secondary wall thickenings start did not show any significant inhibition of differentiation. This result suggests that cysteine protease(s) may be involved in the initiation of morphological changes, including secondary wall formation and cell death. Furthermore, the addition of the inhibitor at any time caused conspicuous inhibition of disruption of the nucleus, although it did not inhibit the vacuole collapse. This result clearly shows that cysteine protease(s) is involved in nuclear degradation. For further analysis of the protease, we isolated two highly similar but different cDNA clones, ZCP1 and ZCP2 (its full size form: ZCP4) which may correspond to the cysteine protease(s) (Minami *et al.* 1996). The sequence of ZCP1 is almost the same as that of p48h-17 isolated from differentiating *Zinnia* cells by Ye and Varner (1996). A database search revealed that these genes are highly similar to cathepsin and papain. In addition, these genes have putative signal peptides which may direct the transport of the proteins into the vacuole. The transcripts for these genes were expressed very transiently around 60 hr of culture, just before autolysis. This timing coincides with the time of maximum activity of the enzyme. Using the cDNA as a probe, we carried out *in situ* hybridization. The probe hybridizes in four limited small area in the hypocotyl of *Zinnia* where differentiating TEs locate, suggesting that this gene is specifically expressed in differentiating TEs, even *in vivo*.

DNase

Several species of nucleases also increase in association with the cell death of TEs (Thelen and Northcote 1989). Among them, a 43 kD nuclease that hydrolyzes both single stranded DNA/RNA and double stranded DNA appears just before autolysis, and therefore this nuclease is thought to be involved in nuclear degradation. Thelen and Northcote (1989) have partially purified this 43 kD nuclease and determined its partial N-terminal sequence, which was similar to that of a barley nuclease that appears in response to gibberellin during endosperm degeneration, which includes a cell death process. Both nucleases have many similarities:

they are endo-type nucleases, hydrolyze single and double stranded DNA and RNA, and require zinc ion for activation. These data indicate that a similar type of endonuclease may be involved commonly in different cell death process in plants. We isolated cDNA clones encoding both enzymes, *ZEN1* from *Zinnia* and *BEN1* from barley (Shigemi Aoyagi, unpublished result). The deduced amino acid sequences of these two cDNAs are very similar (45% identity) and suggest the presence of a putative signal peptide. Because the 43 kD nuclease activity is known to be restricted within the cell, *ZEN1* products must be transported in some intracellular organelle, probably the vacuole. Homology search shows that *ZEN1* nuclease is similar to SI nuclease. The mRNAs for *ZEN1* was expressed only in differentiation-induced culture and very transiently at 60 hr of culture. This timing is just before autolysis and the same as that of *ZCP4*. These results strongly suggest that this nuclease is involved in the cell death process during TE differentiation.

Brassinosteroids

Genes encoding autolysis-related hydrolytic enzymes, DNase, RNase, and cysteine proteases are expressed in very similar patterns just before autolysis starts (Fukuda 1997a). Therefore, genes encoding these enzymes may be regulated to be expressed by the same mechanism, for example, with the same *cis* and *trans* activation factors. Interestingly, *ZPO-C*, a gene for lignin-related peroxidase, is also expressed in very similar pattern to that of the autolysis-related genes, suggesting that secondary wall formation and autolysis may be initiated by the same mechanism, at least in part. To understand the common initiation mechanism, we have surveyed various candidates for the initiation factor. Finally we paid attention to brassinosteroids as a possible factor, based on the report by Iwasaki and Shibaoka (1991) that endogenous brassinosteroids may be involved in the progression of TE differentiation. To elucidate the involvement of brassinosteroids in the progression of TE differentiation in cultured *Zinnia* cells, we analyzed the effects of uniconazole, a inhibitor of brassinosteroid biosynthesis, and brassinolide, an active brassinosteroid on the accumulation of mRNAs for various genes that are expressed at different stages of differentiation (Yamamoto *et al.* 1997). We found that uniconazole did not suppress the accumulation of transcripts that appear in Stage I and Stage II. However, it specifically suppressed the accumulation of transcripts for genes such as *ZCP4* and *ZPO-C* that were induced in Stage III in association with secondary wall formation and cell death. This suppression was recovered with the addition of brassinolide. These results strongly suggest that endogenous brassinosteroids induce the transition from Stage II to Stage III, resulting in cell death.

Concluding Remarks

The programming of TE differentiation from *Zinnia* mesophyll is summarized as follows. The first signal for the initiation of differentiation is exogeneously supplied plant hormones and wounding. After the initiation, mesophyll cells dedifferentiate (Stage I) and then differentiate into TE precursor cells (Stage II). Final determination occurs in the precursor cells to enter Stage III, involving TE-specific events. Brassinosteroids may be involved in the final determination, causing the induction of genes that encode both enzymes functioning in secondary wall formation and in autolysis. Induced autolysis-related enzymes such as cysteine proteases, RNases, and DNases are accumulated in the vacuole. The point of no return is the vacuole collapse. This causes the invasion of the hydrolytic enzymes into the cytoplasm to attack the various organelles. Finally, all cell contents are disrupted, and functional dead cells are formed.

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