Autolysis during In Vitro Tracheary Element Differentiation: Formation and Location of the Perforation

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Tracheary elements differentiated from isolated Zinnia mesophyll cells were observed at various times of culture under a scanning electron microscope. Perforation occurred on the primary wall at one of the longitudinal ends in single tracheary elements. In double tracheary elements, which both of two cells derived from a single cell differentiated into, the pore opened on the primary walls both at the junction of the two tracheary elements and at a longitudinal end of one of the two tracheary elements. These results suggest not only that a single tracheary element has its own program to form a perforation at one end without being affected by neighboring cells, but also that isolated cells indeed hold some traces of polarity and cell-cell communication.

Key words: Perforation — Programmed cell death — Tracheary element differentiation — Zinnia elegans.

Introduction

Cell walls, which are characteristic morphological structures in the plant kingdom, are laid outside the protoplasts. Cell walls are composed of various kinds of polysaccharides such as cellulose, hemicellulose, and pectin; cell wall proteins including arabinogalactan proteins and hydroxy-proline rich glycoproteins; and amorphous polyphenolic polymers such as lignin. Vessels are specially built up of a longitudinal series of individual cells, termed vessel members or elements, to provide the three-dimensional pathway for the ascent of water in plants. For water transport, vessel elements have perforations at both ends, and sometimes also on a side wall, and other regions of the cell walls were reinforced by lignified secondary walls. In general, the water moves freely through perforations in the walls. Therefore, perforation occurs in the last stage of vessel element differentiation as an event of programmed cell death (Esau 1965, Esau and Charvat 1978, Meylan and Butterfield 1981, Buvat 1989). In situ vessel elements are formed successively according to cells derived from the apical meristem. A differentiating vessel element is connected with a mature dead vessel element and a vessel precursor cell at each end, but perforation occurs only at the end wall between the mature dead vessel element and the differentiating vessel element (Esau and Charvat 1978). Therefore, perforation should be regulated temporally and spatially in situ.

An in vitro experimental system, in which isolated Zinnia mesophyll cells differentiate into tracheary elements (TEs), is widely used for physiological, biochemical, and molecular biological studies to elucidate the mechanism of TE formation (Fukuda 1997). This experimental system also offers a great opportunity for the investigation of perforation in vitro. It has been suggested that in intact vascular tissues, end-wall hydrolysis is a sign of the wiping away of the remnants derived from the cytoplasm by the transpiration stream (O’Brien 1981). In isolated Zinnia cells, however, this mechanism is unlikely to explain the formation of perforations. Rather, perforation seems to occur autonomously in single TEs (Burgess and Linstead 1984, Burgess and Linstead 1985, Groover et al. 1997). Furthermore, Burgess and Linstead (1984) reported that the region of the middle lamella is specially resistant to autolysis when it is either on the outside of an isolated cell which differentiates or between neighboring cells in contact, both of which differentiate. These results indicate that active maintenance of the middle lamella by the undifferentiated neighbor may be taking place even in vitro. As a first step toward understanding perforation mechanism during programmed cell death, therefore, we observed in detail perforation of TEs formed in vitro using a scanning electron microscope (SEM) and demonstrated that perforation is formed temporally and spatially at only one end even in single cells.

Materials and Methods

Mesophyll cells of the first true leaves of 14-day old seedlings of Zinnia elegans L. cv. Canary Bird (Takii Shuby Co., Kyoto, Japan) were isolated as described previously (Fukuda and Komamine 1980, Sugiyama et al. 1986), and cultured in differentiation medium. The medium contained 0.1 mg liter -1 of naphthaleneacetic acid (NAA) and 0.2 mg liter -1 of benzyladene (BA) as growth regulators. The population of cells was adjusted to 8.4x10^4 cell ml -1 . Cultured cells were harvested at every 6 h after the secondary wall formation had started, and fixed with 2% (v/v) glutaraldehyde in the differentiation medium for 16 h under a cooled condition according to the procedure of Franklin (1945) with some modifications. That is, half of the cells were sectioned at 20-μm thickness with a sliding microtome equipped with a freezing device, which enables us to investigate the cell walls from inside. All of the cells were washed thoroughly with 1/15 M Sörensen’s phosphate buffer (pH 5.59), dehydrated in a series of ethanol, substi-
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Results and Discussion

Time course of the formation of TEs in the differentiation medium under a light microscope is shown in Fig. 1. TEs were formed at high frequency accounted for up to 54% among the total cells at the end of differentiation. There was high degree of synchrony in the formation of TEs after 48 h of culture. At 72 h, transmission electron microscopic observation revealed that most of TEs lost their cytoplasms, indicating the death of them (data not shown). Figure 2 shows an overview of a typical single TE cultured for 96 h in the differentiation medium. The perforation with a central big hole is observed on the primary wall regions at one longitudinal end. The secondary wall thickenings in the wall are shown as strong reflecting regions which surrounded the primary walls with the mesh-like structure. PW: primary wall; SW: secondary wall thickening.

Figure 1 Time course of tracheary element (TE) differentiation from *Zinnia* mesophyll cells cultured in the differentiation medium, which contains 0.1 mg liter⁻¹ of naphthaleneacetic acid (NAA) and 0.2 mg liter⁻¹ of benzyladenine (BA) as growth regulators. The values represent the average of two samples.

Figure 2 A typical SEM image of a single TE. This scanning electron micrograph shows an overview of the typical single TE cultured for 96 h in the differentiation medium. The perforation with a central big hole is observed on the primary wall regions at one longitudinal end. The secondary wall thickenings in the wall are shown as strong reflecting regions which surrounded the primary walls with the mesh-like structure. PW: primary wall; SW: secondary wall thickening.

Three types of perforations when 100 single TEs were observed at 6 h-intervals after 60 h of culture. At 72 h of culture, perforations were first observed and over 90% of them were classified into the “small numerous pores” type (Fig. 3c, 4). When the maturation of TEs proceeded, the proportion of the “small numerous pores” type drastically decreased, and in turn remarkably increased the proportion of the “central big holes surrounded by small numerous pores” type. At 96 h of culture, the latter type occupied 82% of the TEs in which perforations could be observed. The “simple big holes” type did not appear before 78 h of culture, but thereafter occupied constantly about 15% of the TEs in which perforations could be observed. These results suggest that small numerous pores develop to a few big holes during maturation of TEs and that this development occurs after TEs have lost cell contents. Even at mature stage, most TEs had many small pores in addition to big pores. Such characteristic perforations in TEs appear to differ from TEs formed in situ, which have simple big pores. These results imply that end-wall remnants in situ may be completely swept away by the transpiration water. The percentage of TEs with perforations did not reach 100%, because, in the experimental procedure for SEM, substantial numbers of TEs were stuck onto the specimen carrier stub with the perforation sites. As typically shown in Fig. 5, both ends could be clearly observed in 13 TEs at 90 and 96 h of culture. All TEs had perforations at only one end. These results suggest that the perforations are formed only one longitudinal end on most single TEs.

In the *Zinnia* culture system, some single cells differentiate into two TEs after dividing into two, called double TE. Study of this double TE may give information about perfora-
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When the cells were sectioned with the microtome, some TEs were accidentally fractured and exhibited the cell walls between two TEs of the double TE. Figures 6a and b are two examples of such TEs showing that, after a cell divided almost parallel to the longitudinal axis, the perforation was formed on the primary wall between the two TEs. The perforations on cell walls between the two TEs were always the “simple big holes” type bounded by the secondary wall thickenings. Observation of 13 double TEs showed that all TEs had perforations (at most four) between their two TEs. These perforations are remarkably similar to the perforations observed on the lateral primary walls between two vessel ele-

Figure 3 Three types of perforations of TEs. These scanning electron micrographs represent the magnified view of the portion of perforations in the single TEs cultured for 96 h, allowing us to categorize types of the perforation into three. That is, perforation with the “central big holes (over 2 μm in diameter) surrounded by small numerous pores” type (a), with the “simple big holes” type (b), and with the “small numerous pores” type (c), respectively.

Figure 4 Changes in three types of perforations of TEs during culture. Hundred single TEs were observed at 6 h-intervals after 60 h of culture. Perforations with the “central big holes (over 2 μm in diameter) surrounded by small numerous pores” type are represented in gray columns, perforations with the “simple big holes” type in open columns, and perforations with the “small numerous pores” type in black columns, respectively.

Figure 5 A TE showing both longitudinal ends. This scanning electron micrograph shows a overview of the typical single TE cultured for 96 h in the differentiation medium. Both ends can be clearly observed from this electron micrograph, which suggests that perforations are formed at only one end (arrow).
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Perforations of double TEs that developed after a cell divided almost perpendicularly to the longitudinal axis. The perforations are formed at the two positions, that is, at the junction of the two TEs (arrow a) and at a longitudinal end of one of the two TEs (arrow b). Inlet panel indicates the illustration of the fractured position.

Fig. 7 Perforations of double TEs that developed after a cell divided almost parallel to the longitudinal axes. The perforations are formed on the primary walls between the two TEs. They were always the “simple big holes” type bounded by the secondary wall thickenings (arrows). Inlet panels indicate the illustration of the fractured position.

Fig. 6 Perforations of double TEs that developed after a cell divided almost perpendicularly to the longitudinal axis, perforations occurred at the two positions, that is, at the junction of the two TEs and at the end of only one of the two TEs (Fig. 7). Similar but non-fractured double TEs had perforations at only one longitudinal end. Therefore, it is strongly suggested that a TE perforates only at one end even in divided TEs. Thus, TEs formed in cultured Zinnia cells have the morphological feature of the vessel elements, and most perforations are formed near the longitudinal end of TEs.

These results indicate that single TEs formed in vitro have their own program to form perforations at a given end, which must be one of the features in programmed cell death at the cellular level. These results also suggest the presence of cell-polarity even in a single TE and of well organized cell-cell communication in double TEs. Indeed, an antibody revealed polarized localization of a cell wall component in differentiating single TEs in culture (Shinohara et al. 2000). The fact that auxin transport inhibitors prevent TE formation in cultured Zinnia cells (Burgess and Linstead 1984) also supports the idea that polarity in the cell is involved in vitro TE formation. When the successive differentiation of vascular networks actively progresses, perforations are formed at the side of mature dead TEs, but not of immature living TEs, which is another important characteristic for programmed cell death at the cellular level. Such organized perforations should be carried out by various wall-degrading enzymes. Recently, we found that wall-bound polygalacturonase activity and mRNAs for two polygalacturonase genes are upregulated in association with TE formation (Y. Ohdaira, K. Iwamoto, M. Sugiyama, and H. Fukuda, in preparation). These wall-bound polygalacturonases might be some of the key enzymes which spatially and temporally control the formation of perforations. Efforts are under way to elucidate the involvement of these polygalacturonases in the formation of perforations during programmed cell death.

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