Direct Evidence of Active and Rapid Nuclear Degradation Triggered by Vacuole Rupture during Programmed Cell Death in Zinnia¹

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Differentiation into a tracheary element (TE) is a typical example of programmed cell death (PCD) in the developmental processes of vascular plants. In the PCD process the TE degrades its cellular contents and becomes a hollow corpse that serves as a water conduct. Using a zinnia (Zinnia elegans) cell culture we obtained serial observations of single living cells undergoing TE PCD by confocal laser scanning microscopy. Vital staining was performed and the relative fluorescence intensity was measured, revealing that the tonoplast of the swollen vacuole in TEs loses selective permeability of fluorescein just before its physical rupture. After the vacuole ruptured the nucleus was degraded rapidly within 10 to 20 min. No prominent chromatin condensation or nuclear fragmentation occurred in this process. Nucleoids in chloroplasts were also degraded in a similar time course to that of the nucleus. Degradations did not occur in non-TEs forced to rupture the vacuole by probenecid treatment. These results demonstrate that TE differentiation involves a unique type of PCD in which active and rapid nuclear degradation is triggered by vacuole rupture.

Programmed cell death (PCD) is an active cell death process involved in the selective elimination of unwanted cells, and it is found throughout animal and plant kingdoms (Ellis et al., 1991; Jones and Dangl, 1996). The term apoptosis (Kerr et al., 1972) usually refers to a morphological type often observed in PCD that involves nuclear shrinkage and fragmentation, cellular shrinkage, DNA fragmentation, membrane budding, the formation of apoptotic bodies, and digestion by macrophages (Wyllie, 1980; Kerr and Harmon, 1991). Although none of plant PCDs has been reported to possess all of these apoptotic features, some plant PCD processes show a subset of apoptotic features: Salt stress induces nuclear fragmentation and DNA degradation into oligonucleosomal fragments in barley roots (Katsuhara and Kawanishi, 1996; Katsuhara, 1997). DNA laddering is also reported in Arabidopsis roots and maize cultured cells during PCD induced by Man (Stein and Hansen, 1999). However, this apoptosis-like cell death does not account for the majority of PCD in plants (Fukuda, 1998). Senescence of leaves (Smart, 1994) and ovaries (Vercher et al., 1987) shows the features closer to necrosis than apoptosis. Differentiation into tracheary elements (TEs), a typical example of PCD in higher plants (Pennell and Lamb, 1997), also exhibits morphological features closer to necrosis (Fukuda, 1998).

Extensive studies about PCD during TE differentiation have been performed using the zinnia (Zinnia elegans) culture system established by Fukuda and Komamine (1980a). In the zinnia cell cultures, single mesophyll cells transdifferentiate directly into TEs without cell division (Fukuda and Komamine, 1980b). This system is useful for studies of the processes of PCD because differentiation occurs at a high frequency and because the sequence of events during PCD can be followed in single cells (Chasan, 1994; Fukuda, 1997). Isolated zinnia cells cultured with phytohormones begin to form secondary cell walls and several hours later PCD occurs to become hollow dead cells (Fukuda, 1997; Groover et al., 1997). A number of ultrastructural observations of TE cell death indicated rapid and progressive degeneration of the nucleus, vacuole, plastids, mitochondria, and endoplasmic reticulum and finally, the removal of protoplasts, the plasma membrane, and parts of primary walls (O’Brien and Thimann, 1967; Srivastava and Singh, 1972; Lai and Srivastava, 1976; Esau and Charvat, 1978; Burgess and Linstead, 1984; Groover et al., 1997). Various hydrolytic enzymes including nucleases (Thelen and Northcote, 1989; Ye and Droste, 1996; Aoyagi et al., 1998) and proteases (Minami and Fukuda, 1995; Ye and Varner, 1996; Beers and Freeman, 1997) are synthesized for active degeneration of cellular contents and are thought to accumulate in the vacuole of TEs to sequester them from the cytoplasm. Thus, the collapse of the vacuole is a critical irreversible step to execute the degradation of various organelles (Fukuda, 1996; Groover et al., 1997; Groover

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and Jones, 1999; Kuriyama, 1999). Groover et al. (1997) observed the process of TE differentiation using video camera and revealed that TEs keep physiological activity until vacuole collapse. However, the precise kinetic relationship between tonoplast disruption and degradation of other organelles is unknown.

In this report we present direct evidence that rapid nuclear degradation occurs immediately after vacuole collapse.

RESULTS

Imaging of the Vacuole Rupture and the Morphological Change of the Nucleus in Differentiating TEs

Cells at 54 h of culture were loaded with fluorescein diacetate (FDA) and SYTO16 for 1 h and 10 min, respectively, and were observed by confocal laser scanning microscopy (CLSM). FDA is a hydrophobic molecule that enters cells in a passive way and becomes de-esterified in living cells to become membrane-impermeant fluorescein. Kuriyama (1999) reported that although fluorescein accumulates into the vacuole of most cultured cells after 1 h of FDA loading, it is excluded from the vacuole of highly vacuolated TEs at the late stage of differentiation. Therefore, to catch the moment of vacuole rupture and analyze morphological changes in the nucleus just before and after the vacuole rupture we chose and observed successively highly vacuolated TEs with green fluorescence in the cytoplasm. Figure 1 shows a series of events occurring before and after vacuole rupture. The central vacuole expanded greatly to a point almost occupying the intervening space between thickened secondary cell walls, pressing the nucleus tightly against the plasma membrane and making it almost flat (Fig. 1a). Seven minutes later the fluorescence disappeared from the cytoplasm and there was no longer an obvious boundary between the cytoplasm and the vacuole (Fig. 1b). The nucleus became spherical, probably by the liberation of vacuole pressure. The tonoplast was no longer discernable under bright field microscopy after this event (data not shown). Therefore, this morphological change in the nucleus and the loss of boundary between the cyto-

![Figure 1](https://www.plantphysiol.org)

**Figure 1.** A series of images of a TE that was undergoing vacuole rupture (a–f). This TE was stained with SYTO16 and FDA (a–e). The red autofluorescence of the chloroplasts was merged. a, The green fluorescence of SYTO16 and fluorescein could be observed in the nucleus and cytoplasm (7 min before vacuole rupture). The TE was highly vacuolated and its nucleus was tightly pressed against the plasma membrane. b, Soon after vacuole rupture (0 min), the nucleus was released from the vacuolar turgor pressure and became spherical. The heterochromatin structure could be seen just inside the nucleus. SYTO16 fluorescence in some chloroplasts appeared in this focal plane. The green fluorescence in the cytoplasm disappeared probably because the emission spectra of fluorescein changed following vacuole rupture (see “Discussion”). c, Nuclear fluorescence started decreasing from the central region (6 min after vacuole rupture). d, Nuclear fluorescence of SYTO16 decreased markedly in the central region (10 min after vacuole rupture). e, The fluorescence of SYTO16 disappeared almost completely, whereas the autofluorescence of the chloroplasts was still visible (18 min after vacuole rupture). f, The nuclear envelope could be seen in the light image of the TE even 20 min after vacuole rupture (arrow). The bar indicates 20 μm.
plasm and the vacuole are considered to be markers of tonoplast disruption. Fluorescence in the nucleus disappeared rapidly from the central part (Fig. 1, c and d) and then from the inner edge, and it became undetectable within 20 min after vacuole rupture (Fig. 1e). However, the nuclear envelope was still kept in such nuclei (Fig. 1f). Moreover, the nucleus and chloroplasts did not change the location in the cell in the period of 20 min after vacuole rupture, indicating that these compartments may be anchored to the plasma membrane.

Loss of Tonoplast Integrity Just before Physical Rupture

Differentiating TEs were highly vacuolated and excluded fluorescein from the vacuole before vacuole rupture (Fig. 1a; Groover et al., 1997; Kuriyama, 1999), indicating that the tonoplast still has selective permeability before tonoplast rupture. By observing a living cell successively we found a phase between vacuole swelling and vacuole rupture. In this intermediate phase the nucleus is still pressed against the plasma membrane, indicating that the vacuole still keeps its osmotic pressure, whereas fluorescence in the cytoplasm decreases markedly (Fig. 2, a and b). Propidium iodide, which can stain the nucleus of only cells of which the plasma membrane became leaky, did not label the nucleus of TEs just after the vacuole ruptured, whereas it stained the nucleus of dead non-TEs quickly (Fig. 3). This result indicates that the plasma membrane keeps its integrity at least until vacuole rupture occurs. Therefore the decrease in fluorescence in the cytoplasm prior to the vacuole rupture does not seem to result from the diffusion of fluorescein across the plasma membrane, but across

**Figure 2.** Serial fluorescence images of a TE stained with SYTO16 and FDA (a–e). Red autofluorescence of the chloroplasts was eliminated. a, The fluorescence in the nucleus and cytoplasm was evident in the TE 7 min before vacuole rupture. The TE was so vacuolated that its nucleus was pressed against the plasma membrane and flattened. Boundary between the cytoplasm and vacuole was definite. b, The boundary became obscure and fluorescence intensity in the cytoplasm decreased, whereas that in the vacuole increased (2 min before vacuole rupture). Note that the nucleus was still flattened. c, The nucleus was released from the vacuole turgor pressure soon after vacuole rupture (0 min). d, The fluorescence intensity in the nucleus decreased markedly in the central region (4 min after vacuole rupture). e, The fluorescence of SYTO16 disappeared almost completely (14 min after vacuole rupture). f, Secondary wall thickening had already been obvious even 22 min before vacuole rupture. The bar indicates 20 μm.
the tonoplast. Because fluorescein hardly diffuses across intact membrane, at least in cultured zinnia cells (Kuriyama, 1999), this fluorescein diffusion across the tonoplast represents the loss of tonoplast integrity in blocking charged molecules. Two minutes after the tonoplast lost its selective permeability, the nucleus became almost sphere by liberation from the vacuole pressure (Fig. 2c), indicating that the final loss of tonoplast integrity occurs immediately before its physical rupture.

**Rapid Degradation of the Nucleus after Vacuole Rupture**

To examine whether the disappearance of fluorescence from the nucleus stained with SYTO16 is due to active autolysis we measured the levels of nuclear fluorescence of TEs before and after vacuole rupture (Fig. 4). Each value was shown as a relative value against the value obtained from the nucleus of the cells whose vacuole just ruptured. Changes in the fluorescence in non-TEs were used as an indicator of photobleaching. Before the tonoplast ruptured, fluorescence in TEs and non-TEs did not show substantial decrease (Fig. 4A). This tendency was observed for at least 50 min before vacuole rupture (data not shown). Levels of nuclear fluorescence after vacuole rupture were measured for individual TEs every 2 min. Almost all TEs except one showed a similar decreasing pattern of the fluorescence (Fig. 4B). This decreasing pattern was reproduced in another 10 TEs (data not shown). Therefore, we calculated and represented the average of TE1 to TE5 in Figure 4A. This graph clearly indicated a sudden decrease in nuclear fluorescence.
fluorescence after vacuole rupture. Fluorescence levels decreased approximately by one-half and one-quarter at 4 and 6 min after the vacuole ruptured, respectively. In contrast, fluorescence of the nucleus in non-TEs kept high all the time during observation. To confirm that fluorescence intensity of SYTO16 actually represents DNA contents, nuclei isolated from cultured zinnia cells were stained with SYTO16.

**Figure 4.** Changes in SYTO16 fluorescence intensity in the TE and non-TE nuclei. Fluorescence intensity was determined as the sum of pixel values per area of each organelle. The data are presented as relative values to actual pixel counts on the 0-min image. A, The pattern of changes in nuclear SYTO16 fluorescence intensity in TEs before and after vacuole rupture were compared with that in non-TEs observed in the same visual field. The SYTO16 fluorescence intensity of the TE nuclei decreased dramatically after vacuole rupture. B, The pattern of changes in nuclear fluorescence intensity in individual TEs after vacuole rupture. Five TEs exhibited the similarly the rapid decrease of their nuclear fluorescence following vacuole rupture (represented by TE 1–TE 5), although a TE degraded its nucleus relatively slowly (represented by TE 6). The values of closed square indicates the mean of five values (TE 1–TE 5) ± se. The values of closed lozenge indicates the mean of five non-TEs ± se.
and were incubated with DNase I. The fluorescence of SYTO16 was completely removed by DNase I and the rate of decline in fluorescence intensity after DNase treatment was similar to that of nuclei stained with 4′-6-diamidino-2-phenylindole (DAPI; Fig. 5). These results indicate that the rapid decrease in

![Figure 5](images.png)

**Figure 5.** Images of DNA degradation in isolated nuclei. Differential interference contrast (a–c) and fluorescence (d–l) images of isolated nuclei are shown. Nuclei were stained with SYTO16 (d, e, g, h, j, and k) or DAPI (f, i, and l). Nuclei were treated with DNase I for 0 min (b, c, e, and f), 5 min (h and i), and 15 min (k and l). Images of the nucleus 0 min (a and d), 5 min (g), and 15 min (j) after mock treatment are also shown. Note that SYTO16 fluorescence after DNase I treatment declined similarly to DAPI fluorescence, a marker of DNA content, (e, f, h, i, k, and l), whereas SYTO16 fluorescence remained intensely without DNase I treatment (d, g, and j). The bar indicates 5 μm.
nuclear fluorescence in TEs results from active autolysis of DNA, but not from other factors such as photobleaching.

Next we examined whether the rapid nuclear digestion after vacuole rupture is TE-specific or if it occurs even in non-TE when the vacuole of non-TE is forced to rupture. Probenecid, which is known to inhibit organic anion transport (Cole et al., 1990; Oparka et al., 1991; Wright and Oparka, 1994), not only accelerated vacuole rupture in TEs, but also induced vacuole rupture in non-TEs, although in these, it occurred asynchronously after a long delay (Kuriyama, 1999). Therefore, cells were incubated with 100 μM probenecid for 12 h and were loaded with SYTO16. Non-TEs in which the vacuole had ruptured were recognized by the presence of balloon-like structures composed of fragmented tonoplast replacing the central vacuole. It was difficult to catch the moment of the probenecid-induced vacuole rupture of non-TEs because the timing of vacuole rupture after probenecid treatment varied widely among non-TEs. Therefore, non-TEs that had undergone vacuole rupture, but exhibited relatively intense nuclear SYTO16 fluorescence were chosen and observed successively to examine the changes in the intensity of nuclear fluorescence because such non-TEs were expected to be cells that had lost the vacuole very recently. The intensity of nuclear fluorescence of non-TEs with the vacuole did not change significantly for 30 min (Fig. 6). Although the nuclear fluorescence intensity of four non-TEs of the five that had undergone vacuole rupture decreased during the 30-min period, the decrease was much slower than that of TEs after vacuole rupture. This result suggests that active nuclear digestion does not occur in non-TEs after vacuole rupture and that changes in physiological conditions in the cytoplasm caused by vacuole rupture such as pH and Ca^{2+} concentration are not the reason for the observed decline in nuclear fluorescence.

Rapid Degradation of Nucleic Acids Compared with Chlorophyll in Chloroplasts after Vacuole Rupture

DNA in chloroplasts and mitochondria forms a complex “nucleoid” with proteins (Kuroiwa et al., 1982, 1998). To examine whether degradation of nucleoids in mitochondria and chloroplasts occurs similarly to nuclear degradation or not, cells were loaded with SYTO16 and observed successively with focusing on the surface layer of thin cytoplasm. Nucleoids in mitochondria and chloroplasts in TEs exhibited strong green fluorescence before vacuole rupture, indicating that nucleic acids in these organelles are not degraded markedly before the vacuole ruptures (Fig. 7a). We confirmed the observation by Groover et al. (1997) that cytoplasmic streaming continued until the vacuole ruptured.

After vacuole rupture the nucleoids in chloroplasts appeared to be degraded rapidly, which is similar to the degradation of the nuclear chromatin (Fig. 7, b, c, and d). Fluorescence of nucleic acids in chloroplasts and the nucleus decreased simultaneously by one-half within approximately 6 min and to undetectable level within 16 min after vacuole rupture (Fig. 8). In contrast, chlorophyll fluorescence did not decrease detectably even at 16 min after vacuole rupture (Fig. 8). Moreover, the appearance of chloroplasts detected by autofluorescence did not change even at the time when nucleic acids in them were mostly degraded.

![Figure 6](image-url). Changes in the intensity of nuclear SYTO16 fluorescence in non-TEs that have undergone probenecid-induced vacuole rupture. Because the timing of probenecid-induced vacuole rupture in non-TEs varied widely, non-TEs that had already undergone vacuole rupture and still exhibit intense nuclear SYTO16 fluorescence were chosen and analyzed (represented by non-TE 1–TE 5). The value of closed square indicates the mean of five living non-TEs that had the vacuole. Error bars represent se.
These data indicate a rapid and preferential degradation of nucleic acids in chloroplasts compared with chlorophyll. We could not analyze the kinetics of mitochondrial nucleoids degradation because mitochondria were not stationary.

**DISCUSSION**

Plant PCD does not involve phagocytosis by adjacent cells, which is often observed in animal apoptosis (Greenberg, 1996). In the case of TE PCD, differentiating TEs synthesize newly hydrolytic enzymes and degenerate cellular contents without the assistance of neighboring cells. Our serial observation of SYTO16-stained nuclei provided direct evidence that nuclear degradation occurs only after the disruption of the tonoplast (Fig. 4A). Likewise, in the PCD process during aerenchyma formation in maize (Campbell and Drew, 1983) and senescence of unpolinated ovaries (Vercher et al., 1987), the tonoplast disruption is suggested to play a critical role in autolysis. In animal PCD there is a type of PCD that is suggested to involve the secretion of hydrolases from lysosomes leading to the loss of cytoplasm (Clarke, 1990). Jones (2000) proposed that plant PCD in which vacuole collapse plays a critical role in autolysis may be similar to the lysozomal PCD in animal.

Once the vacuole ruptured, nucleic acids in the nucleus and chloroplasts were degraded rapidly to undetectable levels within approximately 15 min (Figs. 4 and 7d). These data indicate a rapid and preferential degradation of nucleic acids in chloroplasts compared with chlorophyll. We could not analyze the kinetics of mitochondrial nucleoids degradation because mitochondria were not stationary.

**Figure 7.** Fluorescence images of a TE that was undergoing vacuole rupture. The autofluorescence of the several chloroplasts was in focus. a, Nucleic acids in the nucleus, chloroplasts, and mitochondria could be observed in a TE 5 min before vacuole rupture. The nucleus was pressed against the plasma membrane by the large central vacuole. b, The nucleus was released from pressure soon after vacuole rupture (0 min). c, SYTO16 fluorescence in some chloroplasts disappeared within 4 min after vacuole rupture. d, Nucleic acids in the nucleus and chloroplasts were almost completely degraded, whereas chloroplast autofluorescence was still visible. The bar indicates 20 μm.

**Figure 8.** Changes in the intensity of nuclear SYTO16, chloroplast SYTO16, and chlorophyll fluorescence following vacuole rupture. The data are presented as relative values to actual pixel counts per area on the 0-min image. Error bars represent se.
and 8). However, chlorophyll was not degraded and remained intensely fluorescence after SYTO16 fluorescence was undetectable (Fig. 8). Active and rapid digestion of chloroplastic DNA compared with chlorophyll is also observed in chloroplast of male origin in mated Chlamydomonas reinhardtii (Kuroiwa et al., 1982; Nishimura et al., 1999). These results suggest that plants possess a mechanism of digesting unneeded or unwanted nucleic acids actively, rapidly, and selectively. On the other hand, digestion of chloroplast DNA during senescence of rice coleoptiles proceeds gradually and spans days. In this senescence digestion of membrane structure, proteins, and DNA in chloroplasts commences before tonoplast disruption (Inada et al., 1998). In leaf senescence the hydrolysis of the nucleus occurs at a very late stage (Noodén and Guiamet, 1996), which is preceded by breakdown of membrane structure and proteins in the chloroplast (Bate et al., 1990; Bleecker and Patterson, 1997). Therefore, there seems to be different PCD mechanisms in plants, as typically shown in TE PCD and leaf senescence (Fukuda, 2000).

Rapid digestion of nucleic acids after vacuole rupture in differentiating TEs suggests the strong activity of nuclease(s) released from the vacuole. It has been reported that some nucleases accumulated specifically in differentiating TEs (Thelen and Northcote, 1989; Ye and Droste, 1996; Aoyagi et al., 1998). Of these nucleases, ZEN1 (a 43 kD-nuclease) is only the nuclease that can degrade double-strand DNA (Thelen and Northcote, 1989; Aoyagi et al., 1998). This enzyme, which is thought to accumulate in the vacuole, is synthesized just before autolysis of TEs (Aoyagi et al., 1998). Therefore, this nuclease may be responsible for the active degradation of nucleic acids in the nucleus and chloroplasts.

After vacuole rupture the nucleus was degraded from the central region (Fig. 1, b, c, and d). The inner-edge region of the nucleus, which exhibits chromatin condensation, was digested a little later than the central region (Fig. 1, b, c, and d). This delay may be due to the difficulty for the nuclease(s) to attack DNA in these condensed region, heterochromatin. The nucleus kept its spherical form even after the nucleic acids were degraded (Fig. 1f), indicating the absence of nuclear fragmentation in PCD during TE differentiation, unlike apoptosis, and suggesting the presence of the mechanism keeping the nuclear shape. The process of nuclear degradation is summarized in Figure 9.

Our data clearly show that vacuole rupture is a trigger of nuclear degradation in TEs. Then what is the mechanism of vacuole rupture? As shown in a previous paper (Kuriyama, 1999), the vacuole of differentiating TE swells before rupturing in differentiating TEs. We found that the fluorescence of fluorescein in the cytoplasm of differentiating TEs diminished rapidly just before the physical rupture of the vacuole; that is, the tonoplast kept pressing the nucleus against the plasma membrane (Fig. 2b). This rapid decrease in the fluorescence was shown to result from alteration of tonoplast permeability (Fig. 3). As regards the permeability of the tonoplast, the following three possibilities are proposed: (a) fluorescein diffused across the tonoplast; (b) proton was released from the vacuole into the cytoplasm; as a result, cytoplasmic pH...
changed from neutral to acidic, and then green fluorescence from fluorescein decreased greatly; and (c) 1 and 2 occurred simultaneously. At present we do not know which is correct. In any case, however, it is sure that selective permeability of the tonoplast changes at this stage. Although the permeability-change of the tonoplast prior to its rupture is beginning to be revealed, a key mechanism leading to vacuole rupture remains an enigma.

MATERIALS AND METHODS

Plant Material and Culture

The first leaves of 14-d-old seedlings of zinnia (Zinnia elegans cv Canary Bird [Takii Shubyo, Kyoto]) were used for the isolation of mesophyll cells in suspension culture according to the method of Fukuda and Komamine (1980a). All experiments were performed with cells cultured in inducive D medium that contained 0.1 mg/L a-naphthylacetic acid and 0.2 mg/L benzyladenine as phytohormones.

Fluorescence Staining

For CLSM, FDA (Aldrich Chemical, Milwaukee, WI) was added to the medium to final concentration of 0.1 μg mL⁻¹. After 1 h of incubation with rotation, SYTO16 (Molecular Probes, Eugene, OR) was added to the medium at final concentration of 1 μM and was incubated for 10 min. SYTO16 is a vital fluorescence dye that is membrane-permeable, and stains DNA and RNA (Luther and Kametksy, 1996). When the integrity of the plasma membrane was examined, propidium iodide (Wako Pure Chemicals, Osaka), which stains DNA and RNA, but is membrane-impermeable, was added to the medium to final concentration of 1.5 μg mL⁻¹. All staining and incubation was performed at 27°C.

CLSM

After stained cells were transferred onto poly-d-Lys-coated dishes (Glass Bottom Microwell Dishes, MatTek Corporation, Ashland, MA), they were placed on the inverted platform of a confocal laser scanning microscope (Meridian Instruments Far East, Tokyo). Dyes were excited using a 488-nm line from an argon laser, and detection was performed through a band-pass filter allowing the recording of green fluorescence from 515 to 545 nm. A long-pass filter above 630 nm was also used when the red auto-fluorescence of the chlorophyll was to be overlaid.

Measurement of Fluorescence

To quantify degradation of the nucleus, cells were loaded only with SYTO16 to prevent the fluorescence of FDA from overlapping because they have similar fluorescence emission spectra. Time-lapse images were taken for TEs and non-TEs in the same field and analyzed using INSIGHT-IQ system (Meridian Instruments Far East). In each image cells were equally exposed to the laser beam at 25 mW for 0.4 s. Before vacuole rupture images were taken at intervals of 5 or 7 min to avoid too much exposure of the laser beam to cells. Immediately after the vacuole rupture the interval was changed to 2 min. In each image, brightness of pixels in the region drawn around the nucleus of TEs and non-TEs was measured and normalized by the value measured in the image representing the moment of vacuole collapse in individual cells. Fluorescence from the nucleic acids in chloroplasts and auto-fluorescence of chlorophyll were also measured and normalized by the same way.

Isolation of Nuclei

The zinnia cells that had been cultured for 18 h were collected on nylon meshes (pore size of 10 μm) and washed in a mannitol solution composed of 0.7 m mannitol and MES (5 nm 2-morpholinoethanesulfonic acid), pH 5.5. These cells were resuspended in an enzyme solution containing 1% (w/v) cellulase onozuka RS (Yakult, Tokyo), 0.1% (w/v)pectolyase Y-23 (Seishin Pharmaceutical, Tokyo), 0.6 m mannitol, 5 mm MgCl₂, and 20 mm MES, pH 5.5, and were incubated at 27°C for 1 h to produce protoplasts. Protoplasts were collected by centrifuge at 200g for 1 min and resuspended in a solution containing 24% (w/v) Suc and 5 mm MES, pH 5.5. The mannitol solution was overlaid onto the resuspension. After centrifugation at 200g for 5 min, the middle layer containing purified protoplasts was collected. Purified protoplasts were washed in mannitol solution. Isolation of nuclei from purified protoplasts was performed according to the method of Willmitzer and Wagner (1981) with some modifications. The standard buffer used here was composed of 0.25 m Suc, 10 mm NaCl, 5 mm EDTA, 0.15 mm spermine, 0.5 mm spermidine, 20 mm 2-mercaptoethanol, 0.2 mm phenylmethylsulfonyl fluoride, 0.1% (w/v) bovine serum albumin, and 10 mm MES, pH 6.0. Purified protoplasts were agitated in the standard buffer supplemented with 1% (w/v) Triton X-100 and were centrifuged at 1,000g for 10 min. The precipitate was suspended in the standard buffer supplemented with 0.1% (w/v) Triton X-100 and 70% (v/v) percoll (Pharmacia Biotech, Piscataway, NJ), and was overlaid with the standard buffer supplemented with 0.1% (w/v) Triton X-100 and 25% (v/v) percoll. After centrifugation at 600g for 20 min the interphase was collected and diluted with the standard buffer. The subsequent centrifugation at 1,000g for 10 min gave a pellet of nuclei.

DNA Degradation in Isolated Nuclei

Isolated nuclei were resuspended in a DNase reaction buffer containing 15 mm NaCl, 0.15 mm spermine, 0.5 mm spermidine, 10% (w/v) Suc, 10 mm MnSO₄, and MES, pH 6.0, and were incubated with a dye (1 μM SYTO16 or 2 μg/mL DAPI) and 70U/mL DNase I. Fluorescence images of each sample were taken immediately (0 min), and at 5 and 15 min after the addition of DNase I or reaction buffer under an epifluorescence microscope (model BX-50-FLA, Olympus, Tokyo) equipped with charge-coupled device
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