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Morphological changes in the cytoskeleton, nuclei, and vacuoles during cell death of short-lived ray tracheids in the conifer *Pinus densiflora*

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Abstract Morphological changes in the cytoskeleton, nuclei, and vacuoles were monitored during the cell death of short-lived ray tracheids in the conifer *Pinus densiflora*. After formation of the dentate thickenings that occurred at the final stage of formation of cell walls, organelles started to disappear in differentiating ray tracheids. First, the microtubules and vacuoles disappeared. Then actin filaments disappeared in the differentiating ray tracheids adjacent to ray tracheids that lacked nuclei, and, finally, the nuclei disappeared. These features indicate that cell death in ray tracheids might differ from the programmed cell death of tracheary elements that has been studied in vitro in the *Zinnia* culture system.

Key words Cell death · *Pinus densiflora* · Ray tracheid · Xylem differentiation

Introduction

The patterns of cell death in the secondary xylem play an important role in the functions of secondary xylem cells, for example, in the movement of water, the mechanical support of stems, and the transfer and storage of nutrients and metabolites.[1] Tracheary elements of secondary xylem cells, such as longitudinal tracheids and vessel elements, function in the transport of water along the stem. Cell death of longitudinal tracheids occurs successively and is related to the distance of cells from the cambium in the conifer *Abies sachalinensis*.[2] Such cell death of short-lived secondary xylem cells is similar to the time-dependent programmed cell death that occurs in differentiating tracheary elements derived from single cells that have been isolated from the mesophyll of *Zinnia elegans*.[3,4] However, details of the progression of cell death in longitudinal tracheids are not easily determined because the tracheids are long and it is difficult to monitor the organelles in entire cells.

In some conifers, such as *Pinus* and *Larix*, rays consist of two different types of cell, namely, ray parenchyma cells and ray tracheids. Ray tracheids lose their organelles immediately after their differentiation.[5] They form bordered pits and their short lifespan is similar to that of longitudinal tracheids. Moreover, ray tracheids are shorter than longitudinal tracheids. Thus, we postulated that it might be easier to monitor the details of the cell death of ray tracheids, which might provide a suitable model system for studies of the death of short-lived secondary xylem cells in situ. In this potential model system, we observed that cell death of ray tracheids occurred successively and was closely related to the distance from the cambium in *Pinus densiflora* and *Pinus rigida*.[6] However, information about the morphological changes in organelles during the cell death of ray tracheids remains limited.

The purpose of the present study was to investigate the morphological changes in actin filaments, microtubules, nuclei, and vacuoles in short-lived ray tracheids of *Pinus densiflora* in order to characterize the events that occur during the death of short-lived secondary xylem cells in situ. Previous studies of programmed cell death in plants have revealed the disintegration of vacuolar membranes,[7,8] rearrangement of the cytoskeleton,[9,10] and structural changes in nuclei.[11] We postulated that similar changes might also occur during cell death in short-lived ray tracheids.

Materials and methods

Plant materials

Two *Pinus densiflora* trees of approximately 37 years of age, growing in the Karasawayama Experimental Forest of

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Tokyo University of Agriculture and Technology in Sano-Tochigi, Japan, and two trees of approximately 52 years of age, growing in the field nursery of the Tokyo University of Agriculture and Technology in Fuchu-Tokyo, Japan, were used. Small blocks containing secondary phloem, cambium, and secondary xylem were taken from main stems at breast height in May and June in 2005 and 2007, when secondary xylem was actively differentiating.

Specimen preparation

For preparation of 1-μm-thick sections, samples were fixed overnight at room temperature in a 4% solution of glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). After they had been washed with the same buffer, small blocks of samples were dehydrated through a graded ethanol series and embedded in epoxy resin. Radial sections of 1-μm thickness were cut with a glass knife on an ultramicrotome (Ultracut N; Reichert, Vienna, Austria).

For preparation of 50-μm-thick sections, samples for observation of actin were prefixed for 1 h at room temperature in a mixture of 0.2 mM *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester (MBS; Pierce Biotechnology, Rockford, IL, USA) that contained 2% dimethylsulfoxide and 0.05% Nonidet P-40 in distilled water.[12,13] Samples were then fixed overnight at room temperature in a mixture of 3.6% paraformaldehyde and 0.2% glutaraldehyde that contained 10% dimethylsulfoxide and 0.1% Nonidet P-40 in 50 mM piperazine-1,4-bis(2-ethanesulfonic acid) buffer (pH 7.0), supplemented with 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid and 5 mM $MgSO_4$. Radial sections of approximately 50-μm thickness were cut on the freezing stage of a sliding microtome (MA-101; Komatsu Electronics, Tokyo, Japan) and washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.0 mM $NaHPO_4$, adjusted to pH 7.3), as described by Nakaba et al.[2]

Light and fluorescence microscopy

Examination of cell walls and nuclei

Radial sections of 50-μm thickness were stained with a 1% aqueous solution of safranine for observation of cell walls and stained with a 1% aqueous solution of acetocarmine for observation of nuclei. Sections were observed under a light microscope (Axioscop; Carl Zeiss, Oberkochen, Germany). For observations of the autofluorescence of nuclei, radial sections of 1-μm thickness were examined with a fluorescence microscope (BX61; Olympus, Tokyo, Japan) under epifluorescence illumination (excitation/ emission combination, BP 460–495/ BA 510IF).

Visualization of microtubules

For observations of microtubules, we performed immunofluorescence staining with rat monoclonal antibodies against α-tubulin (Harlan Sera-Lab, Belton, UK) that had been diluted 1:10 in PBSB (PBS containing 0.1% NaN_3 and 1 mg ml^{-1} bovine serum albumin).[13–15] Radial sections were incubated in this solution for 60 min at 30°C. Then they were washed with PBS and incubated for 60 min at 30°C with fluorescein isothiocyanate-conjugated (FITC-conjugated) antibodies against rat IgG (Amersham Japan, Tokyo, Japan) that had been diluted 1:10 in PBSB. Then sections were stained with a 0.0005% aqueous solution of propidium iodide for observation of nuclei. These sections mounted on glass slides and examined with a Carl Zeiss LSM 310 confocal laser-scanning microscope (excitation/ emission combination, for FITC: 488/ BP 515–565; and for propidium iodide: 543/ LP 590).

Visualization of actin filaments

For observations of actin filaments, we performed immunofluorescence staining with mouse monoclonal antibodies against actin (MP Biomedicals, Aurora, OH, USA) that had been diluted 1:100 in PBSB.[13] Radial sections were incubated in this solution for 120 min at 30°C and then they were washed with PBS and incubated for 60 min at 30°C with FITC-conjugated antibodies against mouse IgG (Amersham Japan, Tokyo, Japan) that had been diluted 1:10 in PBSB. The immunostained sections were examined as described above for microtubules.

Visualization of vacuoles in living cells

Vacuoles in living cells were visualized with fluorescein diacetate (FDA), a vital stain that fluoresces in the cytoplasm.[16,17] Vacuoles appeared as dark regions surrounded by bright fluorescence. Radial sections of living tissue of approximately 50-μm thickness were cut on a sliding microtome (Yamatokohki, Saitama, Japan). Radial sections were immersed in a solution of 0.02% FDA in 0.2 M aqueous sucrose for 30 min and washed with 0.2 M aqueous sucrose. The sections were mounted on glass slides in 0.2 M aqueous sucrose and examined with a confocal laser-scanning microscope (excitation/ emission combination, 488/ BP 515-565).

Results

Ray tracheids are easily distinguished from ray parenchyma cells by the thick cell walls, bordered pits, and dentate thickenings (Fig. 1). Most differentiating ray tracheids had elliptical nuclei that contained chromatin (arrowhead in Fig. 2a, b). However, deformed nuclei without obvious chromatin were observed in ray tracheids adjacent to ray tracheids that had lacked nuclei (arrows in Fig. 2a, c).

Dense bundles of cortical microtubules, oriented at an angle of approximately 60° to the cell axis, were visible during secondary wall formation in differentiating ray tra-

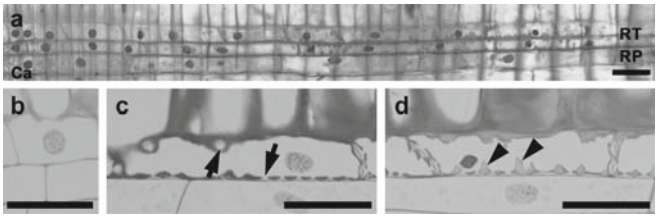

Fig. 1a–d. Differentiating ray tracheids in *Pinus densiflora*. **a** The process of differentiation in ray tracheids can be observed in an individual radial section. **b.** Ray tracheids in the early stage of differentiation have only thin cell walls. **c** Then the ray tracheids form thick cell walls and bordered pits. **d** Finally, the ray tracheids form dentate thickenings. *Arrows* indicate bordered pits; *arrowheads* indicate dentate thickenings. *Ca*, Cambium; *RP*, ray parenchyma cell; *RT*, ray tracheid. *Bars* 50 μm

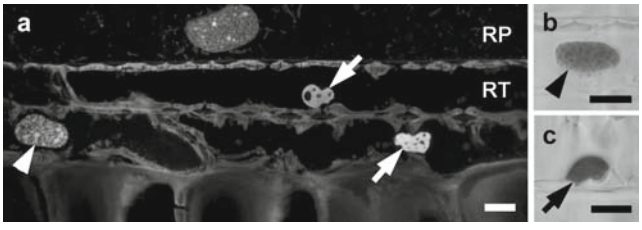

Fig. 2a–c. Nuclei in differentiating ray tracheids in *Pinus densiflora*. **a** Autofluorescence of nuclei in differentiating ray tracheids. **b, c** Nuclei in ray tracheids stained by acetocarmine. *Arrows* indicate deformed nuclei; *arrowheads* indicate elliptical nuclei. *RP*, Ray parenchyma cell; *RT*, ray tracheid. *Bars* 10 μm

cheids (Fig. 3a, b). Circular bands of microtubules were visible around the edges of developing bordered pits (Fig. 3a, b). During the formation of dentate thickening, dense bundles of cortical microtubules were observed at an angle of approximately 90° to the cell axis (Fig. 3c, d). In addition, the orientation of microtubules was disturbed at the periphery of developing dentate thickening (Fig. 3e, f). Microtubules disappeared in ray tracheids after the completion of dentate thickening (Fig. 3g, h).

In differentiating ray tracheids, we observed thick bundles of low-density actin filaments that were oriented at random (Fig. 4a–d), and there was little change in the density and orientation of actin filaments during the differentiation of ray tracheids. Some actin filaments were localized within the periphery of developing bordered pits (Fig. 4a, b), but actin filaments disappeared in ray tracheids that were adjacent to ray tracheids that lacked nuclei (Fig. 4e–g).

After staining with FDA, vacuoles were observed as dark spheres surrounded by the bright fluorescence of the cytoplasm (Fig. 5). In differentiating ray tracheids, vacuoles occupied most of the cell lumen and the cytoplasm was localized at the periphery of the cell (Fig. 5a–d). Signals due to staining with FDA disappeared in ray tracheids when dentate thickening was complete (Fig. 5e, f).

Discussion

The cytoskeleton plays important roles in the cytodifferentiation of secondary xylem cells.[18,19] In the present study, we observed circular bands of microtubules around the edges of developing bordered pits and actin filaments localized within the periphery of developing bordered pits (Figs. 3a and 4a). Similar localization of microtubules and actin filaments has been observed during the formation of bordered pits in longitudinal tracheids.[20–22] Circular bands of cortical microtubules might be involved in the deposition of cellulose microfibrils at pit borders.[18,19] In addition, Chaffey et al.[23,24] proposed that microtubules and actin filaments might be associated with the reduction in the diameter of the aperture of each bordered pit in a differentiating vessel element. Thus, it appears that actin filaments and microtubules play similar important roles in the formation of bordered pits in ray tracheids and in longitudinal tracheary elements.

After the formation of dentate thickenings at the final stage of deposition of the cell wall of ray tracheids of *Pinus densiflora*, the microtubules disappeared (Fig. 3g, h). Furusawa et al.[25] noted that the fluorescence due to microtubules became weaker and then disappeared at the final stage of formation of the cell wall in longitudinal tracheids of *Taxus cuspidata*. Therefore, it appears that microtubules rapidly cease to function after the completion of the formation of the cell wall in the short-lived secondary xylem cells of conifers.

Disappearance of microtubules was accompanied by disappearance of fluorescence due to FDA (Fig. 5a). Rotman and Papermaster[16] reported that FDA readily penetrates the cell membrane, and then esterases remove acetate groups to generate a fluorescent product, namely, fluorescein. Therefore, the disappearance of signals due to FDA suggests that esterases have been inactivated. It is possible that the rupture of vacuoles results in alterations in the intracellular environment, such as a change in pH, and esterases cease to function. Thus, the absence of a signal due to FDA in ray tracheids might reflect the rupture of vacuoles.

Microtubules and signals due to FDA disappeared immediately after completion of the formation of dentate thickenings. However, thick bundles of actin filaments were still visible in the same ray tracheids (Fig. 4f). Actin filaments had disappeared in ray tracheids that were adjacent to ray tracheids that lacked nuclei. Our results suggest that actin filaments retain their function until the final stage of cell death in ray tracheids. An actin network might provide the scaffold for components that control the formation of autophagosomes and it might also direct the movement and the engulfing of the cytoplasm by autophagosomes.[10] Thus, actin filaments might play an important role in the formation of autophagosomes and the transport of materials that are related to cell death at the final stages of cell death in short-lived ray tracheids.

The shapes of nuclei began to change in ray tracheids that were adjacent to ray tracheids that lacked nuclei

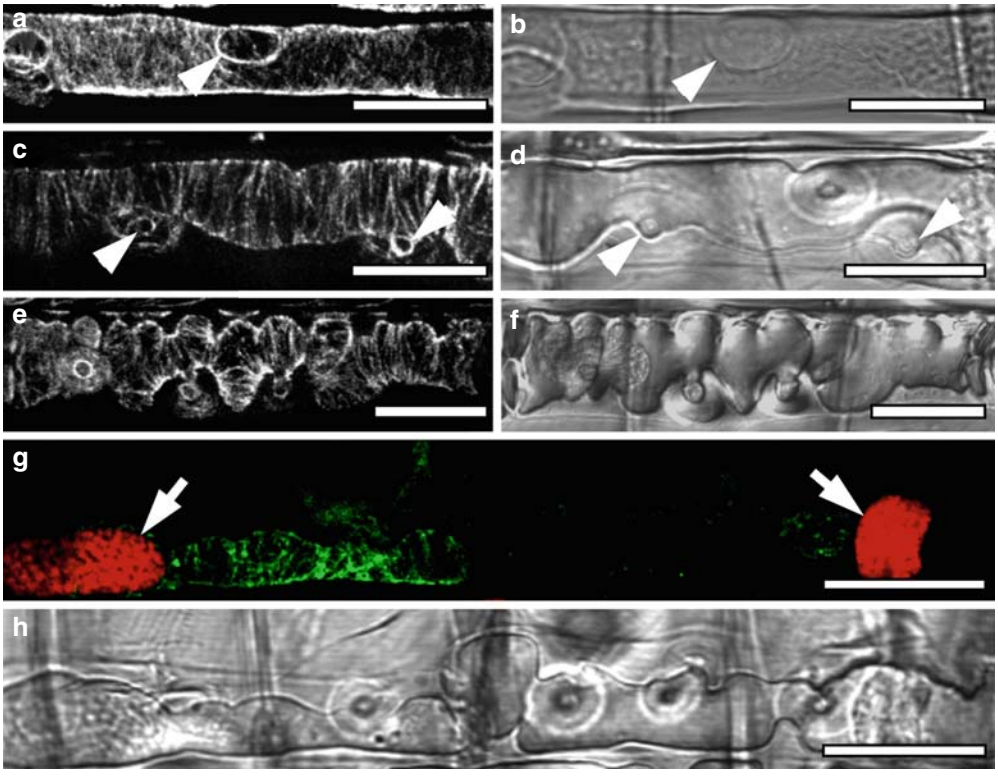

Fig. 3a–h. Microtubules in differentiating ray tracheids in *Pinus densiflora*. **a** Microtubules in ray tracheids that are forming thick cell walls and bordered pits. **b** Normarski differential interference contrast image of section in **a**. **c** Microtubules in ray tracheids that are forming dentate thickenings. **d** Normarski differential interference contrast image of section in **c. e** Microtubules in ray tracheids at the late stage of thickening of the cell wall. **f** Normarski differential interference contrast image of section in **e**. **g** Microtubules in ray tracheids after the formation of dentate thickenings. Normarski differential interference contrast image of section in **h**. *Arrowheads* indicate circular bands of microtubules; *arrows* indicate nuclei in ray tracheids. *Bars* 25 μm

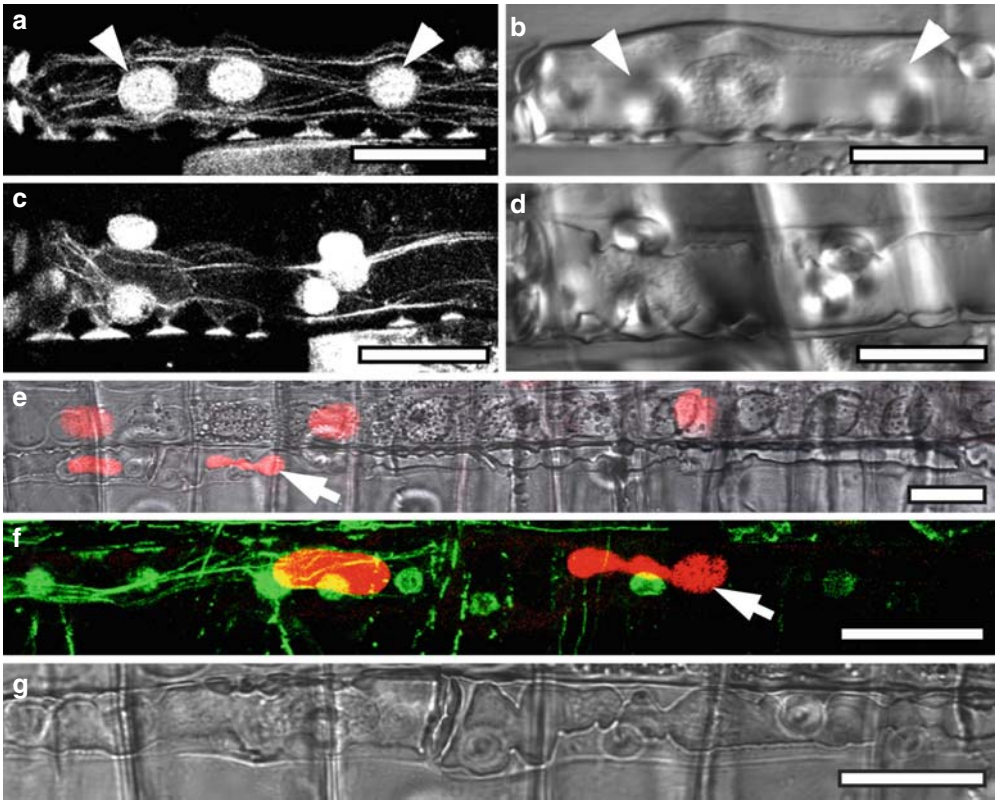

Fig. 4a–g. Actin filaments in differentiating ray tracheids in *Pinus densiflora*. **a** Actin filaments in ray tracheids that are forming dentate thickenings. **b** Normarski differential interference contrast image of section in **a**. **c** Actin filaments in ray tracheids after the formation of dentate thickenings. **d** Normarski differential interference contrast image of section in **c**. **e** Nuclei in ray tracheids adjacent to ray tracheids that lack nuclei. **f** Actin filaments in ray tracheids adjacent to ray tracheids that lack nuclei. **g** Normarski differential interference contrast image of section in **f**. *Arrowheads* indicate actin filaments localized within the periphery of developing bordered pits; *arrows* indicate nuclei in ray tracheids adjacent to ray tracheids that lack nuclei. *Bars* 25 μm

(Fig. 2). In the former cells, nuclei lacked obvious chromatin structures. These results indicate that the disintegration of nuclei occurred in ray tracheids adjacent to ray tracheids that lacked nuclei. It is possible that nuclei might synthesize macromolecules that are involved in the final stage of cell death in ray tracheids.

Our observations revealed that well-ordered morphological changes in the cytoskeleton, nuclei, and vacuoles

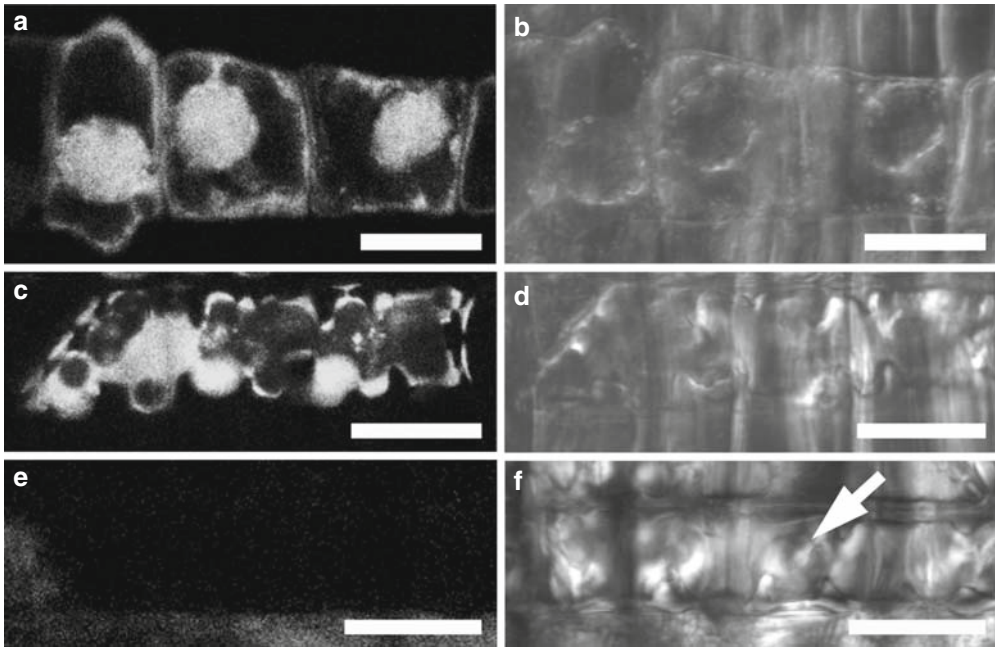

Fig. 5a–f. Vacuoles appearing as dark regions on a field of bright fluorescence in differentiating ray tracheids in *Pinus densiflora*. **a** Vacuoles in ray tracheids that have only a thin cell wall. **b** Normarski differential interference contrast image of section in **a.** **c** Vacuoles in ray tracheids that are forming dentate thickenings. **d** Normarski differential interference contrast image of section in **c**. **e** Vacuoles in ray tracheids after the formation of dentate thickenings. **f** Normarski differential interference contrast image of section in **e**. *Arrow* indicates a nucleus in a ray tracheid that lacks a signal due to fluorescein diacetate. *Bars* 25 μm

occur during the death of short-lived ray tracheids in the conifer, *Pinus densiflora*. The microtubules and vacuoles disappeared immediately after the formation of dentate thickenings at the final stage of cell wall formation. Actin filaments disappeared in ray tracheids adjacent to ray tracheids that already lacked nuclei, and, finally, the nuclei of the former cells disappeared. The differences in timing of the disappearance of the various organelles suggest different roles for these organelles during cell death in short-lived ray tracheids. During the programmed cell death of differentiating tracheary elements derived from single cells of the mesophyll of *Zinnia elegans*, degradation of cell contents begins with the collapse of vacuoles.[10] However, in ray tracheids, actin filaments and nuclei might retain their functions even after vacuoles have collapsed. Therefore, cell death in ray tracheids might differ from the programmed cell death of tracheary elements that has been studied in vitro in the *Zinnia* culture system.

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