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Analysis by Confocal Microscopy of the Structure of Cambium in the Hardwood *Kalopanax pictus*

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An understanding of the morphology and the developmental changes in the shapes and dimensions of cambial cells requires three-dimensional (3-D) analysis of thick slices of tissue. We devised a simple protocol using confocal laser scanning microscopy (CLSM), with safranin and acridine orange as fluorescent dyes and glycerol as the clearing and mounting medium, to examine the 3-D structure of the dormant cambium in *Kalopanax pictus*, a ring-porous hardwood. Optical sections and high contrast images provided clear information about the shapes and nuclear status of cambial cells, which have previously been difficult to determine using conventional microscopy. The axially-oriented cambial cells were found to vary in shape, in particular around the rays, and were not always typically fusiform. We evaluated the reliability of our method by comparing results with those of a parallel study of the same material by standard analysis of serial sections of epoxy-embedded specimens. The images of optical sections obtained by CLSM were of high quality and similar to images obtained by conventional light microscopy of semi-thin mechanical sections. Use of the confocal microscope provided a quick and easy method for visualization of the structure of the cambium in thick hand-cut sections and for studies of the developmental changes in cells from the cambium to the xylem.
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Key words: Confocal laser scanning microscopy, *Kalopanax pictus*, three-dimensional reconstruction, vascular cambium.

INTRODUCTION

Vascular cambium is a lateral meristem that produces the secondary vascular tissues of plants. It consists of a thin layer of cells sandwiched between the phloem and xylem. A full understanding of the structure and function of vascular cambium is a prerequisite for complete understanding of the growth and development of woody plants (Philipson *et al*., 1971; Barnett, 1981; Iqbal and Ghouse, 1990; Catesson, 1994; Larson, 1994).

Straightforward methods for elucidation of the three-dimensional (3-D) structure of cambium might be applicable to taxonomic and genetic studies of woody plants. In recent years, there has been increased interest in the molecular biology of certain woody species (for a review see Chaffey, 1999). Details of the 3-D structure of cambium can help predict the patterns of organization, development and growth of vascular tissues in both wild and transgenic woody plants. They can also be used to characterize various features related to wood quality and structure, such as the length of vessel elements, the types of ray and axial parenchyma, the proportions of various wood elements, and the grain pattern of wood.

In cambium with a storied structure, in which cells are ordered systematically, the architecture of the tissue can easily be understood from an analysis of sections in three different planes, namely, the transverse, radial and tangential planes. However, in species with non-storied cambium, the fusiform cells vary considerably in length and the tips of the adjacent cells, which overlap during intrusive growth, often cannot be seen in a single section. In addition, since fusiform cambial cells are long (Bailey, 1920; Metcalfe and Chalk, 1983; Kitin *et al*., 1999) and the cambial tissue forms only a thin layer, it is difficult to ensure the exact orientation of the sections along the cell axis. The morphology of non-storied cambium can be defined reliably by 3-D analysis based on consecutive (serial) sections of the cambial zone. This method was used to study the shapes of cambial cells in a *Pinus* sp. by Lewis (1935) and Dodd (1948), and in some dicotyledonous woody species by Fujita and co-workers (Fujita *et al*., 1984; Fujita, 1993). Wloch and Zagorska-Marek (1982) and Wloch and Bilczewska (1987) prepared serial transverse and tangential sections of the cambium of a *Tilia* sp. for studies of the intrusive growth of cambial cells and the resulting grain pattern of the wood. Serial mechanical sectioning of cambium has not, however, been widely used by other researchers, probably because of the technical difficulties that arise as a result of the great length and thin walls of cambial cells. It is much easier to prepare serial sections from the rigid cells of the mature xylem or phloem. In a number of studies, the shapes, dimensions and arrangements of cambial cells were characterized indirectly on the basis of the structure of cambial derivatives, on the assumption that the architecture of secondary vascular tissues mirrors that of the cambium (Evert, 1961, 1963; Bannan, 1967, 1970; Cumbie, 1967, 1983).

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Cambial tissue is easily damaged during sampling and sectioning. Reasonable images of cambial cells have been obtained by conventional microscopy using thin sections of resin-embedded tissue (Isebrands and Larson, 1973; Imagawa and Ishida, 1981; Abe *et al*., 1997; Chaffey *et al*., 1997). However, in thin sections, only portions of cells can be visualized. With larger longitudinal sections, which include whole cells, it is difficult to obtain images of sufficiently high quality and, usually, the two ends of a long cell cannot be viewed in a single focal plane.

Recent advances in confocal laser scanning microscopy (CLSM) have provided good tools for the study of plant anatomy, and it is now possible to scan the interior of relatively thick histological sections (Knebel and Schnepf, 1991; Pawley, 1995; Running *et al*., 1995; Running and Meyerowitz, 1996; Chapman and Mulcahy, 1997; Travis *et al*., 1997; Hepler and Gunning, 1998; Gray *et al*., 1999). CLSM has been used to measure cell dimensions (Jang *et al*., 1991; Moss *et al*., 1993; Donaldson and Lausberg, 1998; Gray *et al*., 1999; Kitin *et al*., 1999) and to observe the dynamics of changes in orientation of components of the cytoskeleton in cambium and cambial derivatives in woody plants (Abe *et al*., 1995; Chaffey *et al*., 1997, 1998; Funada *et al*., 1997, 2000; Furusawa *et al*., 1998). Such studies indicate that CLSM might be very useful for 3-D visualization of soft and fragile meristematic cells, such as those of the cambium in woody plants. Large thick sections are particularly useful when attempting to view entire cambial cells (Kitin *et al*., 1999). Reconstruction of 3-D structures using CLSM does not require mechanical sectioning of the specimen. Intact tissue can be studied by optical sectioning, and images can be digitized and analysed by computer. The 3-D structure of tissues can be easily reconstructed from stacked serial images using image analysis software.

One of the major problems associated with the use of a confocal laser scanning microscope is the appropriate choice of fluorescent dyes and the establishment of techniques for inducing fluorescence from particular components of a tissue. As the fluorescence imaging is dependent on numerous factors, protocols for CLSM have to be established and adjusted for optimal performance for each particular plant tissue. The technique used here is a further development of the method we used previously (Kitin *et al*., 1999), which led to a considerable improvement in the contrast of images. We used safranin and acridine orange as fluorescent dyes to examine and define the 3-D structure of the cambium in *Kalopanax pictus*, a ring-porous hardwood. We also studied the morphology of the cambial cells in serial mechanical sections of epoxy-embedded tissue. We then compared the potential utility of CLSM with optical sectioning and mechanical sectioning followed by light microscopy.

MATERIALS AND METHODS

Plant material

A *Kalopanax pictus* (Araliaceae) tree (diameter at breast height, 76 cm) growing on the campus of Hokkaido University, Japan was the source of material for the experiments. Samples, including cambium and adjacent phloem and xylem, were taken from the stem at breast height during the dormant period (7 Nov. 1996 and 5 Dec. 1997). They were fixed in a mixture of 50 % ethanol, acetic acid and formaldehyde (18 : 1 : 1, v/v; FAA) or in an aqueous 4 % solution of glutaraldehyde, degassed briefly under a vacuum and then left overnight in fixative.

Mechanical sectioning

Specimens that had been fixed in glutaraldehyde were post-fixed in 1 % osmium tetroxide and embedded in epoxy resin. Serial tangential semi-thin sections (2 or 3 μm thick) were cut with an ultramicrotome (Ultracut; Reichert-Jung, Vienna, Austria) using a glass knife and were then stained with a 1 % solution of safranin in 30 % ethanol for 1 h in an incubator at 35°C. The stained sections were washed in water and dried on microscope slides in an incubator for 1 h at 35°C. Then they were mounted in mounting medium (Bioleit; Oken-shoji, Tokyo, Japan) for preparation of permanent microscope slides. The samples on slides were observed with a confocal laser scanning microscope (LSM-310: Carl Zeiss, Oberkochen, Germany) using transmitted visible light or excitation by incident-light from an argon ion laser (wavelength, 488 nm) with a band-pass filter (BP; 515–565 nm) or a long-pass filter (LP; 590 nm) or from a helium neon laser (wavelength, 543 nm) with a LP (590 nm) filter.

Optical sectioning

The FAA-fixed materials were frozen on a freezing stage (MA-101; Komatsu Electronics, Tokyo, Japan), which was attached to a sliding microtome, and thick tangential sections (60–80 μm) that included portions of xylem, cambium and phloem were cut in the frozen state to prevent deformation of the thin-walled cambial cells. The same materials, without freezing, were used for preparation of hand-cut transverse sections with a razor blade. The sections were stained with an aqueous 0·1 % solution of acridine orange or with a 1 % solution of safranin in 30 % ethanol for 30 min at room temperature under vacuum. Then they were dehydrated through a graded ethanol series (30, 50, 75, 90 and 100 %; 15–30 min at each concentration) with frequent changes of the respective solutions of ethanol, until no further colour was washed from the specimens. Specimens then were rehydrated by passage through decreasing concentrations of ethanol and finally placed in distilled water. Dehydration of the specimens removed stain from cell vacuoles and reduced the background fluorescence on confocal images. If such dehydration of specimens is not desirable, rinses in buffered solutions can be tested as an alternative method for removing background staining, as recommended by Running *et al*. (1995). However, this procedure can require several days.

The transparency of tissues must then be increased by clearing prior to CLSM (Running *et al*., 1995; Gray *et al*., 1999). We tested glycerol [refractive index (RI), approx. 1·47], xylene (RI approx. 1·50) and methyl salicylate

(RI approx. 1·54) as clearing and mounting media, as follows. The specimens were passed through increasing concentrations of glycerol or methyl salicylate (25, 50, 75, 100 %; 1 h or more per solution) with two or three changes of each solution and left overnight in pure glycerol or methyl salicylate, respectively. Xylene was used to clear samples immediately after the dehydration step, according to the protocol of Running *et al*. (1995). The cleared specimens were mounted on glass slides in the same medium as that used for clearing, and then coverslips were placed on the samples. Incident-light excitation by the argon ion laser (wavelength, 488 nm; BP, 515–565 nm or LP, 590 nm) and the helium neon laser (wavelength, 543 nm; LP, 590 nm) was used to observe samples with the confocal laser scanning microscope. We used a Zeiss Plan-Neofluar 40 × /0·75 objective lens (Carl Zeiss). Consecutive confocal images of tangential and transverse sections of cambium were obtained at intervals of 1, 2 or 3 µm.

Image processing and analysis of 3-D structure

Confocal images were stored digitally on a computer connected to the CLSM system and printed out using a digital colour printer (UP-D8800; Sony, Tokyo, Japan) as described previously (Furusawa *et al*., 1998).

We analysed the 3-D structure of cambium from images of serial mechanical sections and serial optical sections on a computer using the software of the confocal laser scanning microscope.

RESULTS AND DISCUSSION

Techniques for preparation and staining of samples

The usual method for visualization of cambium by conventional microscopy involves examination of semi-thin sections of tissue that has been embedded in resin. In the present study, the soft cell walls and the division plates of cambial cells were relatively well preserved and could be seen clearly by conventional microscopy (Fig. 1A). In previous studies, images have been obtained with the confocal laser scanning microscope after fluorescence has been induced by incident-light excitation of safranin or acridine orange. Safranin was used in conjunction with the confocal laser scanning microscope to produce images of lignified woody cells (Donaldson and Lausberg, 1998) and of mesocarp cells in grapes (Gray *et al*., 1999). Acridine orange has mainly been used to stain DNA, but plant cell walls also emit metachromatic fluorescence after staining with acridine orange (see O'Brien and McCully, 1981). Safranin is a cationic dye that can react electrovalently with various materials in the cytoplasm and cell walls (see O'Brien and McCully, 1981). In general, procedures involving safranin include a rinse in ethanol, which removes some of the dye, especially that associated with non-lignified cell walls. This phenomenon is useful in histological studies by conventional microscopy because tissue components become better differentiated and higher-contrast images are obtained. For CLSM, however, removal of the dye from non-lignified cell walls results in weaker fluorescence and poorer resolution of images of cambium, as compared to those of lignified xylem. Thus, specimens of cambium should not be left in concentrated ethanol for prolonged periods. Specimens should be washed and cleared with the aim of removing safranin from the cell vacuoles and cytoplasm but not from the cell walls.

Both the argon ion laser and the helium neon laser (see above) allowed visualization of cell walls in both safranin- and acridine orange-stained specimens. However, images with the best contrast were obtained with the helium neon laser and safranin-stained specimens (Fig. 1B, C). The structure of the cambium was clearly visible on semi-thin sections (Fig. 1B), as well as on thick hand-cut sections (Fig. 1C). In unstained hand-cut sections of FAA-fixed tissue, primary walls were poorly distinguishable but both nuclei and cytoplasm emitted fluorescence (Fig. 1D). The hand-cut sections shown in Fig. 1 were too thick to allow good images of cambial cells to be obtained with transmitted light with the microscope in the conventional mode. In addition, the thick hand-cut sections often had uneven surfaces or had been damaged by the razor blade during cutting. With the confocal microscope, however, the specimen could be scanned deep beneath its surface, where the tissue was much better preserved.

The metachromatism of acridine orange has been well characterized (Kasten, 1981; O'Brien and McCully, 1981). Using different excitation wavelengths and barrier filter combinations we were able to observe various components of cambial cells. The mechanisms behind the interaction of acridine orange with nucleic acids have been investigated in detail (Kasten, 1981; Gordon *et al*., 1997). By contrast, the specificity of staining by acridine orange is not well known in the primary cell walls of plants, which contain mainly cellulose, glycans, pectins, structural proteins and aromatic substances (see Carpita *et al*., 1996). The cambial cell walls in acridine orange-stained specimens were clearly visible upon excitation by the argon ion laser (Fig. 2A). In our experiments, however, staining with safranin yielded stronger fluorescence than staining with acridine orange and resulted in brighter cell walls and images with slightly better contrast (Fig. 2B).

Techniques for clearing and mounting specimens

It was necessary to clear stained samples to reduce background fluorescence and to increase the transparency of the tissue so that images of adequate quality could be generated. We achieved good results with safranin- and acridine orange-stained specimens of cambium by rinsing them in an ethanol series and then immersing them in increasing concentrations of glycerol. Then cambium could be optically sectioned to a depth of 30–40 µm in the tangential-longitudinal or transverse direction. Other protocols have involved clearing tissues in xylene (Running and Meyerowitz, 1996) and methyl salicylate (Gray *et al*., 1999). Clearing and mounting in xylene or methyl salicylate, both of which are more hazardous than glycerol, did not lead to better-quality images of cambial cells and were not appropriate for our purposes. Little attenuation of fluorescent signals in cambial cells was noticeable until optical

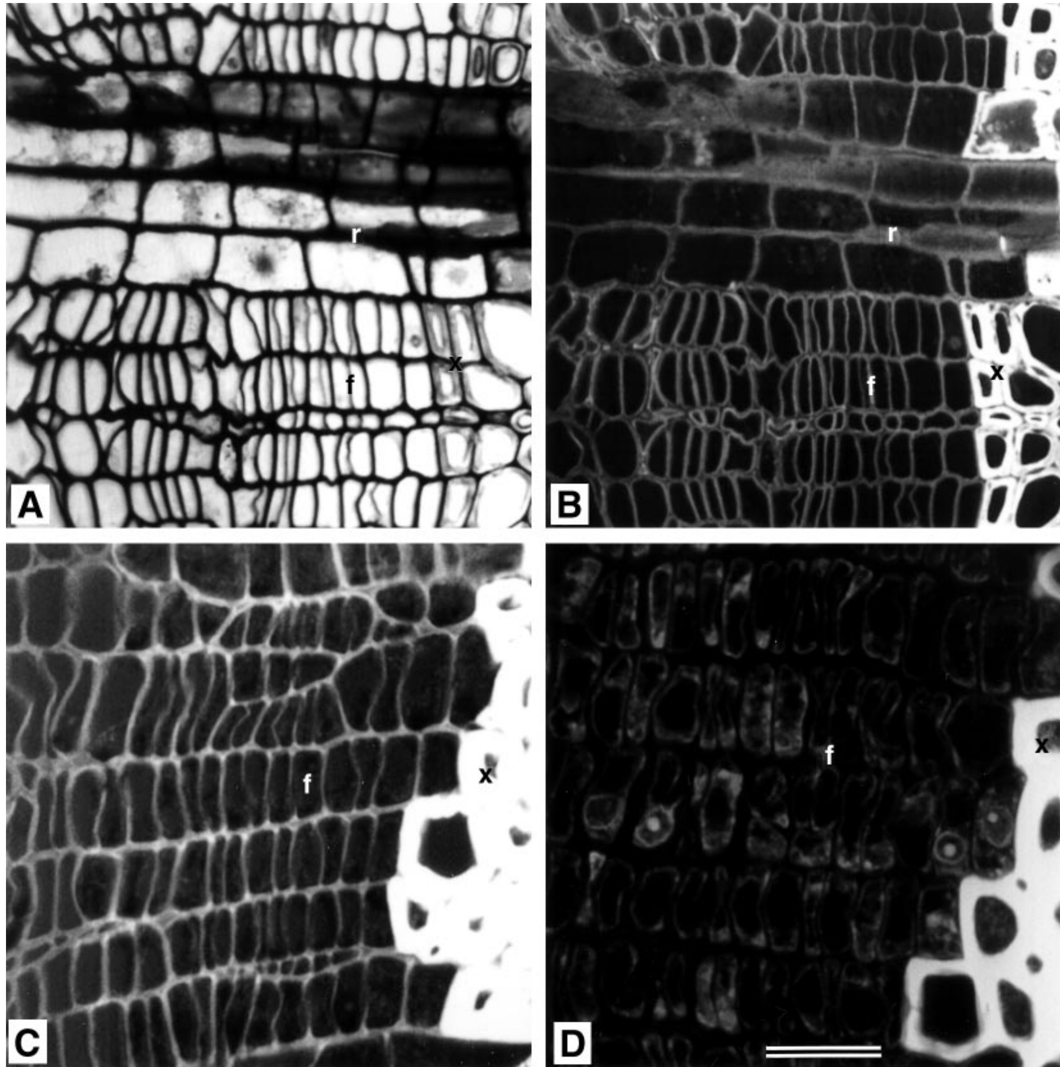

FIG. 1. Comparison of various methods for viewing transverse sections of dormant cambium of *Kalopanax pictus*. A, Transmitted-light image of a 3 μm thick section of an epoxy-embedded specimen stained with safranin. B, Confocal image of the section in A viewed with incident-light excitation by the helium neon laser (543 nm) and the long-pass filter (LP; 590 nm); the primary walls of cambial cells are bright and the cell lumens are dark. C, Confocal image of a hand-cut section, stained with safranin and viewed under the same conditions as in B. D, Confocal image of a hand-cut section, without staining, viewed with excitation by the argon ion laser (488 nm) and the band-pass filter (BP; 515–565 nm). In D the primary walls of cambial cells cannot be seen but fluorescence images of both the nuclei and the cytoplasm are visible. The section shown in A and B was from a specimen that had been fixed in 4 % glutaraldehyde and the sections shown in C and D were from specimens fixed in FAA. Bar = 50 μm. r, Ray cambial cells; f, fusiform cambial cells; x, xylem.

sections reached a depth of 20 to 30 μm (see Fig. 4A–H). At depths greater than 40 μm, the intensity of fluorescence from cambial cells decreased considerably, regardless of the clearing or mounting medium. The attenuation of fluorescent signals leads to errors, in particular when quantitative measurements of cells are made. Gray *et al.* (1999) scanned the mesocarp of grapes to a depth of 150 μm and found that the intensity of fluorescent signals decreased linearly with depth. These authors estimated cell volumes and, to minimize errors, they corrected for the attenuation of fluorescent signals by increasing the intensity threshold sequentially. The width of the dormant cambium of *Kalopanax pictus* is 60–80 μm (Kitin *et al.*, 1999). A depth of 30–40 μm for consecutive optical sections was sufficient for visualization of several adjacent layers of cambial cells, and no corrections of the confocal images for attenuation of signals were necessary. Cambium samples are likely to be more opaque than those of grape mesocarp studied by Gray *et al.* (1999). Individual cambial cells generally have tangential diameters of 6–10 μm. By contrast, the diameters of individual mesocarp cells in grapes are frequently greater than 100 μm. The greater opacity of our specimens of cambium, as compared to that of the specimens of grape mesocarp, might have been due to the greater frequency of cell walls rather than to differences in clearing media used.

Autofluorescence, which was detected as background fluorescence, was emitted by the resin in epoxy-embedded specimens. When the intensity of fluorescence from cell walls was greater than that from the resin, the image

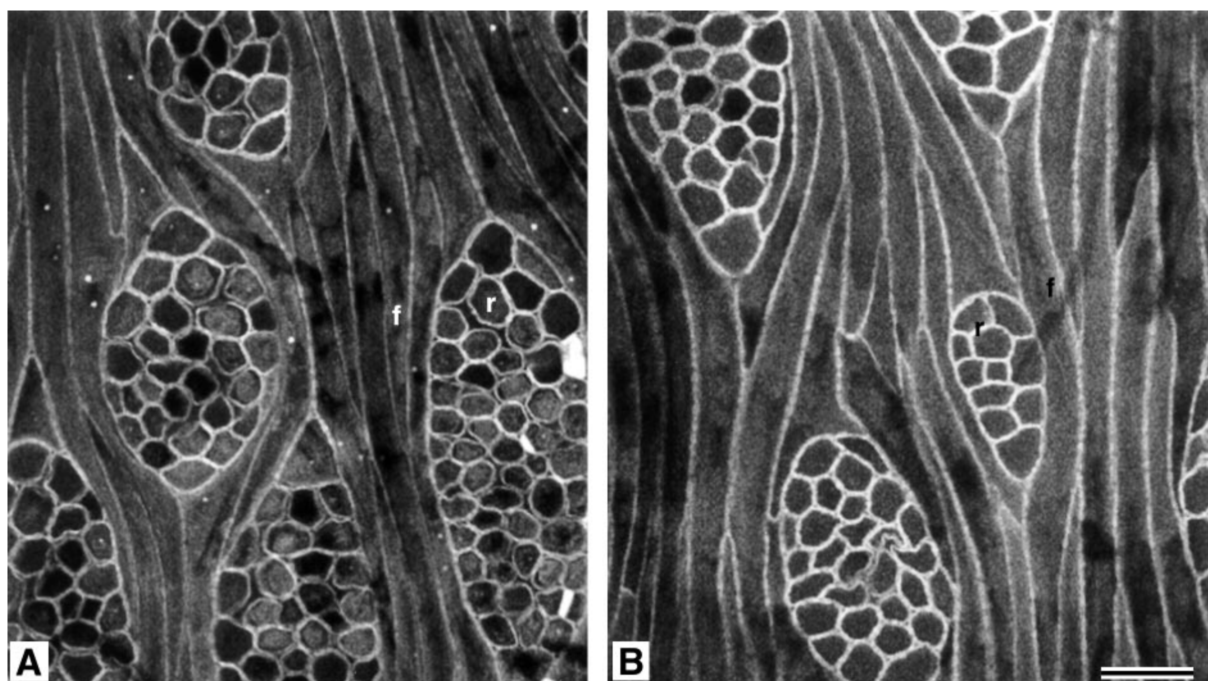

FIG. 2. Confocal images (obtained from a single focal plane) of thick tangential sections (approx. 60 μm thick) of cambium after fixation in FAA. A, An acridine orange-stained sample (excitation by the argon ion laser, 488 nm; band-pass filter, 515–565 nm). B, A safranin-stained sample (excitation by the helium neon laser, 543 nm; long-pass filter, 590 nm). Both dyes reveal the primary walls of cambial cells. Bar = 50 μm. For abbreviations, see legend to Fig. 1.

revealed bright cell walls and dark lumens (Fig. 1B). In 3-μm-thick tangential sections (Fig. 3), images had dark cell walls and bright lumens because of the strong fluorescence from the resin. In both the positive and the negative imaging of cambial cell walls, the shapes and sizes of cambial cells were easily discerned. It is likely that the difference in intensity between the background fluorescence from the resin and the fluorescence from cell walls depends on the intensity of staining and the extent of clearing of the specimen.

Morphology of cambial cells as revealed by serial sections

Investigations of the differentiated tissues of xylem or phloem have often been used to monitor developmental changes in the cambium, such as the changes in the patterns of anticlinal divisions and the dimensions of cambial cells (Evert, 1961, 1963; Bannan, 1967, 1970; Cumbie, 1967, 1983). The lengths of tracheids in conifers and of vessel elements in hardwoods have been used to estimate the lengths of fusiform cambial cells. It is generally considered that the lengths of tracheids and vessel elements do not change significantly during their differentiation. However, this assumption has not been tested adequately and the precision of indirect assessments of the length of fusiform cambial cells remains open to question (Kitin *et al*., 1999). In some cases, determination of the morphology and the dimensions of fusiform cambial cells by conventional microscopy is not possible, in particular in species with non-storied cambium. It is difficult to view the entire length of fusiform cambial cells in a single tangential section because the tips of cells in the section might not be the relevant ones. Studies of serial sections are needed to establish the positions of the actual tips of cells (Kitin *et al*., 1999). Figure 3 shows serial tangential sections through the cambium of an epoxy-embedded specimen. Cells labelled 1–4 are adjacent cells in a radial file of cambial cells. The dark patches and strips between the cells represent portions of the cell walls, which were cut at an angle. The apparent contours of the fusiform cambial cells in each section do not represent the true shapes of cells. However, the consecutive changes in the apparent contours of cells can be followed and the true shapes of cells can be reconstructed from the serial sections. The axially-oriented cambial cells in *Kalopanax pictus* were found to vary considerably in shape, in particular around the rays, where the cells adjust to conform to the shapes and positions of the rays. Thus, the axially-oriented cambial cells were not always typically fusiform. Another elusive issue that requires attention on serial semi-thin sections and serial optical sections is the nuclear status of cambial cells. Under the conventional microscope, with tangential sections of cambium 15 to 20 μm thick, several adjacent fusiform cells might appear as a single cell with several nuclei because of the large depth of focus. Thus, there have been lengthy discussions in the literature as to whether fusiform cambial cells might be multinucleate (see Larson, 1994). The serial sections in Fig. 3 show clearly that each cell has one nucleus.

Developmental changes in the structure of cambium, for example after anticlinal (multiplicative) divisions, can be monitored by studying serial sections. The serial sections in

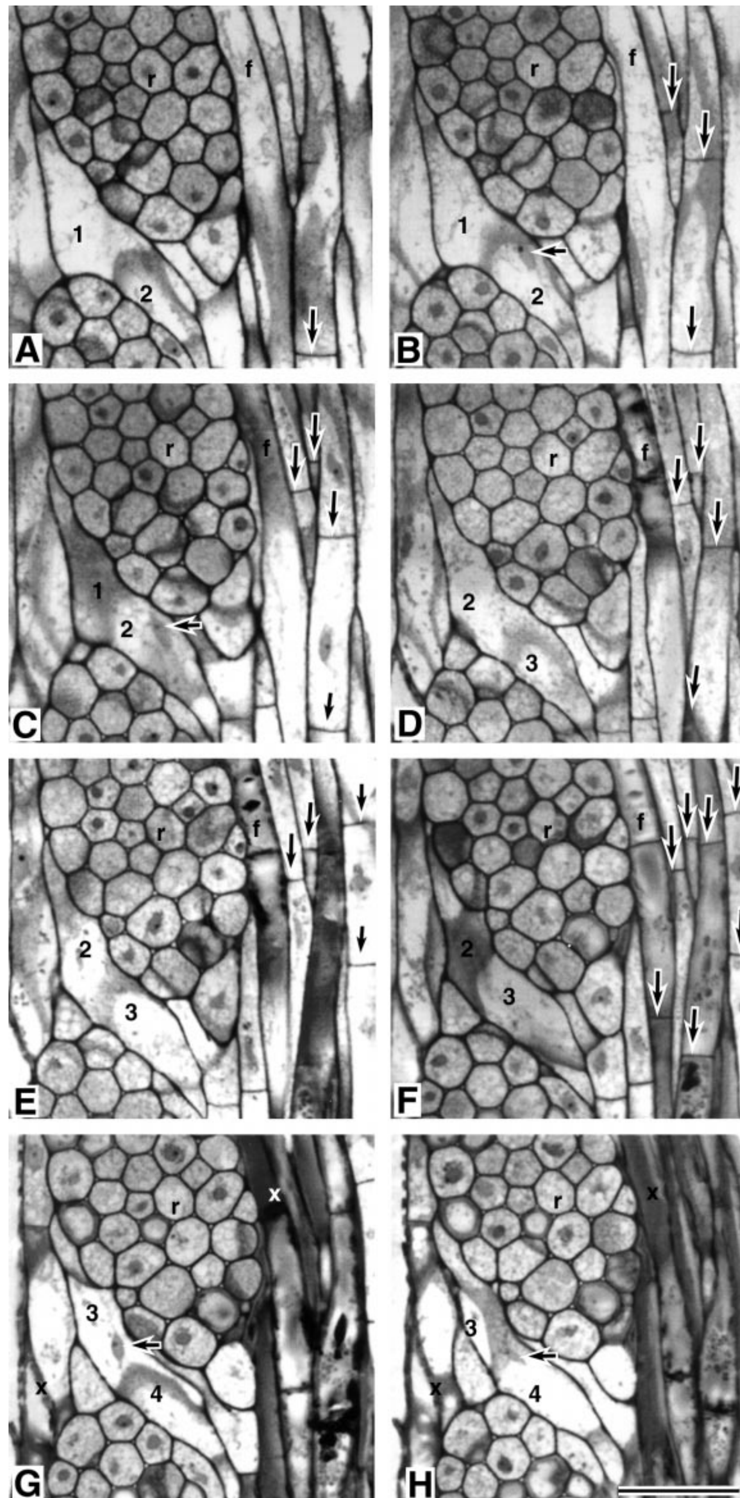

FIG. 3. Serial mechanical tangential sections of 3 μm thickness, from the cambium towards xylem (from A to H), of epoxy-embedded tissue. Fixation in 4 % glutaraldehyde and staining with safranin. Confocal images with the argon ion laser (488 nm) and a band-pass filter (515–565 nm). The images show dark cell walls, nuclei and cytoplasm and bright vacuoles, similar to those observed by conventional light microscopy on semi-thin sections (cf. Fig. 1A). The 3-D structure of the cambium and the sequential changes in the shapes and dimensions of cambial derivatives can be studied on serial sections. Note the increasing frequency of multiplicative divisions (large arrows) towards the xylem (from A to F). The fusiform cell (f) indicated in E and F is divided transversely and would probably have given rise *in situ* to an axial parenchyma strand. Cells designated 1–4 are adjacent cells in a radial file of cambial fusiform cells and probably originated from the same initial. Darker strips between the numbered cells are portions of obliquely cut double cell walls. Small horizontal arrows show nuclei. The nucleus of cell 2 is seen in B and C; the nucleus of cell 3 is seen in G, and the nucleus of cell 4 is seen in H. Note the changes in the apparent shapes of these cells in A to H. Bar = 50 μm. For abbreviations, see legend to Fig. 1.

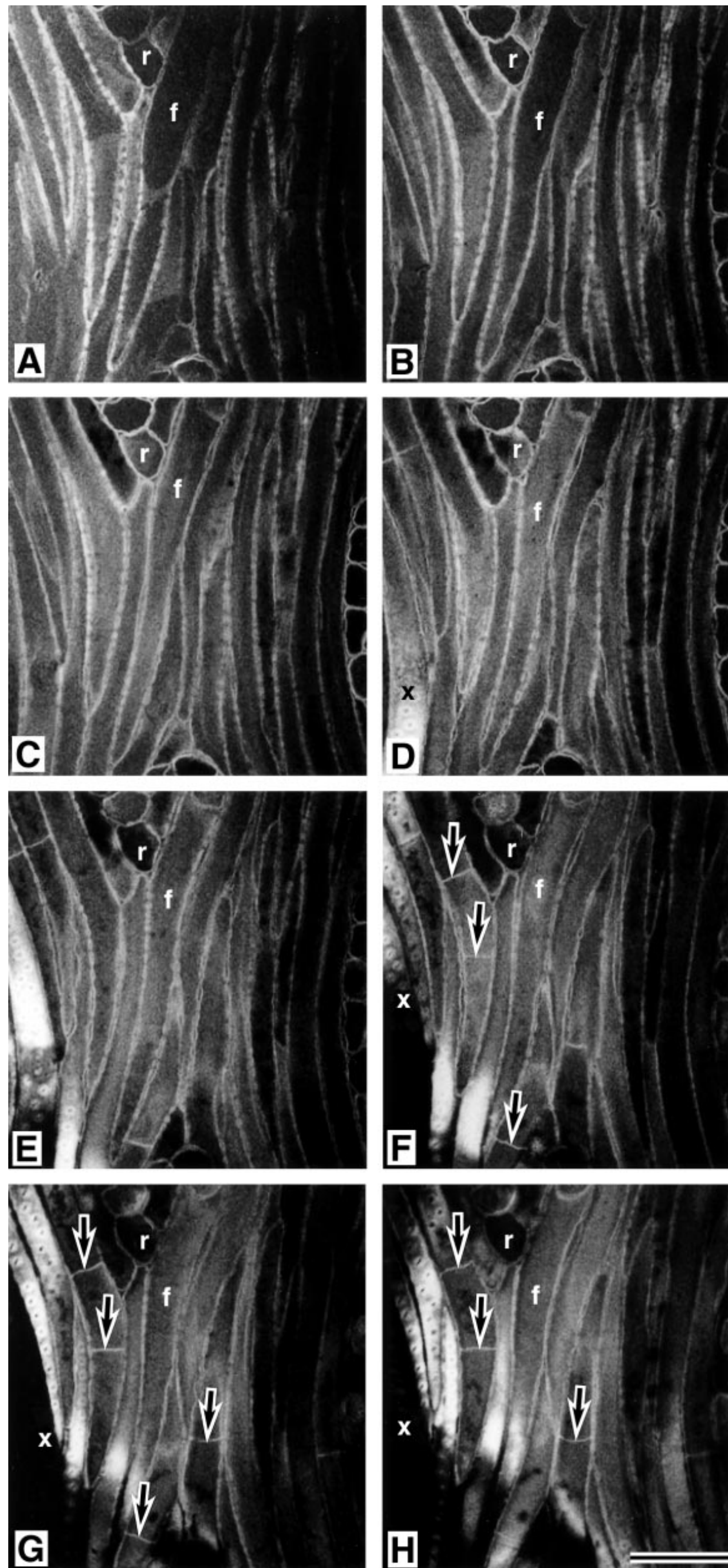

FIG. 4. Consecutive tangential optical sections at 3 μm intervals from the cambium towards xylem (from A to H) of a specimen fixed in FAA and stained with safranin. The sample was excited with the helium neon laser (543 nm) with a long pass-filter (590 nm). Note the beaded structure of the radial walls of fusiform cambial cells, which is generally assumed to be indicative of primary pit fields. Arrows show transverse multiplicative divisions of fusiform cells, which would probably have differentiated *in situ* into axial parenchyma strands. Xylem elements of the annual ring border are visible in D to H. Note the bordered pits at their tangential walls. Bar = 50 μm. For abbreviations, see legend to Fig. 1.

Figs 3 and 4 were made from the cambium through the xylem (from A to H) and they show cambial cells from three to four layers adjacent to the xylem. The boundary between cambial and xylem cells is visible in Figs 3G, H and 4D–H. The lignified walls of the xylem cells appear dark on the sections of epoxy-embedded tissue in Fig. 3 and bright on the optical sections in Fig. 4. The walls of the xylem cells also exhibited strong birefringence under polarized light (data not shown). The large arrows in Figs 3 and 4 indicate anticlinal divisions in fusiform cells, which *in situ* would probably have differentiated into axial parenchyma. The extent of anticlinal divisions and the positions of division plates have been investigated in some species, mainly by analysing the structure of differentiated xylem (Evert, 1961, 1963; Bannan, 1967, 1970; Cumbie, 1967, 1983). The positions of division plates might, however, change during the process of cell differentiation. In addition, it is not possible to determine the extent and the annual duration of multiplicative divisions by monitoring the structure of differentiated xylem because there might be a great difference in terms of timing between the anticlinal divisions in the cambial cells and the differentiation of those cells into xylem elements. Thus, for example, we noted the increased frequency of anticlinal divisions in the fusiform cambial cells at the xylem boundary layer (Figs 3F and 4E–H). Since the sample was a piece of dormant cambium, anticlinal divisions in these cells must have occurred before the cessation of cambial activity in the current year, while the resulting xylem cells would not appear until the beginning of the next growing season. The extent of anticlinal divisions in the cambium can be determined easily in studies by CLSM of cambial specimens collected throughout the year.

Optical vs. *mechanical sectioning of cambial tissue*

A technique for the preparation of ribboned serial sections of epoxy-embedded tissues does exist (Gay and Anderson, 1954; Fisher, 1968; Knobler *et al.*, 1978) but it has not often been used in studies of cambium. Our semi-thin mechanical sections of epoxy-embedded tissues were of relatively consistent quality and the resolution of images was good, so we were able to examine a long series of sections. By contrast, optical sectioning by CLSM was limited by the working distance of the objective lens and the attenuation of fluorescent signals. The drawbacks of serial mechanical sectioning of resin-embedded tissue can be summarized as follows. The procedures for fixation, dehydration, embedding in resin, cutting of sections with an ultramicrotome and, finally, the preparation of numerous microscope slides are laborious and time-consuming. The consecutive sections have to be collected and ordered manually, with proper stacking for 3-D reconstruction. Errors (artifacts) in the 3-D image after manual stacking of sequential images of mechanical sections can occur as a consequence of the irregular thickness of the serial sections and the inappropriate orientation of images. In addition, sections from the sequence are easily damaged or lost during cutting with the microtome and during the preparation of slides. Complete long series are, thus, difficult to obtain. By contrast, the preparation of samples for CLSM is rapid and consecutive images can easily be obtained by optical sectioning (Fig. 4). The confocal images revealed cell shapes and dimensions, as well as details of the structure of the cell wall, such as the primary pit fields and bordered pits (Fig. 4). The 3-D structure of the intact tissue is preserved during optical sectioning and consecutive confocal images can be stacked automatically by the computer software of the CLSM system to produce a 3-D image. The confocal laser scanning microscope is a powerful tool with which to study the 3-D structure and development of cambial cells, which, until now, have been difficult to study by other methods.

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