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THREE-DIMENSIONAL IMAGING AND ANALYSIS OF DIFFERENTIATING SECONDARY XYLEM BY CONFOCAL MICROSCOPY

by

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SUMMARY

We examined the three-dimensional (3-D) structure of differentiating xylem in a hardwood tree, *Kalopanax pictus*, by confocal laser scanning microscopy (CLSM) using relatively thick, hand-cut histological sections. 3-D studies of plant tissues by mechanical serial sectioning with a microtome are very time-consuming. By contrast, the preparation of samples for CLSM is easier and the 3-D structure of intact tissue is preserved during optical sectioning. We obtained extended-focus images of the surface of specimens and these images resembled the stereographic images obtained by scanning electron microscopy. In addition, we observed radial files of cambial derivative cells at various stages of differentiation and the internal structure along the 'z' axis of specimens on serial optical sections. We analysed the developmental changes in the morphology of cambial derivative cells, for example, the 3-D shape and arrangement of cells, the readjustment of the position of cells, and the development of secondary walls, pits and perforation plates. Our results showed that the arrangement of the differentiating xylem cells mirrors that of the cambial cells. Deviations from the longitudinal orientation of vessel elements were specified by similar patterns of orientation of fusiform and ray cambial cells. The development of vessel elements progressed more rapidly than that of other xylem elements. When secondary walls with bordered pits and perforation plates with membranes were present in vessel elements and their expansion ceased, no secondary wall formation was detected in adjacent ray cells. The delay in secondary wall formation by the ray parenchyma cells, as compared to that by vessel elements, might facilitate the readjustment of the position of cells in the developing xylem tissue that is a consequence of the considerable expansion of the vessel elements.

Key words: Cambium, confocal laser scanning microscopy, *Kalopanax pictus*, vessel elements, wood formation.

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INTRODUCTION

The morphogenesis of xylem cells involves rapid developmental changes in structure and chemical composition, namely, the enlargement of cells, the deposition of secondary walls, the formation of pits and perforation plates and, finally, the degeneration of protoplast and cell membranes (for a review, see Mellerowicz et al. 2001). It has been difficult to investigate entire large cells on individual histological sections by conventional light microscopy or transmission electron microscopy because single sections include only parts of such cells. In addition, it is not easy to study the progress of differentiation of vessel elements because the expansion of and developmental changes in these cells are very rapid. Some aspects of the formation of vessel elements, for example the pattern of cell enlargement, the development of perforation plates and the events that trigger apoptosis, are poorly understood even though they have been the focus of extensive research (Barnett 1981; Butterfield & Meylan 1982; Metcalfe & Chalk 1983; Buvat 1989; Iqbal 1990; Aloni 1991; Romberger et al. 1993; Catesson 1994; Larson 1994; Iqbal 1995; Savidge 1996; Fukuda et al. 1998; Aloni et al. 2000). It appears that vessel elements do not elongate during differentiation (Kitin et al. 1999). However, the dynamic processes involved in the formation of individual vessel elements in intact xylem have not been observed. It remains impossible to predict which cells of the cambium will differentiate into vessel elements and whether their positions are determined randomly or by a specific mechanism. Research into xylem differentiation has not yet provided a satisfactory explanation of the mechanism of expansion of vessel elements and the resulting readjustment of the position of cells in the developing xylem tissue.

Unlike analyses based on observations of single histological sections, three-dimensional (3-D) analysis of the structure of the cambium and differentiating xylem can help us to understand patterns of wood formation. Stereographic views of xylem are routinely obtained by scanning electron microscopy (SEM). The depth of focus and the wide range of magnification of SEM make it a powerful tool for studies of wood structure (Butterfield & Meylan 1980; Ohtani 2000). However, SEM does not allow examination of structures beneath the surface of specimens. Studies of internal structures of specimens and the construction of 3-D models of wood features require serial sections through the tissue. The 3-D images derived from serial sections of xylem have allowed investigations of the shapes and dimensions of tracheids (Dodd 1948), as well as vessel networks (Burggraaf 1973; Zimmermann 1983; Fujii et al. 2001) and the origin of the wavy grain in xylem (Hejnowicz & Romberger 1973; Włoch et al. 2001). However, serial sectioning is not only time-consuming but can also lead to artefacts due to the irregular thickness of the serial sections and manual stacking of the series of images (Kitin et al. 2000).

Mechanical sections can be replaced by optical sections of thick slices of tissue when confocal laser scanning microscopy (CLSM) is used for 3-D analysis of cambium (Kitin et al. 2000). CLSM allows analysis of the interior of relatively thick histological sections (Pawley 1995; Running et al. 1995; Hepler & Gunning 1998; Gray et al. 1999; Funada 2000, 2002; Kitin et al. 2000, 2002). Thicker sections and larger areas of

sections can provide very useful information about the morphology and differentiation of cambial derivative cells (Kitin et al. 2000, 2002). In addition, the computer-assisted 3-D reconstruction of structures by optical sectioning is faster and more precise than the 3-D reconstruction that can be achieved by mechanical sectioning.

The application of CLSM to visualization of xylem structure was first described by Knebel and Schnepf (1991) and CLSM was used subsequently to measure the dimensions of xylem cells (Jang et al. 1991; Moss et al. 1993; Donaldson & Lausberg 1998; Moëll & Donaldson 2001), to calculate lignin contents (Donaldson et al. 1999), and to analyse the movement of water in xylem (Matsumura et al. 1998; Abe et al. 2001). In addition, CLSM has been used for immunolocalization of components of the plant cytoskeleton in differentiating xylem cells (Abe et al. 1995; Funada et al. 1997, 2000, 2001; Chaffey et al. 1998; Furusawa et al. 1998; Chaffey 2000; Funada 2000, 2002), and for studies of the length and nuclear status of fusiform cambial cells (Kitin et al. 1999, 2002). However, to our knowledge, CLSM has not been used for anatomical analysis of details of the cell structure of differentiating xylem. Unlike the lignified cells of mature xylem, the cells of differentiating xylem have fragile walls and are easily damaged during the preparation of samples for microscopy (Kitin et al. 2001). It is not easy to avoid deformation of cells during the drying of tissue that is required for SEM. However, drying is not necessary for the preparation of samples for CLSM and, therefore, tissues can be examined in a relatively intact state.

Establishment of optimal conditions for the fluorescence staining, clearing and embedding of specific samples of plant tissue is required for optimal results in each particular case (Kitin et al. 2000). In the present study, we focused on the visualization and analysis by CLSM of the structure of differentiating secondary xylem cells in the hardwood *Kalopanax pictus*. Our goal was to clarify the developmental changes that occur in cambial derivative cells during differentiation and, in particular, the 3-D structure of differentiating vessel elements.

MATERIALS AND METHODS

Plant material

A *Kalopanax pictus* (Araliaceae) tree (diameter at breast height, 76 cm) growing on the campus of Hokkaido University was used as the source of material for the experiments. Sample blocks, including cambium and adjacent phloem and xylem and with dimensions of approximately 2 × 2 × 2 cm, were cut with a sharp knife and a chisel from the stem at breast height. The samples were obtained soon after the beginning of the growing period in early May. They were fixed and stored in a mixture of 50% ethanol, acetic acid, and formaldehyde (18 : 1 : 1, v/v; FAA).

Optical sectioning

Tangential and transverse sections that included portions of xylem, cambium and phloem were hand-cut with a razor blade. The sections were stained with a 1% solution of safranin in 30% ethanol for 30 min at room temperature under a vacuum. Then they were dehydrated by passage through a graded acetone series (30%, 50%, 75%, 90% and 100%, 15–30 min at each concentration) with frequent changes of each respective

solution of acetone until no further colour was extracted from the specimens. Then the specimens were rehydrated by passage through decreasing concentrations of acetone and finally placed in distilled water.

Clearing of cambial tissues with glycerol [refractive index (RI), approximately 1.47] was shown by Kitin et al. (2000) to be appropriate for observations of cambium and xylem tissue by CLSM. Therefore, we soaked the specimens in increasing concentrations of glycerol (25%, 50%, 75%, 100%) for 1 h or more per solution, with two or three changes of each solution, and then left them overnight in pure glycerol. The cleared specimens were mounted in glycerol or water and immediately observed. Incident-light excitation by a helium neon laser (wavelength, 543 nm; long-pass filter, 590 nm) was used for observations with the confocal laser scanning microscope (LSM-310; Carl Zeiss, Oberkochen, Germany), which was equipped with a Zeiss Plan-Neofluar 40×/0.75 air or Plan Neofluar 63×/1.25 oil objective lens (Carl Zeiss). Consecutive confocal images of transverse and tangential sections of cambium were obtained at intervals of 3 μm. The noise in images was reduced by averaging eight times (Kalman averaging).

RESULTS AND DISCUSSION

We cut sections, free-hand, with a sharp razor blade and the preparation of thick sections for 3-D analysis of the large xylem cells was fast and easy. We were able to obtain relatively well-preserved slices of tissue in which cells could be observed at high resolution by CLSM (Fig. 1–3).

Clearing of plant tissues is necessary both to reduce the background of unspecific fluorescence and to increase the transparency of specimens for CLSM (Running et al. 1995; Gray et al. 1999; Kitin et al. 2000). Good results have been achieved with safranin-stained specimens of cambial tissue when they are passed through an ethanol series and then immersed in glycerol (Kitin et al. 2000). When samples are prepared in this way, cambium can be optically sectioned to a depth of 30–40 μm in the tangential, radial or transverse direction and the 3-D structure can be reconstructed. However, clearing of tissues with ethanol washes out much of the safranin dye from unlignified cell walls and this is not appropriate for observations by CLSM, in particular when lignified and unlignified cell walls are to be observed in a single specimen. By contrast, rinsing of safranin-stained tissues in acetone does not remove the dye from unlignified cell walls, as visualized with the naked eye (Kitin et al. 2002). Therefore, in the present study, we rinsed the safranin-stained specimens in acetone rather than ethanol, which resulted in fluorescent images with better contrast and less attenuation of fluorescent signals along the 'z' axis of specimens.

The diameters of vessel elements are much larger than those of fusiform cambial cells, which means that thicker slices of tissue must be scanned. Using our method, we were able to view optical sections of *Kalopanax pictus* to a depth of 144 μm or more from the surface of the sample (Fig. 1). The 3-D reconstruction of the optical sections revealed the large differentiating earlywood vessel elements (Fig. 2 & 3). We should note, however, that strong attenuation of fluorescent signals occurred in regions of

tissue in which cells were present at high density, such as the regions occupied by differentiating ray parenchyma, axial parenchyma and fibrous cells with small diameters. The attenuation of fluorescent signals was particularly pronounced at depths greater than 30 μm (Fig. 1). High-contrast images of all types of cell could be obtained only near the surface of specimens (Fig. 2A & B). The images in Figures 2C and 2D were obtained deeper inside the tissue of the specimen shown in Figures 2A and 2B and did not include fluorescence from cells with small diameters; only fluorescence from cell walls of vessel elements and vessel-associated cells was evident. Regions of tissue with cells with smaller diameters were likely to be more opaque than those with cells with larger diameters, perhaps because of a higher frequency of cell walls along the 'z' axis of optical sections. The absorption of laser light along the 'z' axis of specimens is a critical aspect of CLSM, being directly related to the problem of attenuation of

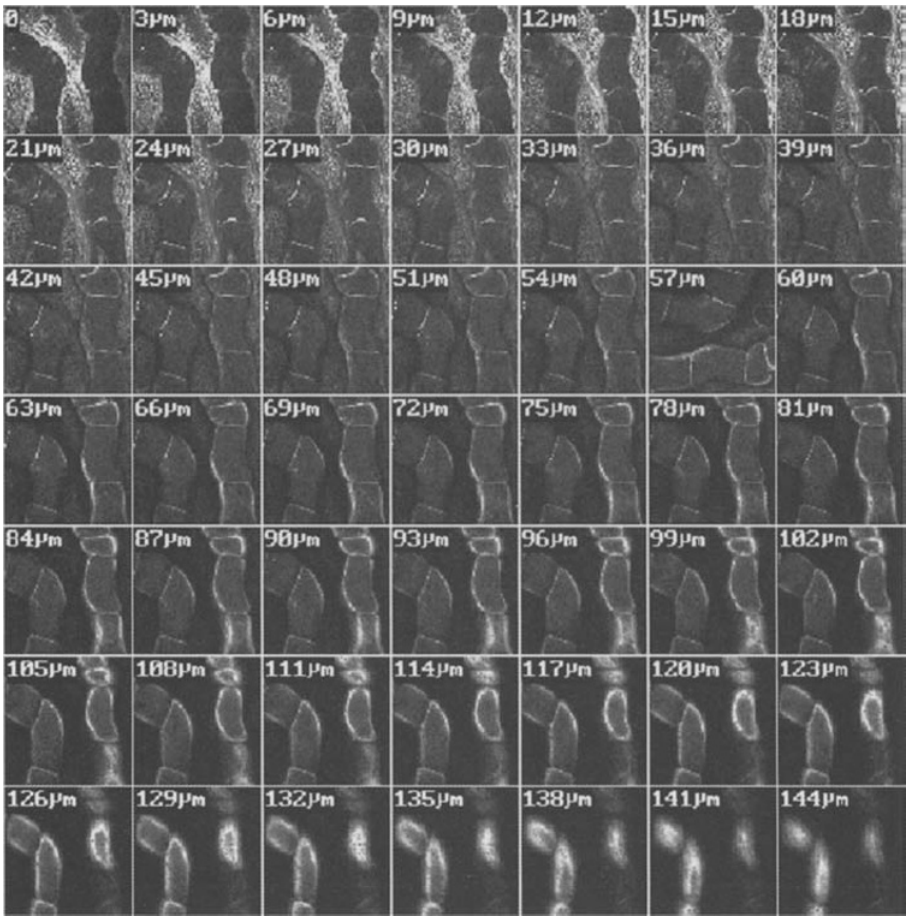

Fig. 1. Tangential optical sections at 3 μm intervals through differentiating xylem (from z = 0 through z = 144 μm). Incident light excitation by a helium neon laser (543 nm) with a long-pass filter (590 nm).

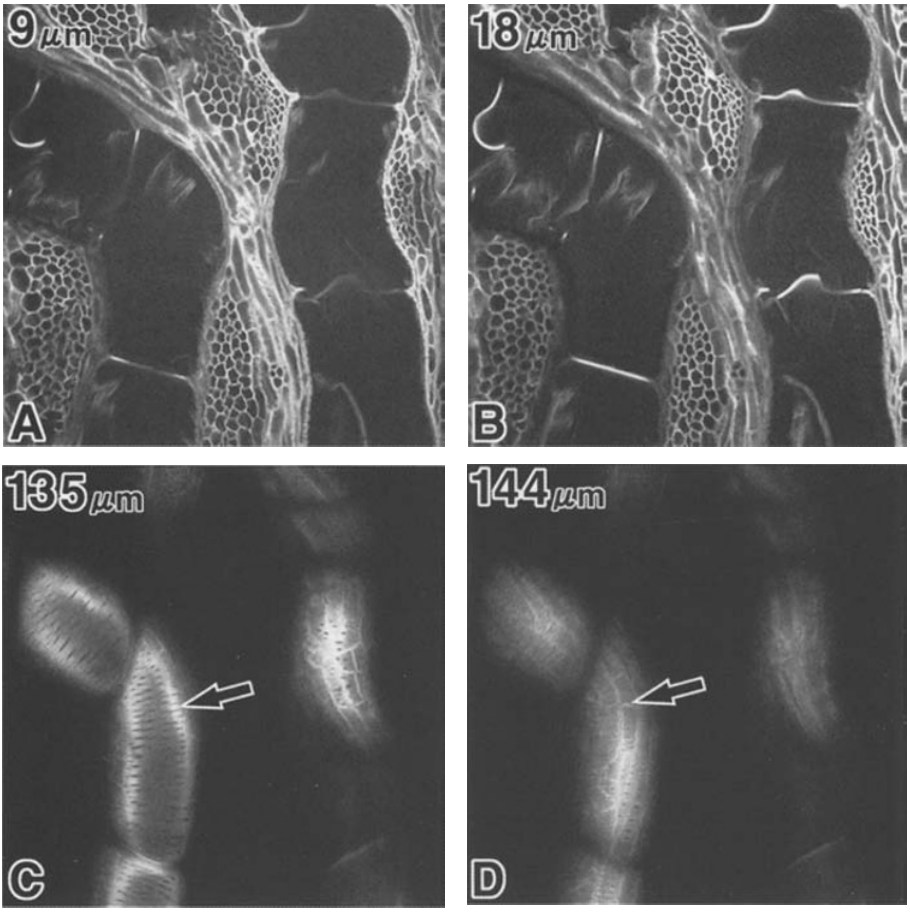

Fig. 2. Optical sections selected from the series shown in Figure 1. The depth of scanning by CLSM is indicated at the upper left corner in each image. At depths of 9 and 18 μm (A and B), images of all cells in the tissue can be clearly observed. In deeper images, however, attenuation of fluorescent signals is evident, especially in areas with a greater density of cells. For example, at depths of 135 and 144 μm (C and D), only the walls of large vessel elements and vessel-associated cells are visible. Note that cell walls can be examined on both sides. At a depth of 135 μm, the inner surface of the wall of the vessel element (arrow) is visible and at 144 μm, the contours of adjacent cells below the vessel element can be seen (arrow).

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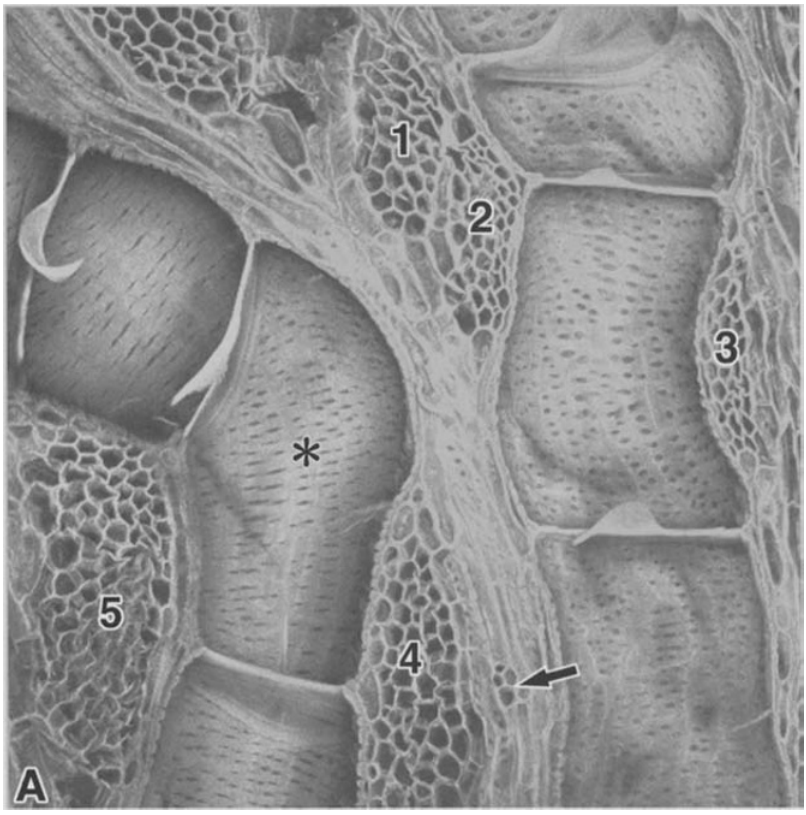
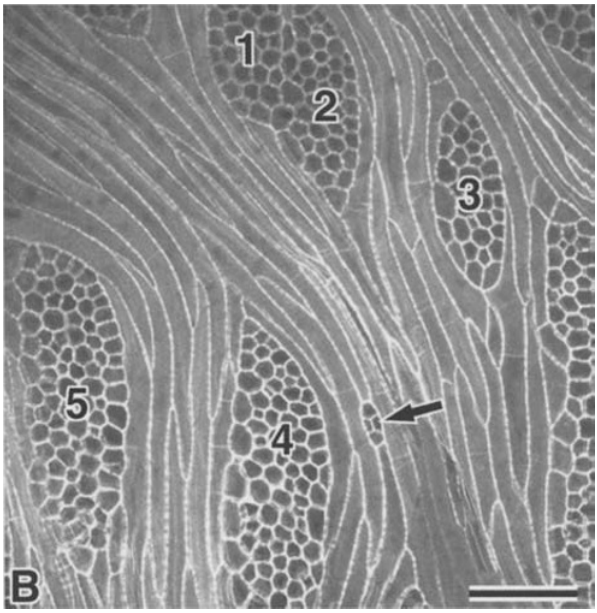
Fig. 3. CLSM images obtained from opposite sides of a thick, hand-cut section, showing a shift in the orientation of cambial fusiform cells and a corresponding shift in the orientation of vessel elements. The specimen was cut from a sample collected on May 11, after bud break. – A: Extended-focus image, 3-D rendering from the series shown in Figure 1. – B: Optical section of cambium obtained on the reverse side of the specimen shown in A, with scanning by CLSM at a depth of 18 μm from the surface of the specimen. Identical numbers in A and B indicate identical rays. The arrows in A and B show the same small ray, composed of four cells. The vessel element indicated by an asterisk in A was derived from cambial cells in the region between rays 4 and 5 in B. — Scale bar = 100 μm.





fluorescent signals (Donaldson & Lausberg 1998). Further investigations are required to determine the way in which the absorption of light depends on the method of staining and on properties of the tissue being examined, such as the dimensions of cells and the thickness and composition of cell walls.

Figures 1 and 2 demonstrate how the depth of optical sectioning by CLSM is limited by the attenuation of fluorescent signals. The structure of deeper parts of a specimen can be analyzed by mechanical serial sectioning with a microtome. However, such mechanical serial sectioning is time-consuming and the possibility of mistakes in 3-D reconstructed images is high, as discussed previously (Kitin et al. 2000). By contrast, the preparation of samples for CLSM was relatively easy and the 3-D structure of the intact tissue was preserved during optical sectioning. It has already been reported that CLSM yields high-quality images of optical sections through thick slices of tissue, which are similar to images of thin sections (thickness, 1–3 μm) of epoxy-embedded tissue (Kitin et al. 2000). Sequential optical sections can be stacked automatically with the computer software of the CLSM system to produce 3-D images. Furthermore, a specimen can be examined more than once if necessary.

Safranin was used for production of fluorescent images of plant cells by Donaldson and Lausberg (1998), Gray et al. (1999) and Kitin et al. (2000). Staining with safranin allows visualization of differentiating xylem and the high-contrast images obtained by CLSM reveal the shapes and dimensions of cells, as well as details of the structure of the cell walls at various stages of development. Figure 3A shows an extended-focus image of differentiating xylem obtained from the optical series shown in Figure 1. The image in Figure 3A provides a stereographic view of the structure, which is similar to images obtained by SEM. However, the images obtained by CLSM, constructed from serial optical sections, have the great advantage that they reveal structures that are not exposed on the surface of a specimen. Such visualization is impossible with a scanning electron microscope. For example, the arrow in Figure 2C points to the cell wall of a vessel element that is visualized from the lumen side. The same arrow in Figure 2D points to cell walls, which are positioned 9 μm beneath the cell wall of the vessel element in Figure 2C.

Another example of the visualization of the internal structure of a specimen along its 'z' axis is shown in Figures 3A and 3B. The image in Figure 3B, including a portion of cambium, shows the opposite side of the thick section in Figure 3A. The images in Figures 3A and 3B show identical radial files of cells of the same sample and reveal the developmental changes in cambial derivatives from the cambium to the xylem. The differentiating xylem in Figure 3A originated from the cambium in Figure 3B. Therefore, the differentiating xylem cells in Figure 3A mirror the arrangement and orientation of the cambial cells in Figure 3B. The deviation of a vessel from the axial orientation on the left side of the image in Figure 3A corresponds to a similar deviation in the orientation of fusiform and ray cambial cells in Figure 3B. Deviations of the orientation of individual vessels from the stem axis have been shown to occur in the structure of vessel networks in hardwoods (Zimmermann 1983; Kanai et al. 1996; Fujii & Hatano 2000; Fujii et al. 2001). The images in Figure 3 show that such deviations can be predicted from the pattern of arrangement of the fusiform and the ray cambial

cells. This observation confirms recent observations that indicate that a change in the inclination of cambial cells in *Picea* leads to a wavy grain in wood (Włoch et al. 2001).

Thick cell walls with pits were seen in the images of differentiating vessel elements and in the vessel-associated cells (Fig. 3A). The vessel elements had ceased to expand and were in the process of forming secondary walls with bordered pits and rims at the developing perforation plates. Although the secondary walls and the bordered pits were well-formed, the membranes of the perforation plates were present and no perforations were seen, an indication that the vessel elements were still developing at the time of sampling and they were not functional with respect to the transport of water. Secondary walls of ray parenchyma cells were not fully developed and they exhibited much weaker birefringence under polarized light than did other elements of the xylem (Fig. 4A & B). Many ray cells were deformed in the sections, perhaps because they had thin and unlignified cell walls. Murakami et al. (1999) demonstrated that secondary wall formation and lignification of isolation ray parenchyma cells is delayed compared to that of adjacent vessel elements in *Populus maximowiczii*. It is likely that the delayed formation of secondary walls of ray parenchyma cells, as compared to that of vessel elements, facilitates the readjustment of the position of the cells in the differentiating xylem that is a consequence of differences in the extent of expansion. Such readjustment of the position of cells and, in particular,

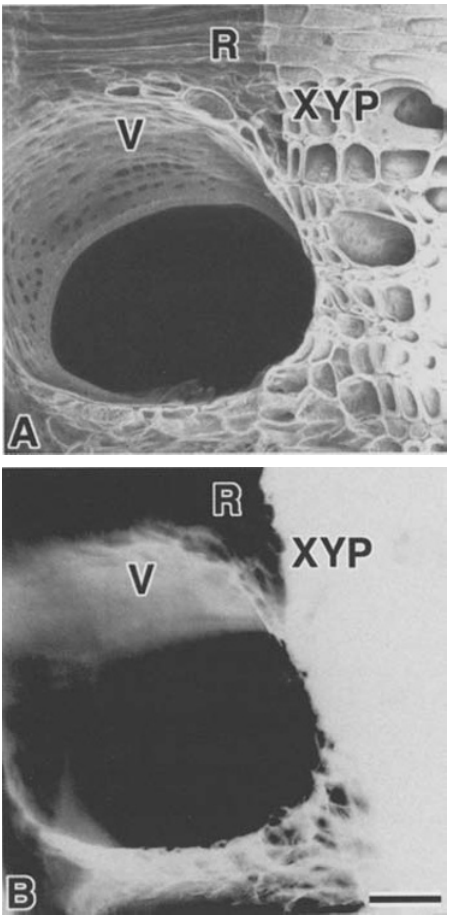


Fig. 4. Images of a thick, hand-cut transverse section, showing the development of an early-wood vessel element and adjacent cells. The specimen came from a sample collected on May 9, after bud break. – A: Extended-focus image, 3-D rendering from 50 optical sections obtained at 3 μm intervals. – B: Polarised-light image of the same section as in A. The xylem cells of the previous annual ring (XYP) and the cell wall of the developing vessel element (V) were strongly birefringent but the differentiating ray cells (R) adjacent to the vessel element were not. — XYP = xylem cells of the previous annual ring; R = differentiating ray cells; V = differentiating vessel element. – Scale bar = 50 μm.

the repositioning of rays, can be seen by comparing the images in Figures 3A and 3B. These images, obtained at the same magnification, show the same rays of the specimen along its 'z' axis (rays indicated by numbers 1 through 5 in Fig. 3). The area occupied by the same differentiating xylem cells in the image in Figure 3A is much larger than that in Figure 3B. In particular, the space between rays is much greater because of the expansion of the new vessel elements.

Our study showed that the arrangement of differentiating xylem cells corresponded to that of their mother cells in the cambium. In addition, vessel elements, fibres, axial parenchyma cells and ray parenchyma cells in the differentiating xylem were visualized at various stages of differentiation. The development of vessel elements was more advanced than that of the other xylem elements, indicating that the time required for maturation differs among the various types of element and, possibly, that the differentiation of various elements is initiated at different times. The biological significance of the sequence in which differentiation of the various xylem elements occurs is still not clearly understood. More studies are needed to clarify the programmed spatial and temporal pattern of differentiation of the xylem cells. Although this problem is difficult to study, CLSM, which allows 3-D analysis of the structures of tissues together with high resolution of images along the 'z' axis, should help expand our understanding of the development of xylem.

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