Journal of Plant Biology, September 2001, 44(3) : 148-156

The Origin and Development of Vascular Cambium in Girdled Stems of *Eucommia ulmoides* Oliv.

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We conducted anatomical studies of girdled stems of *Eucommia ulmoides* at various developmental stages to elucidate the origin and development of callus and the vascular cambium. In the transverse view, ray initial cells in the cambial zone began to divide both periclinally and anticlinally 2 d after girdling. Fusiform initial cells started to enlarge at 3 d, then gradually proliferated via periclinal divisions. Thus, the callus was derived from the ray initial cells of the cambial zone as well as from fusiform initial cells. In the tangential view, callus cells derived from ray initial cells were short while those from fusiform initial cells were long, thereby producing a heterogeneous structure. However, the fusiform initial cells underwent transverse divisions 10 d after girdling, which resulted in shorter cells and a homogeneous callus structure. Afterward, some short cells divided transversely while others elongated, so that a heterogeneous form was regained. Finally, the vascular meristem that was girdled early in its development redifferentiated from short and long cells in the callus. The long cells developed into fusiform initials, with the short ones becoming ray initials.

Keywords: callus, *Eucommia*, fusiform initial, ray initial, vascular cambium

When a tree stem is girdled, a callus forms. Afterward, the vascular cambium begins to differentiate. Warren Wilson and Grange (1984) have identified three phases of regeneration after wounding: 1) the initial phase (lag phase), when no cell division or enlargement is occurring; 2) the division phase, with cell division and enlargement; and 3) the differentiation phase, when tissues or cambia are differentiated. Artificial debarking causes callus formation and differentiation of the vascular cambium and secondary vascular tissues (Dobbins and Fisher, 1986; Fisher and Ewers, 1989). In addition, external pressure can affect differentiation of the vascular cambium (Brown and Sax, 1962; Brown, 1964).

Although the role of immature xylem is negligible (Sharples and Gunnery, 1933), calli develop from immature cells of vascular tissue that already existed on the surface of the exposed vascular cambium. Calli may arise from any of the undifferentiated centripetal products of the vascular cambium; the type of tissue contributing to callus initiation depends on species and the histology of the cambial zone (Noel, 1970). However, theories about the origin of callus formation are contradictory. For example, in a study of *Pinus* stems, Brown and Sax (1962) observed that the new cambium began to differentiate through the callus parenchyma in the fourth week after debarking. Likewise, new vascular cambium was formed within 45 d of girdling in *Fulbernardia*, 76 d in *Trema*. In fully girdled stems of *Arabidaea chica*, new, continuous vascular cambium also differentiated within the massive callus (Dobbins and Fisher, 1986). In contrast, Li and Cui (1988) have studied transverse sections from girdled stems of *Eucommia*. Within the first week, ray cells of the immature xylem swelled, proliferated, and spread laterally under the surface layer. They gradually joined together with neighboring ray cells and were found with other cells derived from the immature xylem. Soon afterward, a cork layer arose near the surface and a newly formed cambium appeared deep within the callus.

These conflicting reports demonstrate that the development of calli in girdled stems has likely been misstated, and that the origin of these vascular cambium initials within the callus has not been studied thoroughly. Therefore, our objective was to determine the origin of callus and vascular cambial initials in girdled *Eucommia ulmoides* stems.

MATERIALS AND METHODS

We used a chisel and a grafting knife to remove

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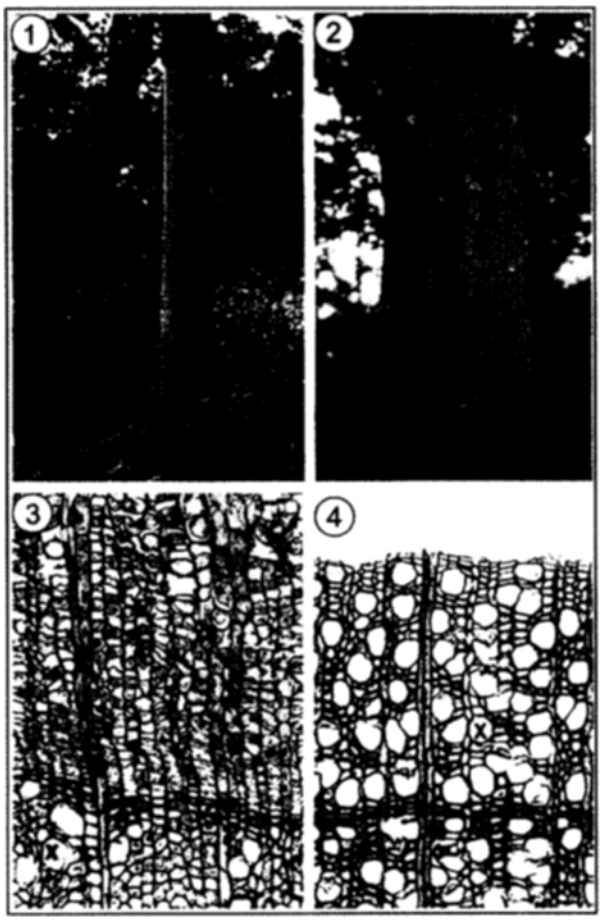


Figure 1. External morphology of *E. ulmoides* stem immediately after girdling. **2.** External morphology with bark formation, 31 d after girdling. **3.** Transverse sections showing secondary xylem (X), cambial zone (CZ), and secondary phloem (P) of intact *Eucommia* stem. Arrow indicates phloem parenchyma and companion cell. R, ray cells; 110X. **4.** Transverse sections of exposed xylem surface on the girdled *Eucommia* stem. Calli were not observed here; 110X.

strips of bark (100 × 47 cm) from the main trunks of 10-year-old *E. ulmoides* Oliv. trees growing in the Hanlim garden in Chungbuk, Korea (Fig. 1). Immediately after being girdled, the trunks were wrapped with a semi-transparent plastic sheet. At various stages during regeneration, we chiseled 1 cm^3 sample blocks from the girdled trunks (Fig. 2). These blocks were fixed in FAA and embedded in paraplast, following routine procedures. We then used a rotary microtome to obtain three-dimensional, 10 μm thick sections from each block. Serial sections were stained with hematoxylin, safranin, and light green (Sass, 1971); then mounted in Canada balsam and observed by light microscope (Leitz Wetzlar, Germany).

RESULTS

Structure of the Vascular Cambium and Its Derivative Tissues in Intact Stems

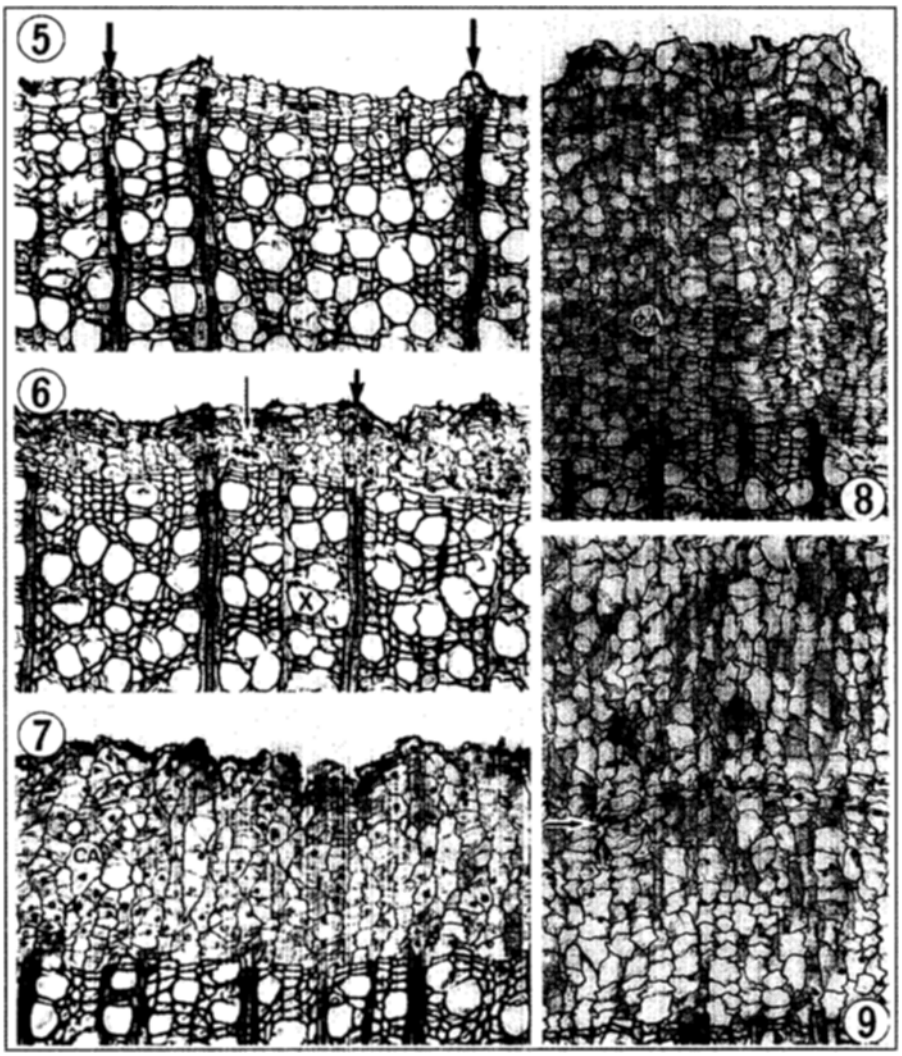
In the transverse view, xylem was situated inside the vascular cambium, with phloem outside the meristem. The xylem was composed of axial parenchyma, vessels, fibers, and ray cells; the phloem comprised sieve tube members with companion cells, phloem parenchyma, successively parallel fibers, and ray cells (Figs. 3 and 4). The cambial zone of the girdled stem was partially eliminated (Fig. 4) but the remaining portion contained one to three cells in a radial file. In the tangential view, fusiform initials averaged 507 μm long. Ray initials were one to seven cells wide and 20 cells (182 μm) long (Fig. 16).

Callus Formation on Girdled Trunks

In the transverse view, the remnant vascular cambial zone was approximately 30 μm thick on the second day after girdling. Ray initial cells in the cambial zone enlarged and protruded earlier than did the fusiform initial cells, which had begun to enlarge without division. Callus was forming on all the preexisting surfaces outside of the vascular cambial zone. This callus surface had a protective layer, and was white on the entire exposed trunk (Fig. 5).

In the tangential view, the callus consisted of ray initial cells that had enlarged and proliferated within the cambial zone. However, fusiform initial cells had not begun to divide by 2 d after girdling, although most of those with prominent nuclei were ready (Fig. 17). Nevertheless, at 3 d post-girdling, enlargement and divisions had resulted in isodiametric ray initial cells, with cells extending into peripheral areas. Fusiform initial cells were shortened through active anticlinal and periclinal divisions, being transformed into globular or isodiametric cells. Their end walls were transverse (Fig. 18). In the radial view, the preexisting ray initial cells in the cambial zone proliferated by periclinal division. However, fusiform initial cells were shortened by periclinal and anticlinal divisions (Fig. 29).

At 4 d post-girdling, the callus was 340 μm thick in the transverse view. The number of cells originating from preexisting ray initial cells in the cambial zone increased through division and enlargement. Fusiform initial cells in the cambial zone increased notice-

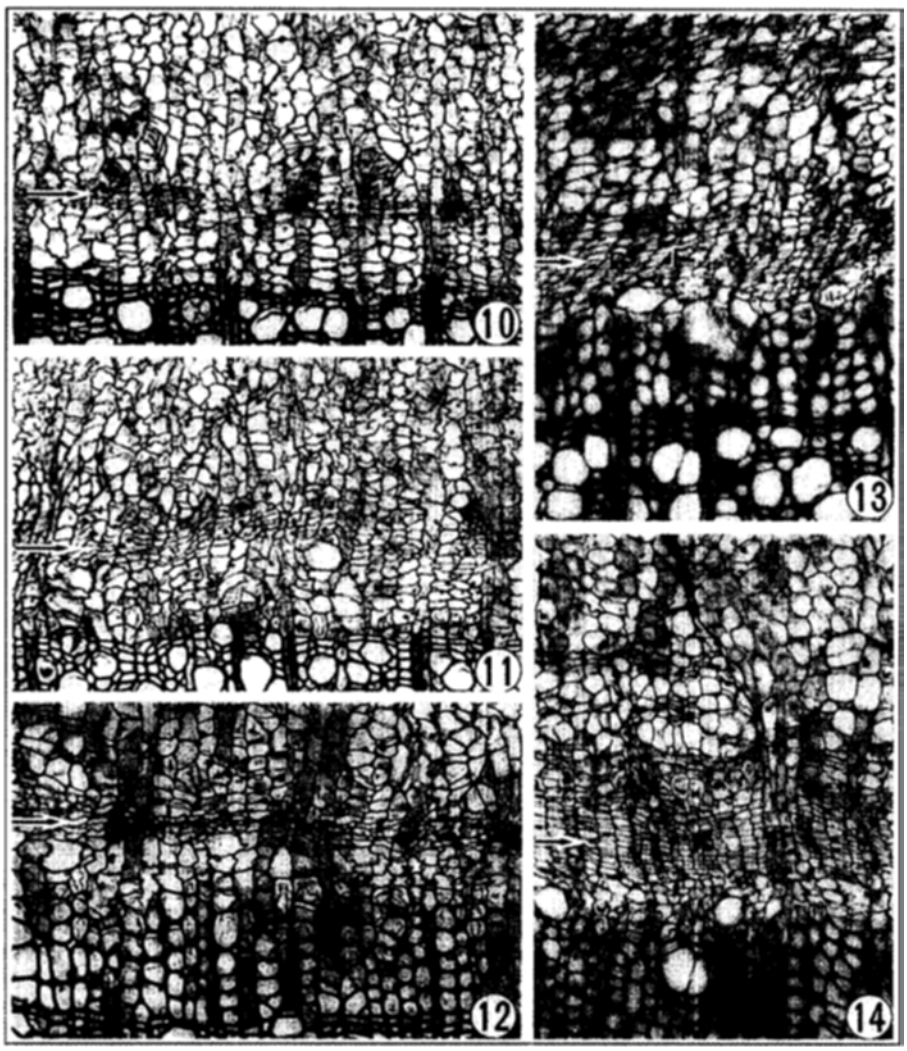


Figures 5-9. Transverse sections of calli from *Eucommia* stems at 2 d (Fig. 5), 4 d (Fig. 6), 6 d (Fig. 7), 10 d (Fig. 8), and 13 d (Fig. 9), respectively, after girdling (all at 110X except where noted). **5.** Enlargement of the cambial zone ray cells (arrows). **6.** Division and proliferation of axial parenchyma cells (long arrow) in cambial zone, and enlargement and proliferation in the cambial zone ray cells (short arrow). **7.** Isodiametric callus cells (CA). **8.** Repeated periclinal divisions of callus cells (CA). **9.** 1 to 4 cells in each radial file in the early vascular meristem stages (arrow); 130X.

ably simply via divisions, with some beginning to divide after enlargement (Fig. 6). In the tangential view, ray initial cells swelled and developed a globular shape through active proliferation. Fusiform initial cells shortened with more frequent divisions. Although most of the cells had transverse end walls (Fig. 19), some were globe-ended. Their cell arrangements became more irregular by gradual transformation (Fig. 7). In the radial view (Fig. 30), fusiform initial cells divided repeatedly through periclinal and anticlinal divisions, while ray initial cells proliferated and enlarged.

Eight days after girdling, the tangential view (Fig. 20) showed that cells originating from ray initial cells within the callus were dividing repeatedly. Fusiform initial cells also divided successively, being transformed into short cells that were only slightly longer than those originating from ray initial cells. The end walls of cells originating from fusiform initial cells were transverse, while ray cells had globe-ended walls.

At 10 d post-girdling, isodiametric cells were regularly arranged in the transverse view. These cells were 650 μm thick. Because cells had become isodiametric, the origin of the parenchyma cells could not be determined in this phase. In contrast, cells located deep in the callus divided periclinally (Fig. 8). There-



Figures 10-14. Transverse sections of calli from on *Eucommia* stems at 16 d (Fig. 10), 19 d (Fig. 11), 22 d (Fig. 12), 28 d (Fig. 13), 31 d (Fig. 14), respectively, after girdling. **10.** Discontinuous structure with 1 to 6 cells in radial file (arrow) at the mid-developmental stage of the vascular meristem; 110X. **11.** Continuous cambium with 2 to 8 cells in each radial file at the late of the vascular meristem stages (arrow) and in lignified secondary xylem; 130X. **12.** Cambium with 2 to 10 cells in each radial file (arrow) by repeated periclinal division; 130X. **13.** Cambium with 7 to 15 cells in each radial file, and newly formed ray initials (R) and fusiform initials (F) in the completed vascular cambial zone (arrow); 110X. **14.** Completed vascular cambium and its derivatives after girdling (arrow); 110X.

fore, this callus tissue had a homogeneous structure that consisted of globular or isodiametric cells (Fig. 21). In the radial view, the callus was composed of both longer and shorter isodiametric cells (Fig. 31).

Regeneration of the Vascular Cambium

Redevelopment of the vascular meristem occurred in four stages, delineated by the number of days after girdling: 1) initial stage (13 d); 2) mid stage (16 d); 3) late stage (19 d); and 4) final stage (25 to 28 d).

Initial Stage

At this stage, the callus was 800 μm thick in the transverse view. Cell arrangements were more regular in some portions than had been seen in the previous phase of callus formation. Ray parenchyma within the callus were easily observed because they were angled and deeply stained (Fig. 9). In the tangential view, the initial vascular meristem teemed with cytoplasm within the callus, with the deeply stained cells averaging 63 μm long. The vascular meristem, transformed

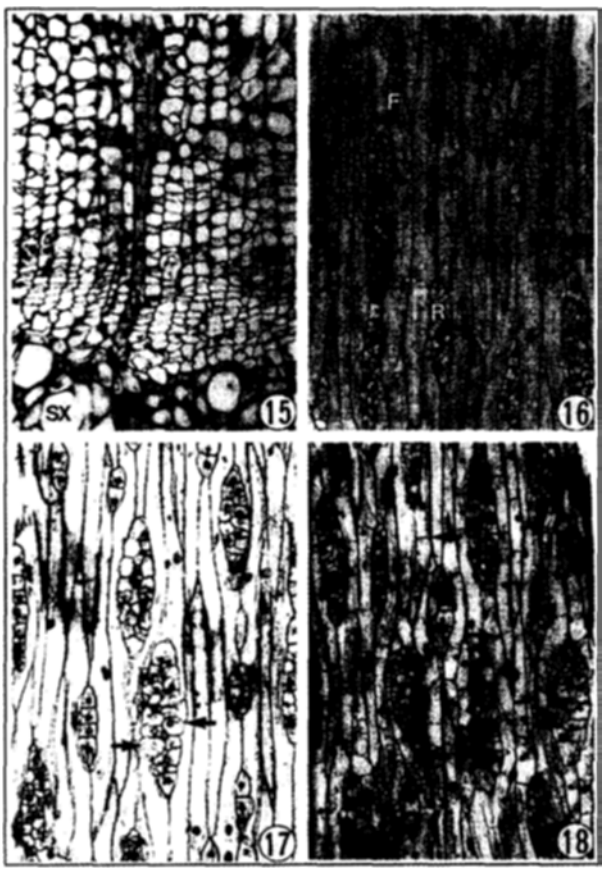
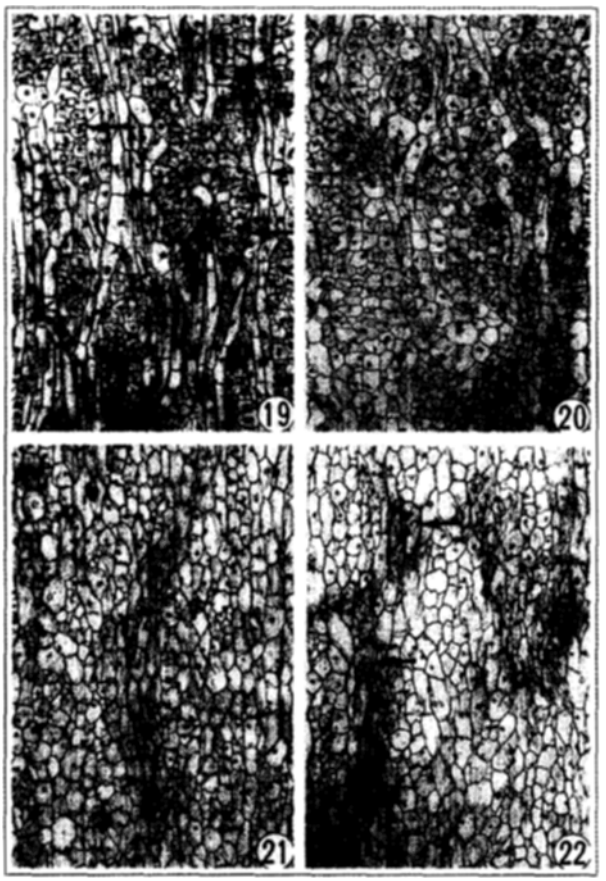


Figure 15. Transverse sections of callus from *Eucommia* stem, 34 d after girdling; vascular cambium with 6 to 11 cells in each radial row formed by periclinal divisions in fusiform initial cells (F) and ray initial cells (R); SX, secondary xylem; 220X. **16.** Tangential sections of the cambial zone of an intact stem of *Eucommia*, composed of fusiform and ray initial cells; 200X.
Figures 17-18. Tangential sections of *Eucommia* at 2 d (Fig. 17) and 3 d (Fig. 18) after girdling. **17.** Vascular cambium ray initial cells showing enlargement and proliferation (arrows). **18.** Initiation of periclinal division of the fusiform initial cells (arrows); 130X.

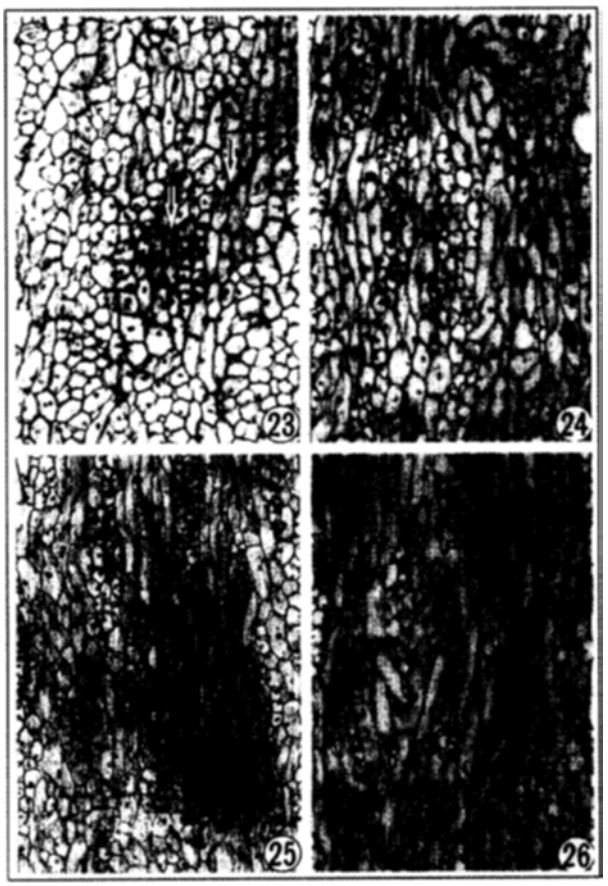


Figures 19-22. Tangential sections of *Eucommia* stems at 4 d (Fig. 19), 8 d (Fig. 20), 10 d (Fig. 21), and 13 d (Fig. 22), respectively, after girdling (all at 110X except where noted). **19.** Gradual callus formation by repeated transverse division of cambial zone axial parenchyma cells in the cambial zone, and division of ray cells. **20.** Callus consisting of somewhat isodiametric cells. **21.** Callus showing an approximately homogeneous structure. **22.** Elongating cells in the early vascular meristem stages; 130X.

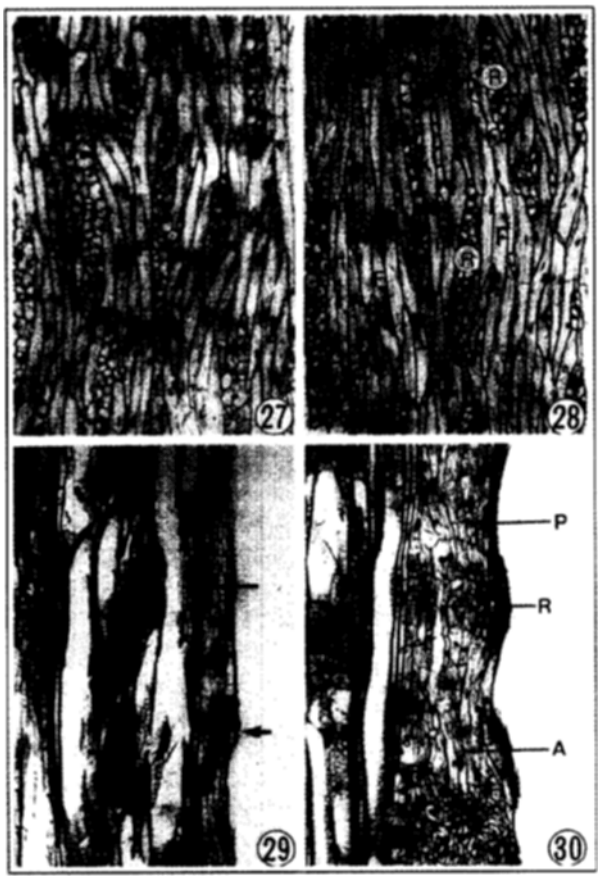
into homogeneous structures comprising global parenchyma cells, also had clear nuclei and was stained green (Fig. 22). In the radial view, a flattened and discontinuous vascular meristem, 150 to 180 μm outside of the preexisting xylem, showed a homogeneous structure composed of short cells. In addition, the vascular meristem consisted of 1 to 3 radially flattened rows of cells that differentiated discontinuously (Fig. 32).

Mid Stage

During the mid stage, the vascular meristem comprised 1 to 6 cells found in a radial file about 175 μm from the xylem. These meristematic cells were arranged discontinuously. The outermost callus had begun to develop cork cambium, and a few phloem ray parenchyma cells also were observed (Fig. 10). In the tangential view, ray parenchyma cells of the vascular meristem were short and clustered. The vascular meristem at this stage showed a heterogeneous structure composed of long and short cells (Fig. 23). Short cells averaged 50 μm in length; long cells were 75 μm long. Their end walls were transverse or tapered. In the radial view, the cambial zone consisted of 1 to 6 cells in each radial file, which resulted from repeated periclinal divisions. The flattened cells in these radial



Figures 23-26. Tangential sections of *Eucommia* stems at 16 d (Fig. 23), 19 d (Fig. 24), 22 d (Fig. 25), and 28 d (Fig. 26), respectively, after girdling (all at 130X). **23.** Partial elongation of some cambial cells (arrows). **24.** Further elongation of fusiform initials, and initiation of the formation of ray initials in late cambial stages. **25.** Continued elongation of fusiform initials and formation of ray initials at the final developmental stage of vascular cambium. **26.** Complete, newly formed vascular cambium composed of fusiform initials and ray initials.



Figures 27-28. Tangential sections of *Eucommia* at 31 d (Fig. 27) and 34 d (Fig. 28), respectively, after girdling. Note the thickening and elongation of fusiform initials and ray initials in the vascular cambium; 130X.

Figures 29-30. Radial sections of *Eucommia* 3 d (Fig. 29) and 4 d (Fig. 30) after girdling. **29.** Origin of callus by periclinal divisions of the fusiform initial (long arrow) in the cambial zone, and proliferation and enlargement of ray initial (short arrow); 110X. **30.** Callus forming by repeated periclinal (P) and anticlinal (A) divisions of the fusiform initial in the cambial zone, and proliferation and enlargement of ray parenchyma cells; 130X.

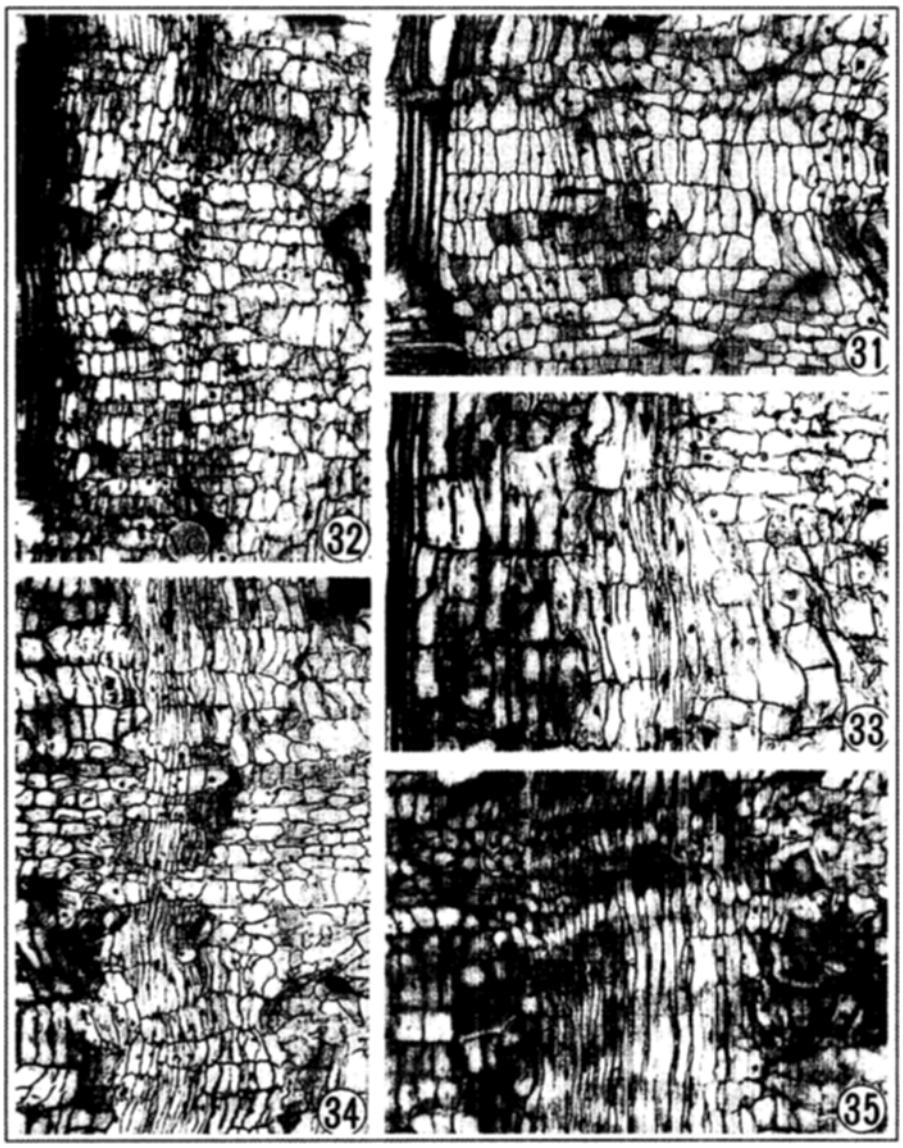
rows had clearer nuclei than those inside or outside the vascular meristem. This meristem developed while the xylem was thickening, at which time the phloem also was beginning to differentiate.

Late Stage

In the transverse view, a more or less continuous vascular meristem zone differentiated outside the preexisting xylem. Activity in this meristem caused the secondary phloem to develop. Ray cells within the secondary phloem began to form more extensively near the epidermis. Callus formation and lignified xylem differentiated simultaneously into the inner side of the vascular meristem (Fig. 11). In the tangential view, the long cells were 128 μm in length, with tapered end walls and a clearly heterogeneous structure (Fig. 24). In the radial view, cells of the vascular meristem were reduced in size due to anticlinal divisions. This continuous meristem was composed of 2- to 8 cells in a radial file. At this stage, the xylem was also beginning to thicken and lignify. Phloem ray cells also were observed (Fig. 33).

Final Stage

In the tangential view of samples after vascular mer-



Figures 31-35. Radial sections of callus on *Eucommia* stems at 10 d (Fig. 31), 13 d (Fig. 32), 19 d (Fig. 33), 28 d (Fig. 34), 34 d (Fig. 35), respectively, after girdling. **31.** Shows process of isodiametric callus (CA) formation, callus derived from fusifom initials by periclinal division (long arrow) and originated from the ray initials (short arrow); 110X. **32.** Initial stage of vascular meristem (VC) within the callus; 130X. **33.** Late stage of vascular meristem (arrow); 200X. **34.** Newly formed vascular cambium; 130X. **35.** Shows fusiform initials (F) and ray initials (R) in vascular cambium; 110X.

istem development was completed, the ray initials were 1 to 4 cells wide and 1 to 16 cells long (Figs. 25 and 26). Fusiform initials had reached lengths of 209 μm through continuous elongation. These cells were arranged only somewhat regularly. The walls of the ray initial cells became thickened and the end walls were a little more tapered (Fig. 26). In the radial view, the vascular meristem had developed into a zone comprising 4 to 13 cells in a radial file. The xylem was thick and lignified, and vessel elements with pits were observed. Ray cells of the xylem were composed of erect and procumbent cells. In addition, the phloem showed sieve tubes and ray cells (Fig. 34). The callus was 1050 μm thick in the transverse view. A vascular cambial zone found outside the xylem was clearly composed of both fusiform and ray initials. Secondary phloem developed outside of the vascular cambium through cambial activity. Likewise, a lignified and thickened secondary xylem was added 10 μm inside the vascular cambium (Figs. 12 and 13).

Activity of the Vascular Cambium

In the tangential view, the radial rows within the vascular cambium zones were decreased through cambial activity. Intrusive growth occurred in multiseriate ray initials. At 31 d after girdling, fusiform initials were 250 μm long (Fig. 27); at 34 d, their average length was 270 μm (Fig. 28). In the radial view, ray initial cells were composed of upright and procumbent cells. Cambial activity also added 3 to 4 radial files in the secondary xylem and phloem. Vessel elements in the secondary xylem were differentiated, while sieve tubes and ray cells were observed in the secondary phloem. Notably, sclereids partly formed during this phase (Fig. 35).

In the transverse view, the callus was 1100 μm thick at 31 d post-girdling. The vascular cambium was composed of 4 to 14 cells in radial files (Fig. 14). In contrast, at 34 d, the thickness of the callus was 1400 μm, with the radial files comprising 4 to 8 cells. Cambial activity caused secondary phloem to differentiate, which consisted of sieve tubes with companion cells, phloem parenchyma, and ray cells. This activity also effected a secondary xylem composed of axial parenchyma, vessels, fiber tracheids, and ray cells inside the vascular cambium (Fig. 15).

DISCUSSION

Based on our tangential views, we propose that in the initial phase of callus formation, cells that originate from cambial zone axial parenchyma are long and have transverse end walls. Those originating from ray cells are isodiametric and short. This produces a callus with a heterogeneous structure. In contrast, the late stage of formation finds a callus that has been transformed into a homogeneous structure composed of isodiametric cells. However, this differentiating timing for inducing homogeneous structures varies by species (Warren Wilson and Pamela, 1961, 1984; Brown and Sax, 1962; Noel, 1968; Sussex et al., 1972; Roberts and McCully, 1979; Dobbins and Fisher, 1986; Li and Cui, 1988).

When the vascular cambium began to differentiate, homogeneous structures with short cells were transformed into heterogeneous structures with either short or long cells. Hence, the sizes of the cambial zone ray cells and axial parenchyma were reduced when cell pressure was eliminated. Kang and Soh (1993) demonstrated that when the procambial cell structures, initiated in *Eucommia* hypocotyls, were compared with the newly formed vascular cambium found in girdled stems, both possessed homogeneous structures with short cells during the initial phase of procambium development. In the late stage, both hypocotyls and girdled stems had the same characteristic heterogeneous structure composed of both short and long cells. In contrast, the structure found in *Eucommia* hypocotyls during the initial stage of vascular cambium development was similar only to that found in girdled stems during the late stages.

After girdling, the procambium cells from hypocotyls were longer than the fusiform initials from the newly formed vascular cambium. Likewise, during the initial phase, end walls were of two types: one transverse, the other globed. Nevertheless, in the late stage, the end walls had similar shapes. However, the vascular cambium from the *Eucommia* girdled stems, without undergoing the process of procambium differentiation, developed directly from either the parenchyma cells or the callus. This has also been reported by Swamy and Krishnamurthy (1980) and Iqbal (1990). In addition, these vascular tissues originated not from the vascular meristem but directly from the callus. This finding confirms that external pressure alone could inhibit cell proliferation, thereby preventing cambial regeneration in callus tissue, and bringing about secondary wall thickening in the callus parenchyma (Brown, 1964).

In most cases, the derivatives from the ray initials are the first to show cellular enlargement and active division. Although some of the fusiform derivatives do separate and undergo repeated periclinal divisions, they contribute relatively little to the formation of callus, compared with the rapidly proliferating ray cell derivatives (Brown and Sax, 1962; Brown, 1964; Fahn 1982). However, we found that the ray cells divided three days earlier than did the immature xylem axial parenchyma. This was because of its advantageous divisibility, which played an important role in initial callus formation. Nevertheless, the axial parenchyma took the lead role in callus formation 3 d after girdling. This indicates a basic difference in the origin of callus formation. Based on transverse views from the initial stages, the radially flattened procambium cells from *Eucommia* hypocotyls, exhibiting 1- to 2 cells in radial files (Kang and Soh, 1993) apparently are smaller than those from girdled stems. This difference in cell numbers is probably caused by activity of the vascular cambium. Cells enlarged in the ray cells and axial parenchyma cells of immature xylem 1 d after girdling. This was also demonstrated by Brown and Sax (1962). However, Warren Wilson and Pamela (1984) have indicated an absence of cell

enlargement. It is considered that these different views have caused by activity of cell division and enlargement, and remaining cell layer and tissue type. Cells derived from axial parenchyma are divided at 3 days after girdling. These results coincide with those found by Warren Wilson and Grange (1984) and Warren Wilson and Pamela (1984). At 4 d post-girdling, the callus cells increased geometrically but individual cell size was reduced through divisions, an observation that also agrees with that of Warren Wilson and Pamela (1984).

Received July 11, 2001; accepted September 12, 2001.

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