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The origin and early evolution of tracheids in vascular plants: integration of palaeobotanical and neobotanical data

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Although there is clear evidence for the establishment of terrestrial plant life by the end of the Ordovician, the fossil record indicates that land plants remained extremely small and structurally simple until the Late Silurian. Among the events associated with this first major radiation of land plants is the evolution of tracheids, complex water-conducting cells defined by the presence of lignified secondary cell wall thickenings. Recent palaeobotanical analyses indicate that Early Devonian tracheids appear to possess secondary cell wall thickenings composed of two distinct layers: a degradation-prone layer adjacent to the primary cell wall and a degradation-resistant (possibly lignified) layer next to the cell lumen. In order to understand better the early evolution of tracheids, developmental and comparative studies of key basal (and potentially plesiomorphic) extant vascular plants have been initiated. Ultrastructural analysis and enzyme degradation studies of wall structure (to approximate diagenetic alterations of fossil tracheid structure) have been conducted on basal members of each of the two major clades of extant vascular plants: *Huperzia* (Lycophytina) and *Equisetum* (Euphyllophytina). This research demonstrates that secondary cell walls of extant basal vascular plants include a degradation-prone layer ('template layer') and a degradation-resistant layer ('resistant layer'). This pattern of secondary cell wall formation in the water-conducting cells of extant vascular plants matches the pattern of wall thickenings in the tracheids of early fossil vascular plants and provides a key evolutionary link between tracheids of living vascular plants and those of their earliest fossil ancestors. Further studies of tracheid development and structure among basal extant vascular plants will lead to a more precise reconstruction of the early evolution of water-conducting tissues in land plants, and will add to the current limited knowledge of spatial, temporal and cytochemical aspects of cell wall formation in tracheary elements of vascular plants.

Keywords: tracheid; xylem; *Huperzia*; *Equisetum*; cell wall; developmental evolution

1. INTRODUCTION

One of the most significant sets of evolutionary events in the history of life on Earth was the migration of complex life forms from water-based environments to land in the Early Palaeozoic. These events began with the migration of aquatic photosynthetic organisms on to land some 475 Myr ago and resulted in a veritable explosion of evolutionary innovation and consequent diversification of terrestrial ecosystems.

Prior to the input of significant energy into terrestrial ecosystems by land plants (embryophytes), terrestrial environments were occupied by various heterotrophic and autotrophic bacteria, protists, fungi, lichens and some simple algae (Gray & Shear 1992; Gray 1993; Knoll 1994; Kenrick & Crane 1997*a*,*b*). Within 75 Myr of the origin of land plants, terrestrial photosynthetic organisms (embryophytes) had undergone a major evolutionary radiation and established conditions for the colonization and subsequent diversification of various metazoan lineages on land. Some 150 Myr after the origin of land plants, the surface of the Earth was dominated by the highly diverse Carboniferous forest ecosystems—a radical change from the pre-Ordovician bacterial, fungal and algal biotic crusts.

Photosynthesis in an aerial environment requires high levels of gas exchange (uptake of CO_2) and is facilitated in the sporophytes of all land plants, with the exception of liverworts, by the formation of stomatal openings in the outer surface of the plant body. These pores, however, also result in significant losses of gas-phase water from the internal tissues of the plant body to the external environment. As a consequence, to remain hydrated terrestrial plants larger than a few centimetres require specialized tissues to transport water from the plant–soil interface to the aerial portions of the plant body (Raven 1993). Biophysical models clearly indicate (Raven 1984, 1993) that any significant increase in the stature of land plants was predicated on the evolution of highly specialized hollow and dead water-conducting cells, whose rates of conductance have been calculated to be 1×10^7 times greater than equivalent living cells with cytoplasmic contents (Raven 1984, 1993).

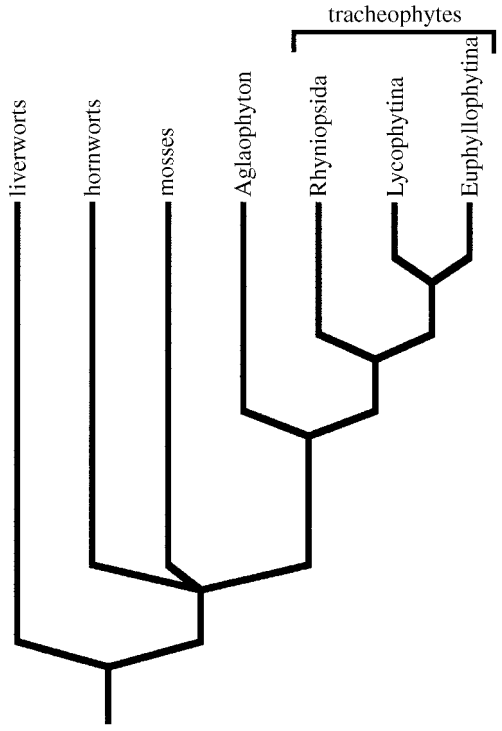

Figure 1. Phylogenetic relationships of land plants. Evidence based on intron distribution indicates that liverworts are the sister group to all other land plants (Qiu *et al.* 1998). The interrelationship of hornworts and mosses is unresolved. *Aglaophyton* does not have tracheids with secondary thickenings, and is hypothesized to be a close (extinct) relative of tracheophytes or vascular plants. Rhyniopsida (all extinct) are hypothesized to be the sister group to the Lycophytina and Euphyllophytina, which together comprise the eutracheophytes.

It has long been known that certain members of the liverworts and mosses possess elongate, non-lignified cells that are dead at maturity and may function in water conduction (Hébant 1977; Scheirer 1980; Mishler & Churchill 1984, 1985; Ligrone & Duckett 1996; Renzaglia *et al.* 1997; Ligrone *et al.*, this issue›). However, much remains to be learned of the structure, development, function and evolutionary homologies of these cells. What is abundantly clear is that the fossil record documents that land plants remained extremely small and structurally simple until the Late Silurian, *ca.* 425 Myr ago (Gensel & Andrews 1987; Gray & Shear 1992). This first burst of structural diversification among land plants was associated with the evolution of tracheids (Knoll & Rothwell 1981; Knoll & Niklas 1987; Raven 1993; Kenrick & Crane 1997*a*), developmentally complex water-conducting cells defined by the presence of secondary cell walls, lignification and programmed cell death.

2. TRACHEIDS OF THE EARLIEST VASCULAR PLANTS

Recent phylogenetic analyses indicate that tracheid-bearing plants (tracheophytes) are monophyletic (figure 1) and defined by the presence of secondary cell wall thickenings in water-conducting cells (Kenrick & Crane 1997*a*,*b*). Evolutionary biologists (Banks 1975; Gensel & Andrews 1987; Taylor & Taylor 1993; Kenrick & Crane 1997*a*,*b*) have hypothesized that a rapid diversification among early vascular plants (tracheophytes) produced three major clades (figure 1), each of which is characterized by a particular tracheid type among its earliest members (Kenrick & Crane 1997*a*,*b*).

Rhyniopsida is hypothesized to be the sister group to a monophyletic eutracheophyte clade that includes all extant vascular plants, as well as many of their extinct relatives (figure 1; Kenrick & Crane (1997*a*,*b*) and references therein). Members of the Rhyniopsida, all of which are extinct, are characterized by the presence of S-type tracheids (after the genus *Sennicaulis*). Two monophyletic lineages have been recognized within the eutracheophyte clade (figure 1): the Lycophytina (lycophytes and their extinct ancestors, the zosterophylls), whose earliest members have G-type tracheids (after the zosterophyll genus *Gosslingia*); and the Euphyllophytina (the extinct trimerophytes, eusporangiate ferns, leptosporangiate ferns, sphenopsids, Psilotaceae, progymnosperms and seed plants), whose earliest (trimerophyte) members have P-type tracheids (after the genus *Psilophyton).*

S-type tracheids of rhyniopsids have annular or helical thickenings and lateral walls that appear to be made of a spongy or reticulate material (figure 2*a–c*). It has been suggested that the 'pockets' in the spongy wall material may represent unlignified portions that were preferentially degraded during fossilization (Kenrick & Crane 1991; Kenrick *et al.* 1991). A very thin degradation-resistant layer of cell wall material with micropores covers the entire spongy layer of wall material adjacent to the cell lumen (figure 2; Kenrick *et al.* 1991; Kenrick & Crane 1991, 1997*a*,*b*). It is unclear whether the micropores are a preservational artefact (i.e. small pockets of unlignified wall material that were preferentially degraded during fossilization) or were a real structural component of this innermost wall layer.

G-type tracheids of early zosterophylls have annular or helical secondary cell wall thickenings that exhibit two layers: a carbonaceous dark layer closest to the cell lumen, and a light layer that appears to represent a mineralized hollow core of each gyre (figure 2*d–f*) (Kenrick & Edwards 1988; Kenrick & Crane 1991; Kenrick *et al.* 1991). The hollow core in wall thickenings of these fossil cells may represent an unlignified portion of the thickening that was preferentially degraded during fossilization (Kenrick & Edwards 1988; Kenrick & Crane 1991). Between gyres of secondary thickenings, lateral walls in G-type tracheids exhibit holes ranging in size from less than 1 μm to 4 μm (Kenrick & Edwards 1988). These holes have been interpreted as perforations in the primary wall (Hartman 1981; Hueber 1983; Rayner 1984) or as small pits in a layer of lignified secondary wall that is continuous with the degradation-resistant dark layer of the annular or helical thickenings (Brauer 1980; Taylor 1986; Kenrick & Edwards 1988; Edwards 1993; Kenrick *et al.* 1991; Kenrick & Crane 1991, 1997*a*). Alternatively, these holes could represent small pockets of non-lignified cell wall material that were degraded and lost during the process of fossilization.

P-type tracheids (Kenrick & Crane 1997*a*,*b*) are found in early euphyllophytes, and have bordered pits (figure 2*g–i*). Strands of secondary wall material traverse the pit

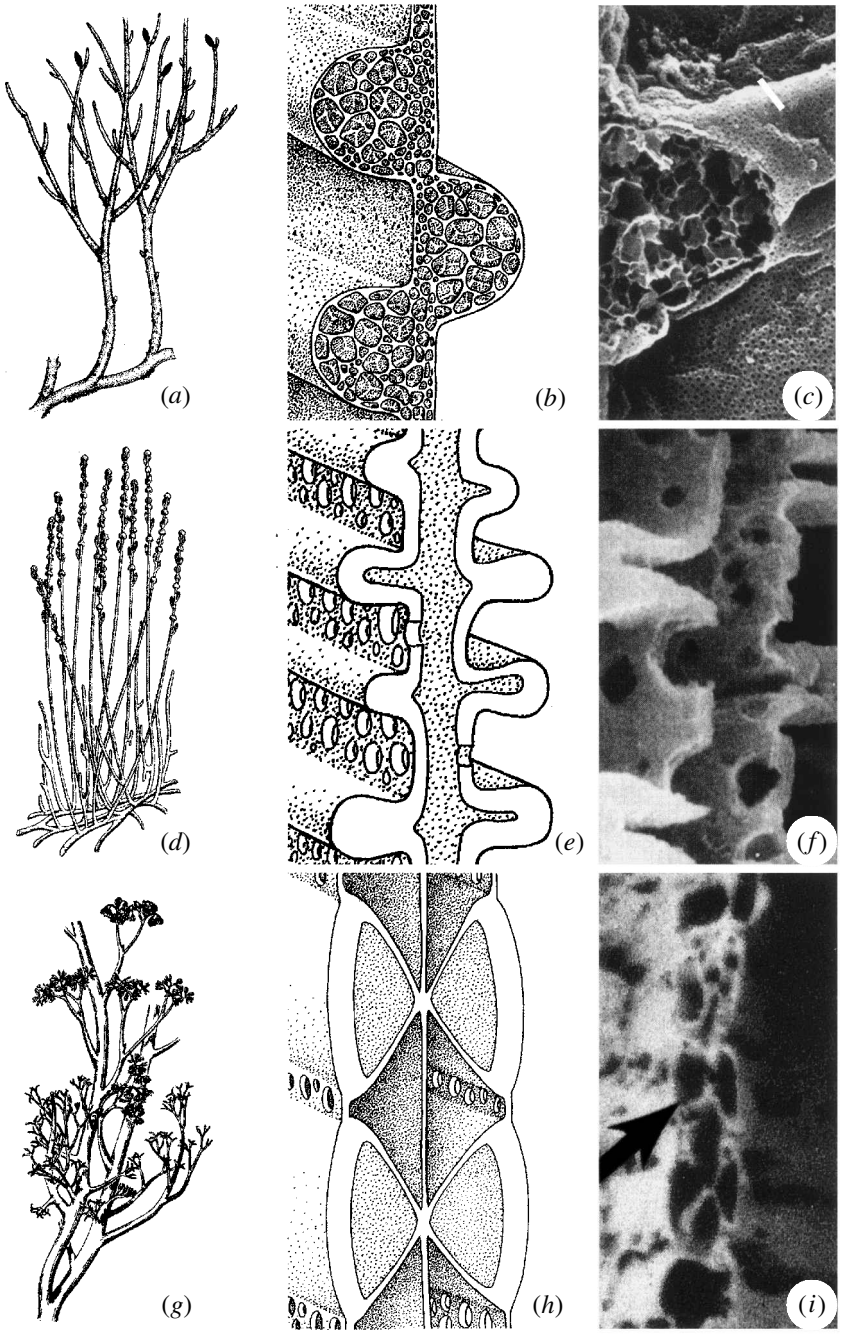

Figure 2. The three major types of early tracheids. (*a*) Reconstruction of *Rhynia*, a member of the Rhyniopsida that produced S-type water-conducting cells. Reproduced from Kenrick & Crane (1997*a*). Copyright permission of the Smithsonian Institution Press. (*b*) Drawing of the wall of an S-type water-conducting cell showing the wall thickenings with a spongy (alveolate) structure and a thin microporate surface on the lumen face of the tracheid. Reproduced from Kenrick & Crane (1997*a*). Copyright permission of the Smithsonian Institution Press. (*c*) SEM of a single gyre from an S-type cell of *Sennicaulis*. Note the spongy wall construction within the gyre at left, and the microporate nature of the inner surface of the tracheid. Reproduced from Kenrick *et al*. (1991). Copyright permission of the Palaeontological Association. (*d*) Reconstruction of *Zosterophyllum*, a member of the Lycophytina that produced G-type water-conducting cells. Reproduced from Kenrick & Crane (1997*a*). Copyright permission of the Smithsonian Institution Press. (*e*) Drawing of the wall of a G-type water-conducting cell showing the wall thickenings that lack material in the core and holes in the surface of the lumen face of the tracheid. Reproduced from Kenrick & Crane (1997*a*). Copyright permission of the Smithsonian Institution Press. (*f*) SEM of a portion of a G-type cell of *Gosslingia*. Note the spongy wall construction within the gyre at left, and the microporate nature of the inner surface of the tracheid. Reproduced from Kenrick & Crane (1997*a*). Copyright permission of the Smithsonian Institution Press. (*g*) Reconstruction of *Psilophyton*, a member of the Euphyllophytina that produced P-type water-conducting cells. Reproduced from Kenrick & Crane (1997*a*). Copyright permission of the Smithsonian Institution Press. (*h*) Drawing of the wall of a P-type water-conducting cell showing the wall thickenings that lack material in the core and continuity of the wall material that overlies the pits and has holes. Reproduced from Kenrick & Crane (1997*a*). Copyright permission of the Smithsonian Institution Press. (*i*) SEM of a portion of a P-type cell of *Psilophyton*. Note the secondary cell wall thickenings appear hollow (arrow). Reproduced from Hartman & Banks (1980). Copyright permission of the Botanical Society of America.

apertures and connect the pit borders that overlie the pit cavity (Gensel 1979; Hartman & Banks 1980). Holes occur in this area of additional secondary cell wall ornamentation (Gensel 1979; Hartman & Banks 1980; Kenrick & Crane 1997*a*,*b*). In the fossil record, P-type tracheids are similar to G-type cells in possessing secondary wall thickenings (between the pits) that appear hollow (figure 2).

Although various aspects of secondary cell wall patterning differ between fossil S-, G- and P-type tracheids (figure 2), all of these early water-conducting cells possess secondary cell wall thickenings composed of two distinct layers: a degradation-resistant (possibly lignified) layer next to the cell lumen and a degradation-prone layer closest to the primary cell wall (Kenrick & Edwards 1988; Kenrick & Crane 1991; Kenrick *et al*. 1991; Edwards 1993). S-, G- and P-type tracheids may all be evolutionarily homologous and represent developmental transformations of a rudimentary tracheid type from a common ancestor of all vascular plants (Kenrick & Crane 1991 1997*a*,*b*; Cook & Friedman 1998). Alternatively, there could have been two or three separate origins of water-conducting cells with secondary wall thickenings (i.e. 'tracheids' are homoplasious) (Kenrick & Crane 1991).

3. TRACHEARY ELEMENTS OF EXTANT VASCULAR PLANTS

Until quite recently (Cook and Friedman 1998), all studies of tracheary element (tracheid and vessel element) differentiation and fine structure had been conducted on highly derived vascular plants, namely conifers and flowering plants (Esau *et al*. 1963, 1966*a*,*b*; Wooding & Northcote 1964; Cronshaw & Bouck 1965; O'Brien & Thimann 1967; Hepler & Fosket 1970; Esau 1978; Daniel & Nilsson 1984; Uehara & Hogetsu 1993; Fineran 1997). As a result, current biochemical and cell biological models of tracheary element development (Boudet *et al*. 1995; Barceló 1997) are based solely on seed plants (Cook & Friedman 1998).

Electron micrographs of tracheary elements in conifers and angiosperms depict secondary wall thickenings that are essentially homogeneous. Although seed plant tracheids typically have a three-layered secondary cell wall (S1, S2, and S3 layers), these layers of cell wall are all heavily lignified and differ mostly in the orientation (angle) of microfibril deposition (Esau 1977; Boudet *et al*. 1995). In seed plants that have been studied, lignification begins at the cell periphery and gradually progresses towards the cell lumen as centripetal wall deposition progresses (Hepler & Fosket 1970; Liu *et al*. 1994; Boudet *et al*. 1995; Barceló 1997). Most significantly, tracheary elements of extant seed plants are not known to exhibit a degradation-prone (possibly unlignified) layer of cell wall material between an outer wall and an innermost degradation-resistant layer of cell wall, as is characteristic of tracheids in early tracheophytes. Thus, despite major progress in reconstructing the structural diversity of early vascular plant tracheids, interpretation of the developmental and evolutionary relationships between these early fossil tracheids and those of extant vascular plants has remained uncertain (Cook & Friedman 1998).

Patterns of tracheid differentiation and mature fine structure are virtually unknown in basal vascular plants (i.e. the pteridophytes). Most basic information on tracheid secondary wall patterning in pteridophytes can be traced to the studies of Bierhorst (1958, 1960), Wilder (1970), Morrow & Dute (1997), Carlquist & Schneider (1997*a*,*b*, 1998*a*,*b*, 1999), Schneider & Carlquist (1997, 1998*a*,*b*, 1999*a*,*b*) and Carlquist *et al*. (1999). Interestingly, Bierhorst reported an 'unlignified or very faintly lignified' core at the base of secondary cell wall thickenings in *Lycopodium*, *Equisetum*, *Psilotum* and various ferns (Bierhorst 1958, 1960). Unfortunately, photomicrographs of this supposedly unlignified core in the tracheid walls of primitive vascular plants were never published, so it is difficult to evaluate these observations. Nevertheless, Bierhorst's reports remained intriguing and several palaeobotanists suggested a relationship between the purported 'unlignified core' and the missing layers of wall material in early fossil tracheids (Brauer 1980; Taylor 1986; Kenrick & Crane 1991; Kenrick *et al*. 1991).

Comparative studies of basal and potentially plesiomorphic vascular plants are essential if we are to understand better the early evolution of tracheids. Knowledge of the relationship between development and mature tracheid wall structure in primitive extant vascular plants can be used to aid in the interpretation of the diverse tracheid wall structures of early fossil vascular plants. Indeed, recent (Cook & Friedman 1998) and ongoing developmental studies of tracheid wall structure in basal extant vascular plants have begun to yield information on critical intermediate character states that link the structures of water-conducting cells of extant vascular plants to those of their 400 Myr-old ancestors. In addition, this research is beginning to provide the requisite information for an explicit model for the evolution of cellular differentiation patterns that could have produced the tracheids of the earliest vascular plants and their extant evolutionary descendants.

4. BACKGROUND PHYLOGENETIC INFORMATION

Phylogenetic analyses indicate that extant vascular plants are distributed in two major sister clades (Raubeson & Jansen 1992), the Lycophytina and the Euphyllophytina (figure 3). Among extant Lycophytina (Lycopodiaceae, Selaginellaceae, Isoetaceae), homosporous Lycopodiaceae are sister to Selaginellaceae plus Isoetaceae (figure 3) (Therrien & Haufler 1997); while within the Lycopodiaceae, terrestrial (non-epiphytic) species of *Huperzia* are basal (Wagner & Beitel 1992; Wikström & Kenrick 1997). Among members of the Euphyllophytina, the phylogenetic positions of sphenopsids (*Equisetum* and its extinct relatives), Psilotaceae (*Psilotum* and *Tmesipteris*), Marattiales, Ophioglossales and Filicales (leptosporangiate ferns) remain unresolved (figure 3), although Psilotaceae may be closely related to Ophioglossales, a result found in several recent phylogenetic analyses (Manhart 1994, 1995; Hasebe *et al*. 1995; Pryer *et al*. 1995; Wolf 1997).

To reconstruct the characters associated with the common ancestors of extant vascular plants, it is important to study basal representatives of both the Lycophytina and the Euphyllophytina. Homosporous lycopods

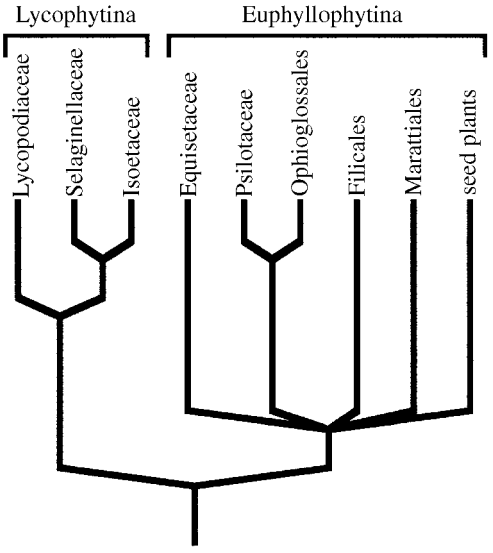

Figure 3. Current hypothesis of phylogenetic relationships among extant vascular plants. See text for additional information.

such as *Huperzia* may well provide critical information about the plesiomorphic structural features of the common ancestors of extant lycophytes. Given the uncertainties of euphyllophyte interrelationships, information on tracheid structure and development from *Equisetum*, *Psilotum* and *Ophioglossum* (or *Botrychium*) will be critical to reconstructing those features of tracheids that are likely to have characterized the common ancestors of extant euphyllophytes.

5. TRACHEID DEVELOPMENT AND STRUCTURE IN *HUPERZIA*

Huperzia lucidula, a member of the most basal extant clade within the Lycopodiaceae, is highly plesiomorphic among vascular plants and can be considered a true living fossil. Morphologically, it is almost indistinguishable from the Lower Devonian fossil lycopod *Drepanophycus*. It has recently been discovered that secondary cell wall structure in tracheids of *Huperzia* is significantly different from what is known of 'model systems' among conifers and angiosperms (Cook & Friedman 1998). More importantly, there is compelling evidence (see below) that tracheid secondary cell wall structure in *Huperzia* shows a high degree of structural correspondence to the secondary wall thickenings of early fossil tracheids.

In *Huperzia*, like many lycophytes, the vascular cylinder of the stem takes the form of an actinostele, with protoxylem at the ends of xylem arms and phloem and parenchyma located between the arms of xylem. Protoxylem elements are few and small in diameter, in comparison with metaxylem elements. At maturity, protoxylem tracheids of stems exhibit annular secondary wall thickenings that are occasionally interconnected via vertical or slanted thickenings, forming a loose reticulum (figure 4*a*). Short, helical thickenings are sometimes seen in cells with annular thickenings. Longitudinal sections of metaxylem tracheids demonstrate that bordered pits of various sizes, shapes and patterns appear in secondary walls. Early metaxylem elements are small in diameter and possess circular or oval pits with borders. Oval or elongate pits are found in later-formed tracheids of larger diameter (figure 4*b*).

Differentiation of lateral walls in protoxylem and metaxylem tracheids of *Huperzia* involves three discrete stages of cell wall deposition, each of which produces a cell wall layer with distinct properties (Cook & Friedman 1998). As is true for all plants, cell wall formation is centripetal. The first layer to mature is the primary cell wall, which is smooth, homogeneous, and assumes a light grey appearance under the transmission electron microscope (TEM). Deposition of the primary cell wall is completed before synthesis of the two layers that compose the secondary cell wall is initiated.

Secondary cell wall deposition begins at the lumen surface of the primary cell wall. A first-formed layer of secondary cell wall is deposited over the surface of the primary cell wall (figure 4*c*,*e*), except in areas that will develop into pit membranes (metaxylem) or that lie between gyres of secondary wall material (protoxylem). In *Huperzia*, the first-formed secondary cell wall layer determines the pattern of further secondary cell wall deposition and has been named the 'template layer' (Cook & Friedman 1998). The template layer exhibits dark, mottled staining under the TEM (figure 4), which matches the contents of dictyosome-derived vesicles (figure 4*c*) that fuse with the plasmalemma and contribute to the synthesis of this layer of secondary cell wall. Each vesicle contains a single, electron-opaque particle, and it is likely that this structure is directly related to the electron-opaque particles found within the template layer (figure 4*d*,*f*).

After deposition of the template layer of secondary cell wall is completed in *Huperzia*, an additional and structurally distinct layer of secondary cell wall (the 'resistant layer') is deposited on the lumen surface of the template layer. This later-formed layer is first discernible next to the cell lumen as a thin, very lightly stained layer of newly synthesized cell wall material (figure 4*g*). The TEM shows that dictyosome-derived vesicles (figure 4*d*) that appear to be contributing to this second phase of secondary wall formation differ markedly from those associated with the synthesis of the template layer. The contents of these vesicles lack the electron-opaque particles associated with formation of the template layer. As the later-formed resistant wall layer continues to increase in thickness (figure 4*g–i*) and to mature, it stains more darkly.

Cell autolysis completes tracheid differentiation in *Huperzia* (figure 4*j*). At maturity, three distinct layers of cell wall can be discerned in *Huperzia lucidula*: a homogeneous primary cell wall; a mottled, heterogeneous template layer that covers much of the primary cell wall; and a homogeneous layer of secondary cell wall that overlies the template layer. The structural distinctness of the two layers of secondary thickenings in *Huperzia* is also apparent in longitudinal sections of water-conducting cells that have been prepared for scanning electron microscopy (SEM) (figure 5). Often, as a result of mechanical stress associated with microtomy, the inner resistant layer of the secondary cell wall becomes physically detached from the template layer, which remains attached to the underlying primary cell wall (figure 5).

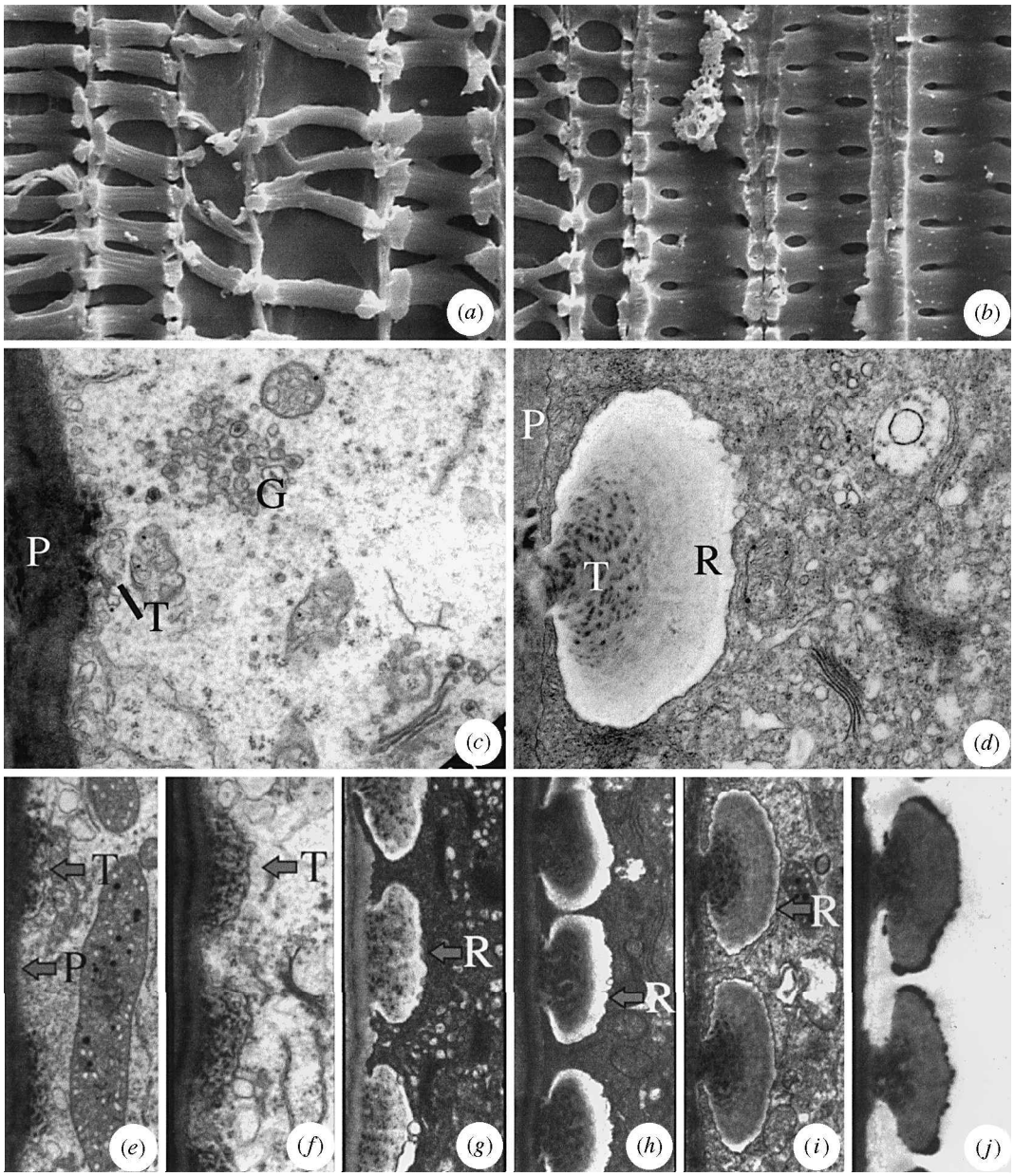

Figure 4. (*a*) SEM of a longitudinal section of protoxylem elements of a stem of *Huperzia*. (*b*) SEM of a longitudinal section of metaxylem elements of a stem of *Huperzia*. Early metaxylem is to the left (reticulate secondary cell walls). (*c*) Earliest stage of secondary cell wall deposition on to the primary cell wall (P) in *Huperzia*. The budding face of a Golgi body (G) can be seen, as well as vesicles apparently fusing with the cell wall to form the template layer (T). These vesicles each contain a single electron-dense particle that appears to be incorporated into the template layer. (*d*) Deposition of the resistant layer (R) of secondary cell wall material in a tracheid of *Huperzia*. Note that the vesicles derived from Golgi bodies are now electron-translucent. (*e–j*) Development of secondary wall thickenings in *Huperzia* (longitudinal views). A first-formed layer of secondary wall, the template layer, is deposited on the primary cell wall (*e*,*f*). Subsequently, a later-formed layer of secondary wall, the resistant layer, is deposited on the surface of the template layer (*g–i*). The resistant layer appears unstained or very lightly stained when first deposited, but later appears grey. In mature, dead tracheids (*j*), the template layer is distinct from the resistant layer of secondary cell wall, and can be recognized by the inclusion of electron-dense particles.

Huperzia remains the sole extant basal vascular plant for which there is ultrastructural developmental information on cell wall formation in tracheids. Further developmental studies of other lycophytes and various basal euphyllophytes will be central to determining how general the pattern of secondary cell wall formation in *Huperzia* may be among extant vascular plants.

6. EXPERIMENTAL CELL WALL DEGRADATION IN TRACHEIDS IN *HUPERZIA* AND *EQUISETUM*

S-, G- and P-type tracheids preserved in the Silurian and Early Devonian were invariably subject to diagenetic alterations. During the time between plant death and permanent incorporation in the fossil record, plant parts are often attacked by a variety of cell-wall-degrading

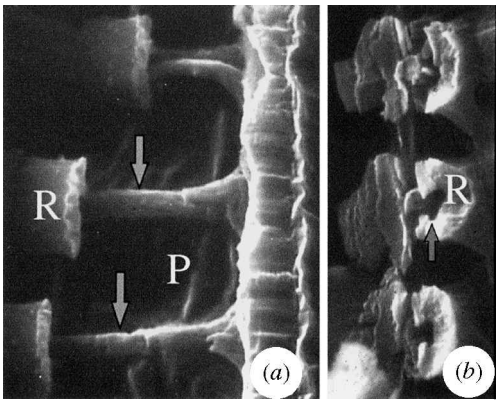

Figure 5. (*a*,*b*) SEM images of the secondary thickenings of tracheids in *Huperzia* in which the resistant layer (R) of the secondary cell wall has become physically separated from the template layer (arrows). The primary cell wall (P) can be seen in both cells.

enzymes of fungi and bacteria. To simulate degradation processes and patterns experienced by fossilized cells, *Huperzia* stem segments have been experimentally treated with cell-wall-degrading enzymes (Cook & Friedman 1998). Although it is not possible to replicate exactly the conditions of preservation in the fossil record, enzyme degradation studies can help determine if there is correspondence between the different layers of cell walls of tracheids in extant *Huperzia* and the reported patterns of differential tracheid wall preservation in early vascular plants.

Stem segments 2 mm long were immersed for two to four weeks in an aqueous solution of 2% pectinase (from the fungus *Rhizopus*) and 2% cellulase (from the fungus *Penicillium*), rinsed in water and treated for 2 h in aqueous 2% osmium tetroxide, before usual preparation for electron microscopy. These experiments demonstrated that the two layers of secondary cell wall material in tracheids of *Huperzia* are distinct chemically, as well as structurally and developmentally. The primary cell wall and first-formed layer of secondary cell wall (at the cell periphery) are significantly degraded by a mixture of pectinase and cellulase. After enzyme treatment, the primary cell wall and the first-formed layer of secondary cell wall (template layer) were either entirely missing or were represented by a delicate reticulum of remaining cell wall material (figure 6*b*). The later-formed secondary cell wall layer (next to the cell lumen) was not degraded by enzyme treatment. This portion of the secondary wall in *Huperzia* has been named the 'resistant layer' (Cook & Friedman 1998).

Experimental evidence for a degradation-prone template layer in *Huperzia* (Cook and Friedman 1998), is the first conclusive report for this type of secondary cell wall organization in an extant vascular plant. Cell wall degradation experiments have now been extended to the tracheids of *Equisetum*. When subjected to a solution of pectinase and cellulase, secondary thickenings in *Equisetum* tracheids show clear evidence of degradation of the primary cell wall and the base of the secondary cell wall (figure 7). An overlying cap of secondary cell wall (shaped much like a mushroom in sectional view)

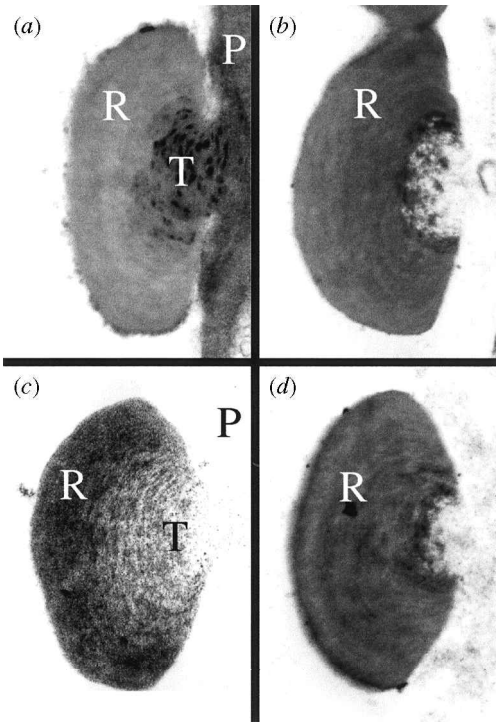

Figure 6. Comparison of wall layers in tracheids of *Huperzia*. (*a*) Traditional TEM view (control) of a single secondary cell wall thickening with a template layer (T) and a resistant layer (R). The primary cell wall (P) is at the right. (*b*) In cells subjected to enzyme treatment (see text), the template layer has been degraded and appears reticulate, while the resistant layer remains intact. The primary cell wall is largely degraded and is not apparent. (*c*) Secondary cell wall of tracheid which has been post-stained with potassium permanganate, an indicator of the presence of lignin. The primary cell wall appears to lack significant amounts of lignin, while the template layer is partially stained, suggesting the presence of some lignin. The resistant layer is heavily stained, indicating the presence of large amounts of lignin. (*d*) Secondary cell wall of tracheid which has been subjected to enzyme treatment and then analysed cytochemically for the presence of lignin. The resistant layer is intact and heavily lignified. The template layer is largely degraded, but shows signs of a reticulate distribution of lignin.

adjacent to the lumen does not show evidence of degradation by a combination of cellulase and pectinase. These experiments provide, for the first time, compelling evidence that a degradation-prone (template) layer and a degradation-resistant layer are present in the secondary thickenings of tracheids in a member of the euphyllophyte clade.

The clear implication of these results from *Equisetum*, coupled with the recent findings in *Huperzia* (Cook & Friedman 1998), is that the common ancestor of the Lycophytina and Euphyllophytina is likely to have produced tracheids with secondary cell wall thickenings, comprised of an underlying degradation-prone template layer and a subsequently formed degradation-resistant layer.

7. CYTOCHEMICAL LOCALIZATION OF LIGNIN IN THE TRACHEIDS OF *HUPERZIA*

Lignin is one of the most degradation-resistant biopolymers on Earth (Graham 1993). This suggests that those

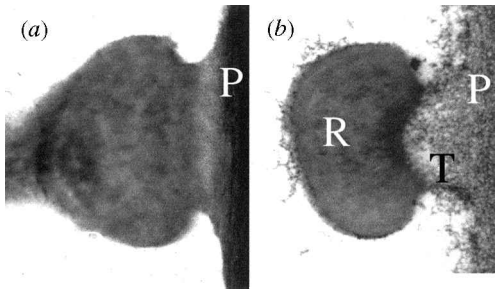

Figure 7. Secondary cell wall thickenings in the tracheids of *Equisetum*. (*a*) Traditional TEM view (control) of a single secondary cell wall thickening. The primary cell wall (P) is on the right. (*b*) In cells subjected to enzyme treatment (see text), a template layer (T) is apparent and has been partially degraded. A resistant layer (R) is also quite clearly seen. The primary cell wall is partially degraded. These data indicate that at least in one member of the euphyllophyte clade, an organization of secondary cell walls may involve a first-formed partially lignified template layer and a later-formed heavily lignified resistant layer.

portions of the cell walls of early fossil tracheids that were not degraded during preservation in the Silurian and Devonian may have been heavily lignified. We sought to determine whether there is a correspondence between the results of our laboratory-based enzyme degradation studies and patterns of lignin distribution within the cell walls of tracheids of extant basal vascular plants.

Potassium permanganate has been widely used as a fixative or a post-fixation stain to distinguish lignified cell walls from those that are unlignified (Hepler & Fosket 1970; Barceló 1997; Fineran 1997). Thin sections of enzyme-degraded *Huperzia* tracheids were post-stained with permanganate and examined under the TEM for cytochemical analysis of lignin distribution. The resistant layer of secondary wall stained darkly and homogeneously, while the template layer stained in a reticulate manner (figure 6*d*). What remains of the primary wall appears to contain little, if any, lignin.

To cross-correlate cytochemical data for enzyme-degraded cells with untreated tracheids, TEM-level cytochemical localization of lignin in *Huperzia* was performed (based on a modification of the 'Coppick and Fowler' method, see Fineran (1997) for methods) on intact tracheids (figure 6*c*). These data indicate that the resistant layer is heavily lignified, that the template layer is partially lignified in a reticulate manner, and that the primary cell wall of *Huperzia* tracheids appears to lack significant quantities of lignin.

8. RELATIONSHIPS BETWEEN THE TRACHEIDS OF EXTANT BASAL VASCULAR PLANTS AND THE EARLIEST FOSSIL VASCULAR PLANTS

Extant lycopods are more closely related to zosterophylls and fossil lycopods than they are to other vascular plants (figure 1). Therefore, one might expect tracheids of *Huperzia* to be more similar to the G-type cells typical of zosterophylls and early fossil lycopods than to fossil S- and P-type cells or the tracheids of other extant plants. A prominent feature of G-type cells is the presence of holes in resistant lateral walls between annular or helical secondary thickenings (figure 8). These holes have been interpreted as perforations in the primary wall (Hartman 1981; Hueber 1983; Rayner 1984) or as small pits in a layer of lignified secondary wall that is continuous with the degradation-resistant dark layer of the annular or helical thickenings (Brauer 1980; Taylor 1986; Kenrick & Edwards 1988; Edwards 1993; Kenrick *et al*. 1991; Kenrick & Crane 1991, 1997*a*). Tracheids of *Huperzia* do not possess holes in lateral secondary walls comparable with those between thickenings in G-type tracheids (figure 8).

The biochemical nature of the template layer in *Huperzia*, its susceptibility to degradation and its position at the base of secondary cell wall thickenings strongly suggest developmental and structural homology with the degradation-prone wall layer in the secondary cell wall thickenings of the G-type tracheids characteristic of early members of the Lycophytina. For many years, palaeobotanists suggested that the absent layer of wall material in the secondary cell walls of early fossil tracheids represents an unlignified or poorly lignified wall layer that did not survive the fossilization process (Kenrick & Crane 1991, 1997*a*,*b*; Kenrick & Edwards 1988; Brauer 1980). Documentation of a degradation-prone, partially lignified template layer and a heavily lignified resistant layer in the secondary cell wall thickenings of *Huperzia* (Cook & Friedman 1998) provides a critical link between the water-conducting cells of Late Silurian and Early Devonian lycophytes and those of their extant descendants (figure 8).

Structural correspondence of secondary cell wall layers of basal extant vascular plant tracheids can be extended beyond the ancestral G-type cells of the early Lycophytina to the P-type cells representative of the earliest members of the Euphyllophytina (the sister group to lycophytes). Secondary cell wall thickenings of fossil P-type tracheids also have an absent core layer (figure 8; Hartman & Banks 1980; Edwards 1993). Results of experimental degradation studies of tracheids of extant *Equisetum* show a degradation-prone layer is present in the secondary thickenings that positionally matches the template layer of *Huperzia* and underlies a resistant layer (figure 8). The clear implication of these findings is that the common ancestor of lycophytes and euphyllophytes (together the eutracheophyte clade) produced tracheids with secondary thickenings composed of a first-formed poorly lignified template layer that was susceptible to degradation during fossilization and a second-formed heavily lignified resistant layer that is still present in 390 Myr-old tracheids.

Many features of development of tracheary element walls at the cellular level appear to be the same in *Huperzia* and seed plants: the primary wall reaches maximum thickness before secondary wall layers are laid down; microtubules, endoplasmic reticulum and dictyosomes line up along the forming secondary wall; dictyosome-derived vesicles fuse with the plasma membrane near the developing secondary thickenings; the cell undergoes programmed cell death (Baucher *et al*. 1998). Developmental differences do exist, however, between tracheary elements of seed plants and those of *Huperzia*. Most significant is that in those seed plants studied, secondary cell wall thickenings of mature tracheary elements appear homogeneous (figure 8) when

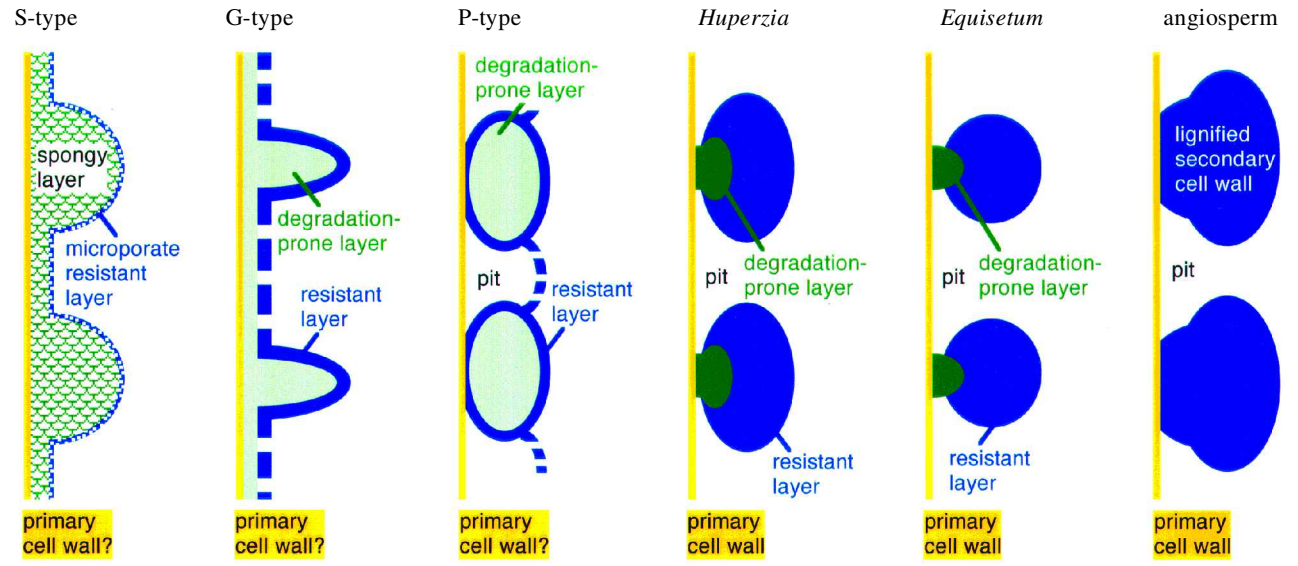

Figure 8. Schematic of longitudinal view of cell wall thickenings in fossil S-, G- and P-type tracheids and in tracheids of *Huperzia*, *Equisetum* and extant angiosperms (and conifers). The initial diversification of vascular plants is hypothesized to have produced an early divergent Rhyniopsida (all extinct) with S-type tracheids; the Lycophytina (lycopsids and zosterophylls) with G-type tracheids (in Early Devonian members); and the Euphyllophytina (all other vascular plants), with P-type tracheids (in Early Devonian members). The cell lumen is to the right in each diagram. Cell wall thickenings in S-type cells have a thick, partially resistant spongy layer that is covered by a thin microporate resistant layer adjacent to the cell lumen. Cell wall thickenings in G- and P-type cells appear to have a core of degradation-prone wall material that is covered by a thick, resistant layer adjacent to the cell lumen. The degradation-prone layer of G- and P-type cells is absent from tracheids recovered from Lower Devonian fossils. Holes are present between gyres in the degradation-resistant layer of G-type tracheids. In P-type tracheids holes are apparent in a layer of degradation-resistant cell wall that overlies pits. In *Huperzia* and *Equisetum*, secondary cell wall thickenings are composed of a degradation-prone layer that is likely to be poorly lignified and a degradation-resistant layer that is heavily lignified. Secondary cell wall thickenings of extant seed plant tracheary elements appear to be homogeneous and lack any equivalent to a degradation-prone layer.

viewed with light microscopy and TEM. (This also appears to be true of secondary cell wall thickenings in *Equisetum*, even though degradation studies indicated that the distribution of lignin is not homogeneous.) Seed plants typically have secondary cell walls with three layers (S1, S2 and S3) that differ in the orientation of cellulose microfibril deposition, but are all heavily lignified (Boudet *et al*. 1995). Lignification begins at the cell periphery (middle lamella and primary cell wall) and progresses towards the cell lumen as centripetal wall deposition continues (Boudet *et al*. 1995; Barceló 1997). In contrast, in *Huperzia* and *Equisetum*, lignification appears weak or absent in the primary cell wall, heterogeneous and somewhat more prominent in the template layer, and is heaviest in the layer closest to the cell lumen.

Ultimately it will be essential to study the development, biochemical nature and degradation properties of other extant euphyllophyte clades, such as Psilotaceae, Ophioglossales, Marattiales, leptosporangiate ferns, *Ginkgo* and cycads, to determine how widespread the pattern of tracheid wall structure seen in *Huperzia* and *Equisetum* is among extant vascular plants. Given that conifers and angiosperms appear to lack a template layer similar to that which has been found in basal vascular plants, phylogenetically based comparative analyses of tracheid structure among diverse euphyllophyte clades will be necessary to address the question of whether the template layer of secondary thickenings in tracheary elements was lost prior to the origin of seed plants or within seed plants.

Developmental analysis and enzyme degradation experiments also suggest a possible connection between the tracheids of *Huperzia* and *Equisetum*, and the more primitive fossil S-type cells. It has been suggested that the spongy layer of S-type cell walls survived in the fossil record because it was partially resistant to degradation, perhaps due to thin veins of resistant biopolymers running through a mass of less resistant material (figure 8; Kenrick *et al*. 1991). Similarly, the template layers of *Huperzia* and *Equisetum* do not completely disappear under conditions of experimental degradation, but instead yield a reticulate condition that bears considerable resemblance (figures 6*b* and 7*b*) to the spongy wall layer of S-type cells (figure 2*c*). Thus, the template layer of *Huperzia* and *Equisetum*, as well as the missing core of secondary cell wall in P-type and G-type tracheids, may be structurally and evolutionarily homologous to the spongy layer of S-type cells.

9. WORKING HYPOTHESIS FOR THE EARLY EVOLUTION OF TRACHEIDS

Documentation of a degradation-prone template layer and a degradation-resistant layer in the secondary cell wall thickenings of the primitive vascular plants *Huperzia* and *Equisetum* provides the critical link between the water-conducting cells of Early Devonian tracheophytes and those of their extant descendants. While the secondary cell wall thickenings of tracheids of previously studied extant vascular plants (Esau *et al*. 1963, 1966*a*,*b*;

Wooding & Northcote 1964; Cronshaw & Bouck 1965; O'Brien & Thimann 1967; Hepler & Fosket 1970; Esau 1978; Daniel & Nilsson 1984; Uehara & Hogetsu 1993; Fineran 1997) appear to differ significantly from those of early fossil vascular plants, developmental data from *Huperzia*, and the results of enzyme degradation studies in *Huperzia* and *Equisetum*, demonstrate a clear structural correspondence between the tracheids of extinct and extant primitive vascular plants. Moreover, tracheid development in *Huperzia* provides the essential information needed to propose an explicit model for the evolution of secondary wall thickenings in vascular plants.

The evolution of complex water-conducting cells is unlikely to have occurred in a single step. Rather, tracheids almost certainly evolved through a series of innovations and modifications of development. Although 'recapitulation' explanations for the evolution of organismal development are frequently problematic (Alberch & Blanco 1996 and references therein), the developmental sequence of secondary cell wall patterning associated with tracheid development in the basal lycopod *Huperzia* (and almost certainly in *Equisetum*, a basal euphyllophyte) may indeed recapitulate aspects of the developmental evolution of tracheids in early vascular plants.

Our developmental data are congruent with the hypothesis that evolution of water-conducting cells in land plants commenced with the programmed autolysis of cells. We hypothesize that the origin of secondary cell wall thickenings in tracheids began with deposition of a partially degradation-resistant template layer of cell wall on to the inner surface of the primary cell wall prior to cell autolysis. A further innovation, a more heavily lignified resistant layer, was added to the process of secondary cell wall deposition, and this secondary cell wall organization can be found as a thin, innermost (next to the lumen) layer in mature S-type conducting cells of Rhyniopsida.

At some point in the Late Silurian or Early Devonian, modification of the process of tracheid cell wall deposition led to a decrease in the proportion of the degradation-prone template layer and an increase in the proportion of the resistant layer within secondary cell walls, as found in G- and P-type fossil tracheids. The resistant layer represents 2% of the thickness of secondary wall thickenings in S-type cells, 30% in G- and P-type cells (Cook & Friedman 1998), and well over 50% in extant *Huperzia* and *Equisetum*. This trend towards increasing amounts of degradation-resistant (lignified) secondary cell wall material has previously been noted (Kenrick & Edwards 1988; Kenrick & Crane 1997*a*). Continuation of the trend towards reduction of the template layer and augmentation of the resistant layer has produced the characteristic secondary thickenings of tracheids in extant seed plants, in which no equivalent of a template layer has been reported and the entire secondary wall is highly lignified and resistant to degradation.

10. SUMMARY

The origin of vascular plants occurred during the Silurian, well over 400 Myr ago. This evolutionary transition in structural and physiological complexity is believed to have been one of the most significant evolutionary events during the entire history of land plants. The development of lignified water-conducting tissues played a major role in the remarkable morphological and anatomical radiation of land plants during the Silurian and the Devonian (Raven 1993). Following the evolution of lignified vascular tissues, greater stature of the primary plant body was achieved, secondary growth evolved and a host of physiological modifications were likely to have been possible as a consequence of the improved efficiencies of lignified water-conducting tissues. Through integration of phylogenetically based palaeobotanical and neobotanical studies of tracheid structure we are beginning to address explicitly how, in a historical and mechanistic sense, lignified vascular tissues evolved. What is clearly needed is far more information about the structure, development and biochemical nature of cell walls in tracheids among extant basal vascular plants.

Beyond the evolutionary significance of studying tracheid structure in plesiomorphic vascular plants, additional developmental information on the course of lignification in basal vascular plant tracheids will be central to the formulation of more inclusive 'models' of tracheid structure and differentiation. Our current knowledge of spatial and temporal aspects of lignin deposition during cell wall differentiation for all vascular plants has been limited to seed plants (Boudet *et al*. 1995; Barceló 1997). If we are to ever fully understand the molecular and cellular basis for differentiation of tracheids, more phylogenetically global models of development will be essential, and these models will most certainly depend on a thorough understanding of the tracheids of basal vascular plants.

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Discussion

W. G. Chaloner (*Department ofGeology, Royal Holloway University of London, UK*). It is now widely accepted that the conducting elements at the core of the axis of the Devonian plant *Aglaophyton* are not technically tracheids, since they show no evidence of gyres of (annular or helical) wall thickenings. On these grounds, most authors have excluded that genus from the tracheophytes (see, for example, the 'protracheophytes' of Kenrick & Crane 1997).

Nonetheless, the walls of these presumed water-conducting cells of *Aglaophyton* have the same distinctive black coloration that characterizes the presumably lignified xylem elements of the other true tracheophytes (e.g. *Rhynia*, *Asteroxylon*) in the Rhynie Chert. Inevitably this suggests similarity of the original wall chemistry of all these tissues. Do you think that it is possible that these walls in *Aglaophyton* were indeed lignified, but perhaps rather late in their ontogeny, after axial elongation was completed? If this was the case, no provision for wall stretching—no gyres of secondary wall thickening—would have developed. If these cells were lignified, would they then qualify as tracheids? Has the difference between *Aglaophyton* and *Rhynia* perhaps been overrated?

W. E. Friedman. It is entirely possible that the walls of water-conducting elements in *Aglaophyton* were lignified. As you suggest, these smooth-walled metaxylem cells that lack evidence of gyres could have been stretched during axis elongation, and lignified after elongation of the immediately proximal tissues of the axis had ceased. If this is the case, these water-conducting cells of *Aglaophyton* would differ from the similar (and potentially homologous) hydroids of some mosses by virtue of the presence of lignified walls. Although never explicitly discussed in the literature, it is possible that the water-conducting elements of *Aglaophyton* could represent a character reversal from lignified tracheids with gyres to tracheids that lack gyres, but retain lignin. If this were the case, the distinction (structurally and phylogenetically) between *Rhynia* and *Aglaophyton* might be significantly less than presently assumed, and both taxa might be members of the same rhyniophyte clade. A possibly analogous case can be found in *Nothia*, which Kenrick and Crane hypothesize to be a lycophyte. The water-conducting cells of *Nothia* lack gyres and if the phylogenetic placement is correct, this would represent a secondary reversion in this character.

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