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Cluster root development in *Grevillea robusta* (Proteaceae)

II. The development of the endodermis in a determinate root and in an indeterminate, lateral root

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SUMMARY

Light, fluorescence and electron microscopy were employed to follow the development of the endodermis in cluster roots and lateral roots of *Grevillea robusta* A. Cunn. ex R. Br. Endodermal cells had three different origins: rootlet endodermis arose from the rootlet meristem; endodermis covering the primordium shortly after initiation came from division of parental endodermis; cells at the junction between parent and rootlet endodermis developed from re-differentiated rootlet cortical cells. In the cluster root, the Casparian band formed in three ways, and was not initially present opposite the two sets of single xylem elements in the rootlet stele. A new clearing technique was developed that allowed visualization of xylem, suberized endodermis, Casparian band formation and phenolic compounds. In lateral roots, endodermal differentiation was asynchronous, but was related to position relative to protoxylem poles. However, the observed delay began before these poles had differentiated. At the tip of mature rootlets, which are determinate, the endodermis terminates in a 'dome' of cells, with the initial cell differentiating as an endodermal cell. Results are discussed in terms of determinate development in roots and the spatial and temporal contexts within which this development takes place.

Key words: *Grevillea robusta* A. Cunn. ex R. Br., Proteaceae, cluster roots, endodermis, lateral roots.

INTRODUCTION

The lateral root primordia of gymnosperms and angiosperms generally arise in the pericycle of the parent root (Greulach, 1973). The early development of a lateral root, from primordial formation to emergence through the epidermis, represents a major challenge to the maintenance of structural integrity within the parent root (Peterson & Moon, 1993). This is particularly the case for cluster roots in the Proteaceae, which are composed of lateral roots of determinate growth, produced in vast numbers (Purnell, 1960), density ranging from 35 rootlets per cm of parent root in *Lupinus albus* (Dinkelaker, Römheld & Marschner, 1989) to 1000 rootlets per cm of parent root in *Hakea prostrata* (Lamont, 1972). Studies of endodermal development in dicotyledonous species have been very limited (Scott & Peterson, 1979*b*), with no such investigation known on a dicotyledonous tree species.

The debate over the function of the endodermis has been continuing for at least a century (Sutherland, 1976). While attention has focused on histochemistry, less emphasis has been placed on the overall structural development of the endodermis in conjunction with neighbouring tissue during lateral root outgrowth. McCully (1995) has stated that 'The way plants work can be understood only in terms of the way they are put together'.

Cluster roots offer ideal material from which to learn how the plant is 'put together'. In addition to providing many rootlets for examination in a relatively small volume, the regular occurrence of cluster roots along a given parent root allows prediction of where the next cluster will occur (Skene *et al.*, 1996). This, in turn, provides a method by which young primordia, not yet visible at the surface of the parent root, can be located and examined.

In this study we investigated the development of

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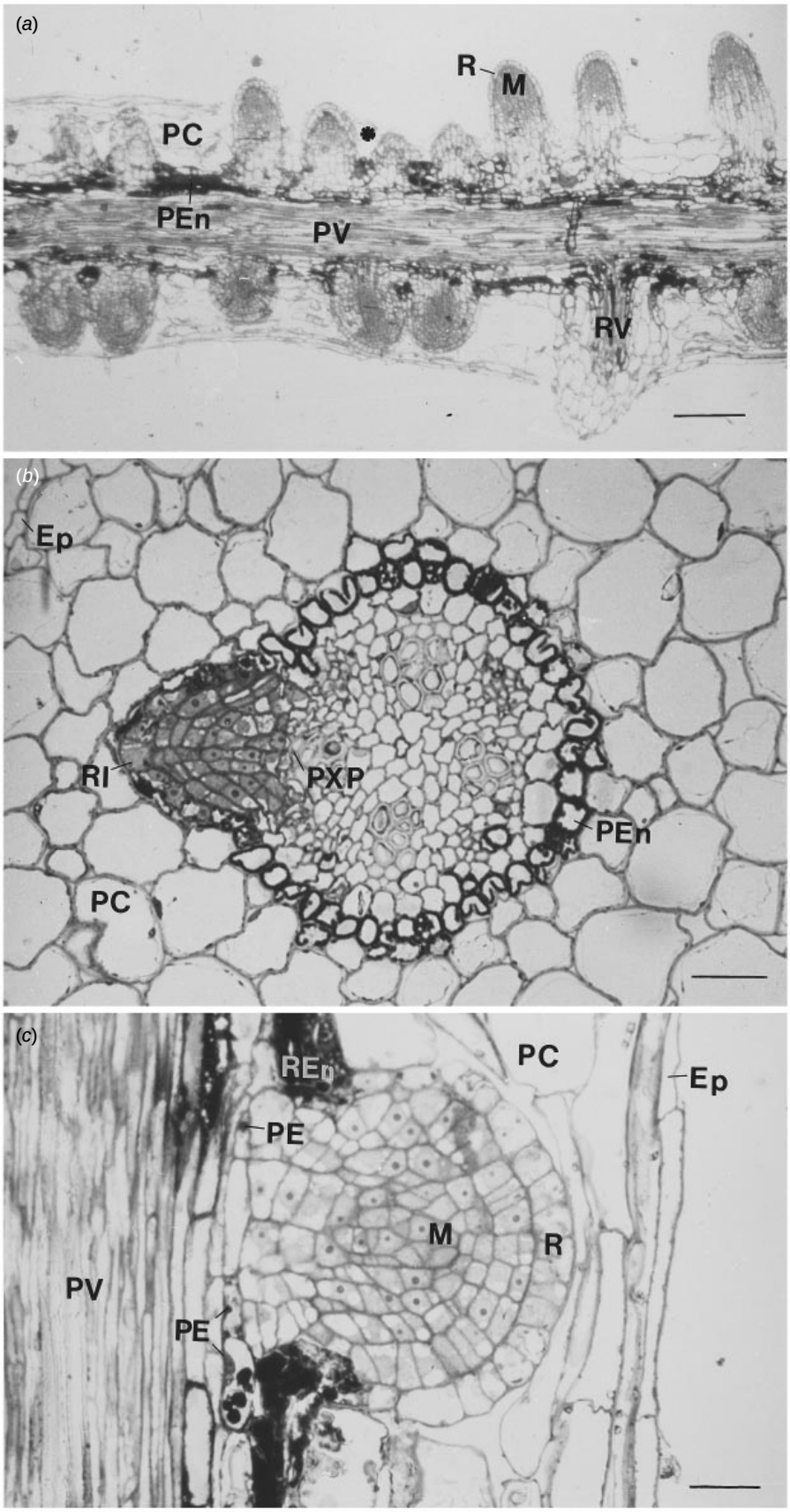

Figure 1. Cluster rootlet initiation and development in *Grevillea robusta*. (*a*) L. S. of a young cluster root. There is evidence of cortical breakdown (*). Bar, 140 μm. (*b*) T. S. of a rootlet primordium, just before meristem formation. Bar, 30 μm. (*c*) T.S. of a young cluster rootlet. Young endodermal cells containing large, osmiophilic vesicles (PE) are visible at the base of the cluster rootlet. Bar, 30 μm. Ep, epidermis; M, meristem; PC, parental cortex; PEn, parental endodermis; PV, parental vascular tissue; PXP, protoxylem pole; R, root cap; RI, rootcap initial; RV, rootlet vascular tissue.

the endodermis in cluster roots of *Grevillea robusta*, which are determinate, and compared this with the development of endodermis in lateral roots, which are not determinate. This is the first detailed study of endodermal development in cluster roots of any species.

MATERIALS AND METHODS

Plant material

Grevillea robusta A. Cunn. ex R. Br. seedlings (Provenance Loitokitok, Kenya Forestry Seed Centre) were grown and prepared for light and transmission electron microscopy as described in Skene, Raven & Sprent (1998).

Hand-cut sections through cluster roots were made by immobilizing roots in 1-cm^3 pieces of polystyrene, and slicing with half of a double-edged razor blade. Sections were observed using an Olympus® BH2 microscope. For fluorescence, a BH2-RFL reflected light fluorescence attachment was used, with an excitation filter UG1, barrier filter L-420 and dichroic mirror DM400.

Staining and clearing procedures

The following procedures were used.

1. Berberine–aniline blue fluorescent staining. This was as described by Brundrett, Enstone & Peterson (1988), and stained lignin and suberin. Furthermore, it allowed the distinction between Casparian bands and suberin lamellae based on differentiation between encrusted and lamellar suberin.
2. Sudan red 7B (fat red) staining procedure (Brundrett, Kendrick & Peterson, 1991).
3. Lactic acid clearing (Peterson & Fletcher, 1973). Although developed for demonstrating sieve tubes, this technique proved useful in the visualization of endodermis and Casparian band.
4. Novel clearing procedure. A new clearing method, bringing together several recognized clearing techniques, was developed. This allowed visualization of xylem and Casparian bands in intact, cleared roots. Specimens were initially placed in 1 % (w/v) NaOH for 24 h. Following a 30-min rinse in H_2O, segments were placed in 0·6 % (v/v) H_2O_2 for 1 h at 60 °C. They were then rinsed three times in H_2O (10 min, 30 min, 18 h), placed in glacial acetic acid–95 % ethanol (1 : 3) for 72 h, then rinsed in H_2O (3 × 5 min). Specimens were cleared in 85 % lactic acid at 100 °C for 1 h, rinsed in H_2O (3 × 15 min) and stained with aqueous 0·04 % water-soluble aniline blue. Under u.v. fluorescence, xylem, Casparian bands and phenolic compounds can be visualized together.

RESULTS

Endodermal development in juvenile cluster rootlets

Cluster roots developing on *G. robusta* are composed of a number of rootlets growing through the endodermis, cortex and epidermis into the growth medium (Fig. 1*a*). Although mature cluster roots have a classic elliptical form, rootlet development is asynchronous, as can be seen in Figure 1*a*, although only rootlets in the same plane of section can be compared. Parental epidermis and cortex have been completely disrupted. Cluster rootlets develop from a primordium which originates in the pericycle opposite the protoxylem pole (Fig. 1*b*). In Figure 1*b*, the parental endodermis, darkly stained by OsO_4, is not complete. One apical cell in the rootlet has undergone greater expansion than neighbouring cells and is breaking through the parental endodermis. This cell becomes the rootlet cap and epidermis initial (see also Skene *et al*. (1998)). Later, a meristem forms, and a rootlet cap is visible (Fig. 1*c*). Cluster rootlet cells are becoming vacuolate, and young rootlet endodermal cells (identifiable because of their unique, large osmiophilic vesicles) start to differentiate at the base of the rootlet (PE, in Fig. 1*c*) from cells that were initially rootlet cortex cells. This occurs before any rootlet endodermis has begun to differentiate from the rootlet meristem. As the rootlets develop and grow through the parent cortex, there is, initially, a collar of parental endodermal cells surrounding each of them, visible in T.S. (Fig. 2*a*) and L.S. (Fig. 2*e*). This collar is made up of young, newly differentiated cells with osmiophilic vesicles, and other, older, cells from the original parental endodermis. The presence of large osmiophilic vesicles is invaluable for locating young, newly differentiated endodermal cells. These vesicles provide evidence that cells in the parental endodermis, lying over new primordia, undergo division. A little later, the new rootlet endodermis begins to form within the rootlet (REn), and at the same time the outer layer of parental origin begins to break down (Fig. 2*b*). At the base of the rootlet, cortical cells begin to transform into endodermal cells as the parental and rootlet endodermal layers unite (Fig. 2*c*). At the tip of developing cluster rootlets that have penetrated the growth medium, the meristem forms a dense group of cells, surrounded by a root cap (Fig. 2*d*). In a newly emerged rootlet (Fig. 2*e*), the rootlet endodermis is linked with the parental endodermis by the re-differentiation of basal cortical cells in the rootlet, while the collar of parental endodermis (PEC) still surrounds the outer, basal region of the cluster root. Endodermal development

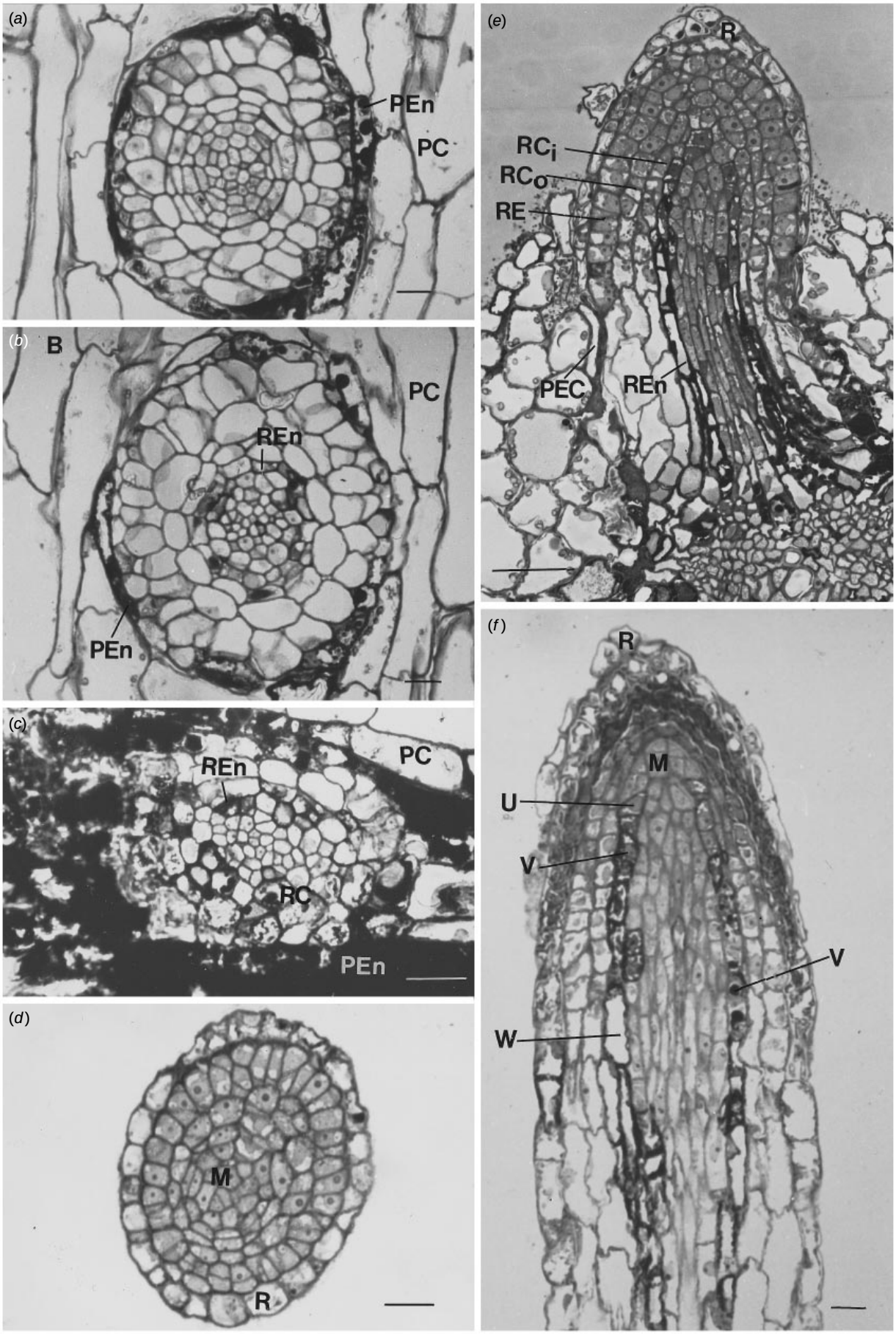

Figure 2. For legend see opposite.

in lateral roots in *G. robusta* resembles that in cluster roots. Three stages of endodermal development can be seen: an undifferentiated stage (U); a vesicular stage (V); and an osmiophilic wall stage (W) (Fig. 2*f*).

Suberization and Casparian band development in lateral and cluster roots

The endodermis and epidermis of the parent root stains positively with Sudan red 7B for suberin (Fig. 3*a*, *b*). In Figure 3*a*, the first periclinal divisions in the pericycle of the parent root can be seen. The cells resulting from these divisions do not exhibit significant asymmetry, although outer cells appear slightly smaller than inner cells. Furthermore, endodermal cells opposite the protoxylem poles are less suberized than those opposite protophloem poles. The Casparian bands are clearly visualized by a berberine–aniline blue staining procedure in both lateral roots (Fig. 3*c*) and cluster rootlets (Fig. 3*d* and inset). In Figure 3*d* inset, it can be seen that suberization and Casparian band formation are retarded opposite the xylem elements, and that the Casparian band is seen to develop in one of three ways (Figs 3*d* inset and 6): first, a localized point on the endodermal radial wall towards the cortex; second, a double Casparian band; and third, a larger continuous band. Since all three types are seen in one section, it is not thought to be a staining artefact.

Casparian band formation in lateral roots

Using the combined clearing method, fluorescence studies allow the visualization of Casparian band development in lateral roots of *G. robusta*. (Fig. 3*e–g*). There is an asynchronous development of the endodermis (Fig. 3*f*) relative to the position of the protoxylem pole. In mature endodermal cells, orange fluorescence, thought to be from phenolic compounds, is visible (Fig. 3*g*). At junctions between cluster rootlets and the parent root there is a concentration of this orange fluorescence (Fig. 3*h*). Near the tip of the lateral root, a central cell that will subsequently develop into a metaxylem element begins to differentiate at the same time as the endodermis (Fig. 4*a*, *b*). However, endodermal development is asynchronous (Fig. 4*a*). In Figure 4*a*, one of the protoxylem cells has begun to differentiate (arrowhead). This allows prediction of the future location of the four protoxylem poles (po) that will eventually develop (this was elucidated on the basis of sections taken further back in the root).

Mature endodermal cells in cluster rootlets

Mature rootlet endodermis possesses a primary wall, suberin lamellae and a secondary wall (Fig. 4*c*, *d*). Thus we see all the four stages of development described by Kroemer (1903, in Scott & Peterson (1979*a*)), that is: pro-endodermis (Fig. 2*f*), suberin lamellae (Fig. 4*c*, *d*), Casparian banding (Fig. 3*d*) and secondary thickening (Fig. 4*c*, *d*). The three layers of the wall are visible both between endodermal and pericycle cells (Fig. 4*c*) and between endodermal cells (Fig. 4*d*).

Endodermal differentiation in the determinate tips of mature cluster roots

When the cluster root meristem itself is differentiated, endodermal development continues in an acropetal direction towards a single initial cell which differentiates as an endodermal cell (Fig. 5*a–c*). In Figure 5*c*, only five derivatives are visible, because of the plane of section and the different sizes of the derivative cells. Also visible is the inner cortical initial, which differentiates as a cortical cell (Fig. 5*a*). It can be seen that these layers both form domes, culminating in their initial cells. The endodermal cells, and the endodermal initial change in appearance with age. In Figure 5*a*, *b* cells are younger, and show three distinct regions within the cell, whereas older cells in Figure 5*c* and inset show more even staining throughout the cell.

Figure 2. Cluster rootlet development in *Grevillea robusta*. (*a*) Tangential longitudinal section (T.L.S.) showing a young rootlet passing through the cortex (PC) of the parent root. This section was taken just behind the rootlet meristem. Remnants of the parental endodermis (PEn) are visible, surrounding the rootlet. Bar, 20 μm. (*b*) T.L.S. showing a cluster rootlet growing through the cortex of the parent root. This section shows a more mature rootlet than that in (*a*). Rootlet endodermis (REn) is beginning to differentiate and the parental collar (PEn) is less prominent. Bar, 20 μm. (*c*) T.L.S. through the endodermis of the parent root (PEn) showing the base of an emerging cluster rootlet. Much of the rootlet cortex (RC) is undergoing suberization. Bar, 30 μm. (*d*) T.S. of a cluster rootlet, outside the parent root. This section was taken through the meristem (M), and shows the rootcap (R) and dense meristematic zone (M). Bar, 25 μm. (*e*) T.S. lateral root with emerging cluster rootlet. In the rootlet, distinct layers of epidermis (RE), outer cortex (RC_o), inner cortex (RC_i) and endodermis (REn) can be followed to their initial cells. The parental endodermis collar (PEC) can be seen at the base of the rootlet. Bar, 75 μm. (*f*) L.S. of a lateral root of *G. robusta*, showing the rootcap (R) and meristem (M). The endodermis can be seen to develop through three characteristic stages: an undifferentiated stage (U), a vesicular stage (V) and an osmiophilic wall stage (W). Bar, 70 μm. PC, parental cortex; R, root cap; REn, rootlet endodermis.

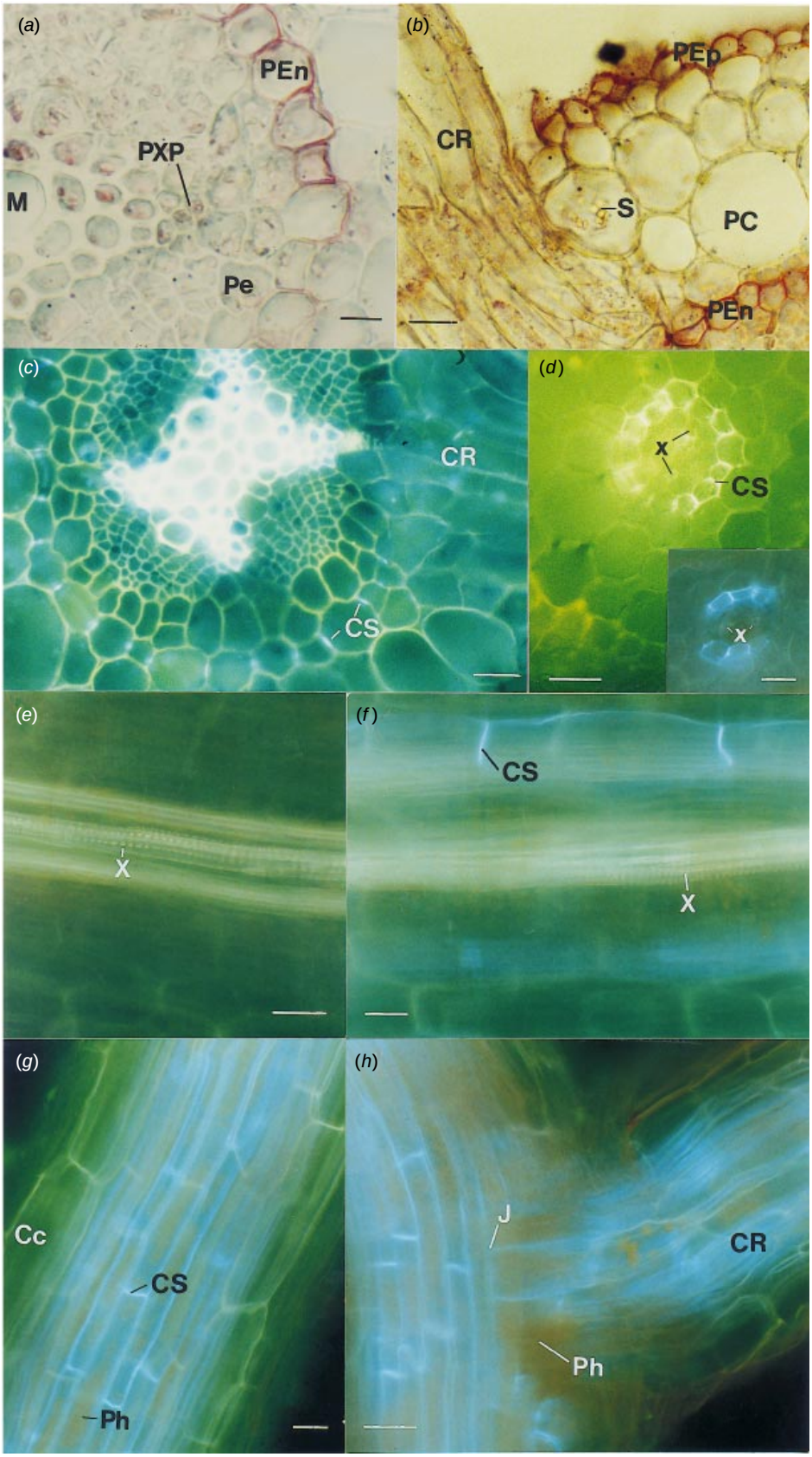

Figure 3. Endodermal characteristics in lateral roots and cluster rootlets. (*a*) T.S. fresh, hand-cut section with sudan red 7B staining of suberin lamellae within endodermal walls (PEn). The three cells of the pericycle (Pe) opposite the protoxylem pole have undergone a periclinal division. M, metaxylem element. Bar, 20 μm. (*b*) T.S. fresh hand-cut section of a lateral root, stained as in (*a*). Epidermis (PEp) and endodermis (PEn) stains red. S, starch; CR, cluster rootlet. Bar, 40 μm. (*c*) U.v. fluorescence. T. S. fresh, hand-cut section of lateral root, stained with the berberine–aniline blue procedure. The Casparian bands (CS) are clearly visible as turquoise strips between adjacent endodermal cells. CR, cluster rootlet. Bar, 50 μm. (*d*) U.v. fluorescence. T.S. fresh,

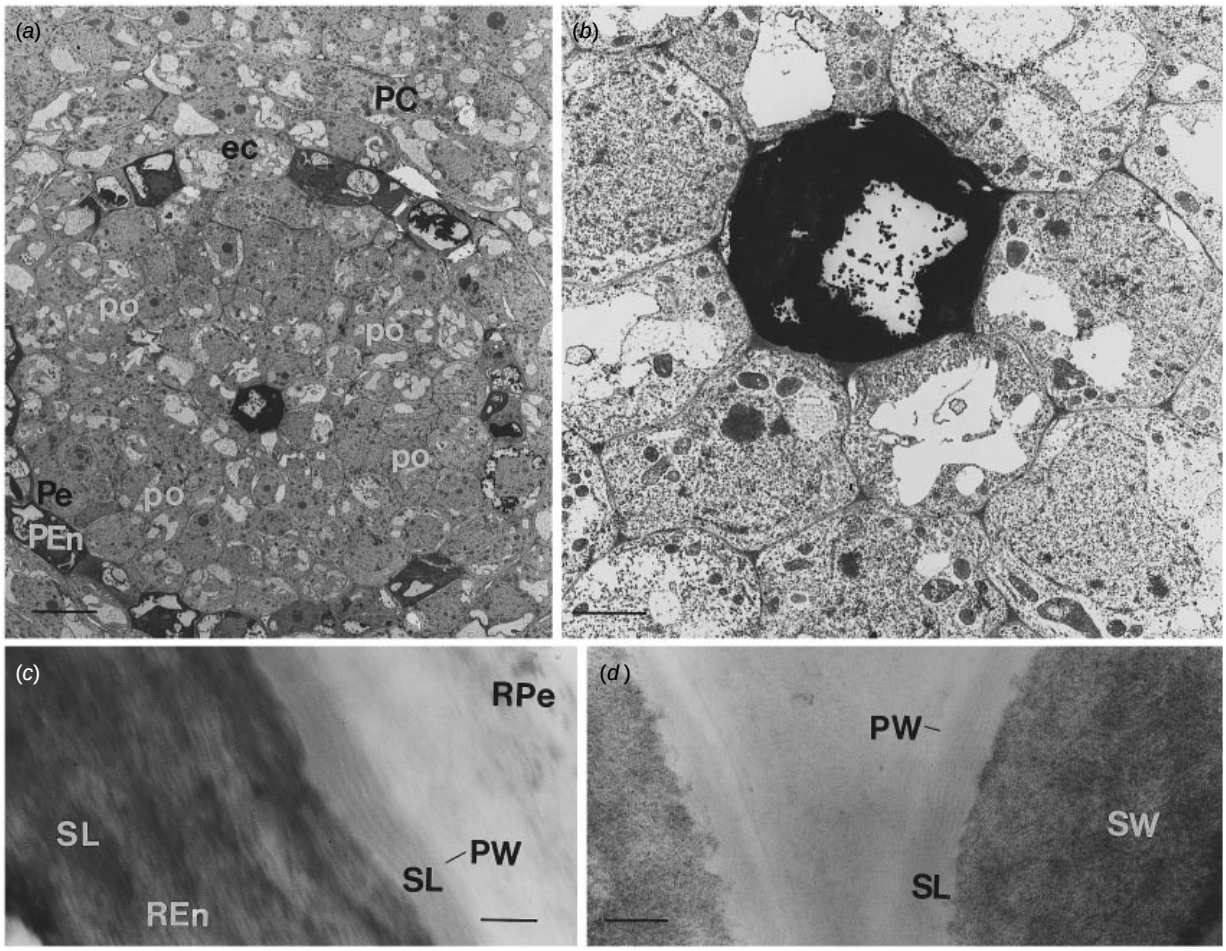

Figure 4. Endodermal development in a lateral root of *Grevillea robusta*. (*a*) TEM. T.S. of lateral root, 300 μm behind tip, showing endodermis (PEn) developing asynchronously around the stele. In the centre of the stele can be seen a differentiating metaxylem vessel. A xylem element in the mid-stele is discernible (arrow). Using it as a marker for a protoxylem pole, the positions of the four poles (po) can be predicted. Bar, 10 μm. ec, exceptional cell of late development not opposite a future protoxylem pole; Pe, pericycle; PC, parental cortex. (*b*) Detail from (*a*). The central metaxylem element is surrounded by seven other cells. Bar, 2 μm. (*c*) T.S. of junction between a rootlet endodermal cell (REn) and a pericycle cell (RPe). PW, primary wall; SL, suberin lamellae; SW, secondary wall. Bar, 50 nm. (*d*) T.S. Junction between two mature cluster rootlet endodermis cells showing primary wall (PW), suberin lamellae (SL) and a thick secondary wall (SW). Bar, 100 nm.

DISCUSSION

In this discussion, we have adopted Esau's definition of endodermis (1960): 'a cell layer constituting a boundary between vascular and non-vascular tissues and having Casparian strips or other types of suberized wall layers'. The endodermis does not develop all these characteristics simultaneously. Kroemer (1903, in Scott & Peterson (1979*a*)) defined four stages of endodermal development: formation of the pro-endodermis; the primary stage endodermis (with Casparian bands); the secondary stage endodermis (with suberin lamellae); and the tertiary stage endodermis (with additional cellulosic wall material).

In *G. robusta*, as a lateral root develops, young

hand-cut section of cluster rootlet, stained as in (*c*). Casparian band development (CS) can be clearly seen. x, protoxylem poles. Bar, 15 γm. Inset: section through a young rootlet, showing reduced suberization and Casparian band development opposite the xylem elements. Note the different types of Casparian band formation. Bar, 25 μm. (*e*)–(*h*) U.v. fluorescence micrographs of entire, cleared lateral roots using the combined clearing method. (*e*) 2500 μm from the apex. Both suberization, and a Casparian band, are absent. X, xylem. Bar, 60 μm. (*f*) 3600 μm from the apex, suberization has started and the Casparian band (CS) has begun to form, but not around the entire stele. X, xylem. Bar, 60 μm. (*g*) 4500 μm from the apex, all cells are suberized and the Casparian band (CS) is entire. Material (Ph), thought to be phenolic in nature, can be seen in the endodermal cells as orange fluorescence. Cc, cortical cells. Bar, 140 μm. (*h*) Site of cluster rootlet (CR) emergence from a parent, lateral root. The endodermis can be seen to join at a junction (J) with much orange fluorescence at this junction (Ph). Bar, 120 μm.

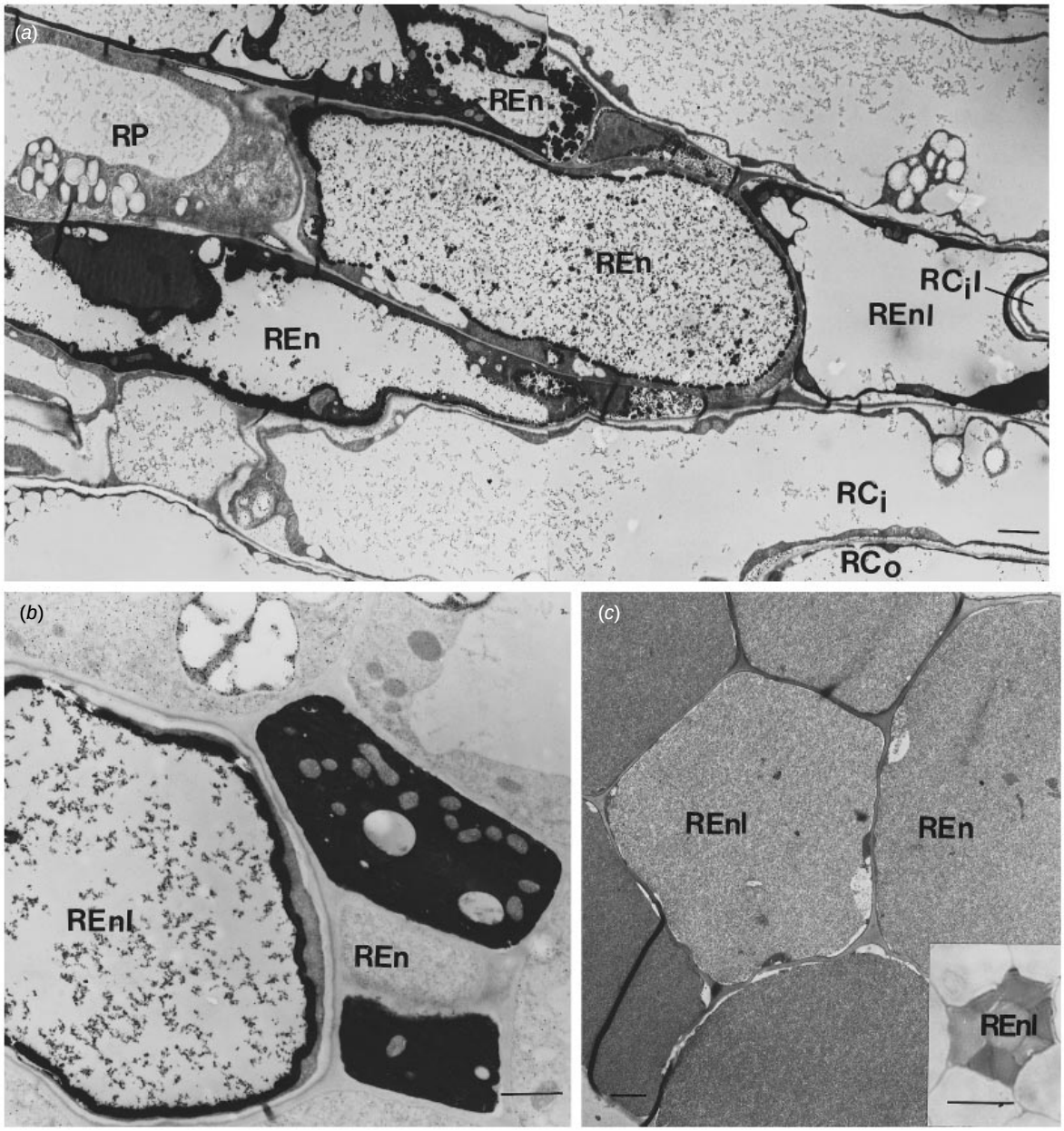

Figure 5. Apex of a mature cluster root. (*a*) Near median section (M.S.) of mature cluster rootlet, showing how the endodermis forms a dome around the tip of the stele. The endodermis initial (REnI) has differentiated as an endodermal cell. Three of the most recent derivatives from this initial cell can be seen (REn). The section is not truly median, and the initial cell continues between the derivatives. To the right of the endodermis initial, lies the inner cortex initial (RC_iI). RC_i, rootlet inner cortex; RC_o, rootlet outer cortical cell; RPe, rootlet pericycle cell. Bar, 3 μm. (*b*) LM. T.S. of rootlet, 28 μm from the tip. The endodermis initial (REnI) with one derivative cell (REn) visible. Bar, 2 μm. (*c*) T.S. of mature rootlet, 30 μm from tip. The initial cell (REnI), in the centre, has five derivative cells (REn) around it, all of which have dense osmiophilic cell contents. There are six inner cortical cells outside this. Bar, 2 μm. (*c*) Inset: LM, T.S. of mature rootlet, 30 μm from the tip. There are six endodermal cells around this initial (REnI). There are also six inner cortical cells outside, and 12 outer cortical cells (not shown) beyond this. Bar, 16 μm.

endodermal cells contain large osmiophilic bodies (Fig. 2*f*). These are similar to the lipid accumulations in *Ranunculus acris*, reported by Scott & Peterson (1979*b*), but are much larger. Casparian bands had always formed in lateral roots before cluster root development.

Asynchronous development

The question of asynchrony in development is of great interest. At the endodermal level, the reduced rate of differentiation opposite protoxylem poles (or, indeed, the accelerated rate opposite phloem

regions), is clear even before the poles appear (Fig. 4*a*), indicating that the asynchrony is a product, not of xylem or phloem differentiation, but of an earlier signal, either from the apex, more mature tissue, a xylem (or phloem) cell in its early stages of differentiation, or a combination of some or all of these.

Endodermal development in the young cluster rootlet

There has been much discussion over the contribution of parental endodermis to the emerging lateral root (see McCully (1975); Clowes (1978)). Our study has highlighted several important stages in endodermal development:

the reduced suberization of endodermis at the onset of pericycle division;
the breaking of this layer as the rootlet meristem develops;
the development of rootlet endodermis coinciding with the breakdown of the parental endodermal 'sleeve';
the re-differentiation of basal rootlet cortical cells into endodermal cells.

In Figure 3*a*, pericycle cell division is asymmetric, but not to the extent reported by McCully (1975) in *Zea*. The reduced staining for suberin in the endodermal layer adjacent to these divisions might be related to the division event in the pericycle. Barnabas & Peterson (1992) suggest that, in onion, passage cells might be the cause of delayed development of suberin lamellae.

Different types of Casparian band

Cluster rootlet endodermis differentiates in the standard way, with Casparian band, suberin lamellae and secondary thickening. The berberine–aniline blue staining procedure revealed three different types of Casparian band development: first, a localized point on the endodermal radial wall towards the cortex; second, a double Casparian band; and third, a larger, continuous band (Fig. 6). In three dimensions this band encircles cells in their radial and transverse walls. It is not known whether these three types are part of a single developmental sequence or whether they occur around all the radial walls. The Casparian band in the onion root first forms in the middle of the radial wall, at *c*. 10 mm from the apex. Later, at 70 mm from the apex, the band expands until, at 80 mm, it fills the entire radial wall (Barnabas & Peterson, 1992). Haas & Carothers (1975) report that in *Zea*, Casparian band formation is at a single, precisely determined site, with no shift in position. This has also been reported for *Hordeum vulgare* (Robards *et al*., 1973). Van Fleet (1961) reported that in *Smilax glauca* the Casparian band

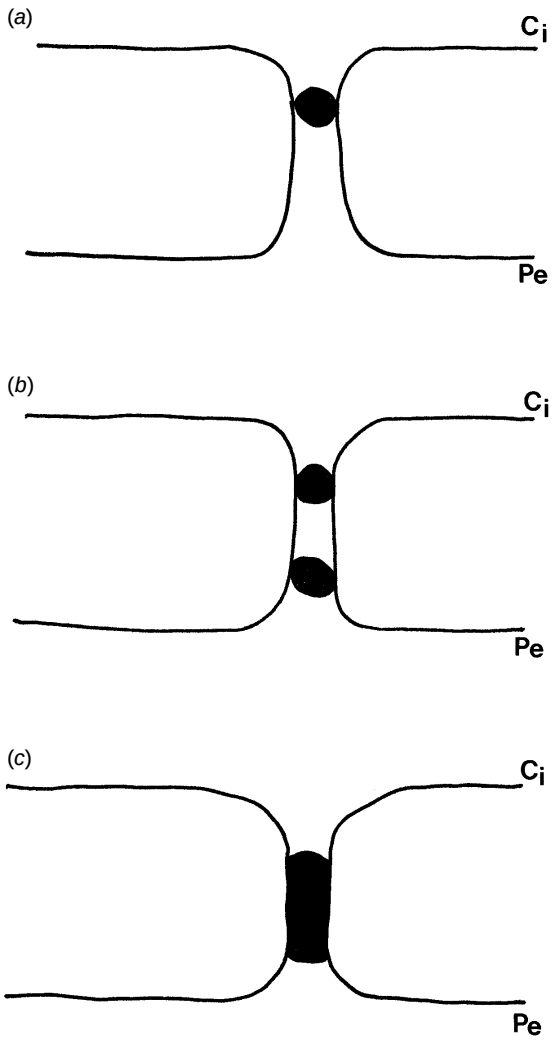

Figure 6. A schematic representation of the three different types of Casparian band development in cluster rootlets of *Grevillea robusta*. (*a*) A single localized point on the endodermal radial wall towards the cortex. (*b*) A double Casparian band. (*c*) A single band, wider than in (*a*). Ci, cortex; Pe, pericycle.

appeared to shift from a marginal to a more central position, but explained this in terms of radial enlargement of the cells involved. Schreiber *et al*. (1994), in work involving cellulase/pectinase digestion, showed that the Casparian band in *Clivia miniata* had a second cell-wall layer, apparently added onto the surface of the Casparian band, from the outer and inner side of the anticlinal wall. This might be what we have observed, with the second wall layer eventually forming a bridge, thus explaining the 'fusion' event.

Another possibility is that there might be two Casparian bands such as those noted by Brundrett *et al*. (1988) in onion and *Lilium* exodermis. The occurrence of single and double Casparian bands in adjacent cells as seen here in *Grevillea* and in the work by Brundrett *et al*. (1988) indicates either a developmental progression or some difference in development between these cells. The double Casparian band in onion exodermis does not seem to

be an early developmental stage because both double and single bands are found throughout the length of the root (Barnabas & Peterson, 1992). Carol Peterson (pers. comm.) has suggested that the presence of passage cells could be a basis for such differences in Casparian band formation. It is of interest that this anomaly, only reported previously in exodermis, has been observed in the endodermal tissue of cluster rootlets.

A further feature of endodermal development is that the Casparian band had not yet formed opposite the two xylem elements in the rootlet (the term 'protoxylem pole' seems irrelevant in that there are only two single files of xylem elements in each rootlet (inset, Fig. 3*d*)). Scott & Peterson (1979*a*) noted this non-uniformity in Casparian band formation at all levels of the root in *Ranunculus acris*. It has been suggested that endodermal cells opposite the protoxylem poles remain at the primary stage of development (Scott & Peterson, 1979*a*), acting as 'passage cells'. However in *G. robusta*, more mature cluster roots were found to have an entire Casparian band system and suberization around the stele.

Determinate development

Cluster rootlets are determinate (Purnell, 1960; Skene *et al.*, 1996). The final question relating to endodermal development stems from this determinate growth, namely, if the rootlet meristem itself differentiates, how does the endodermis terminate? Endodermal development continues as far as the 'initial' cell, which itself differentiates as an endodermal cell. This raises interesting questions concerning the fate of undifferentiated cells. Do they differentiate based on their lineage alone, or does cell position relative to neighbouring cell types also play a role? Certainly the endodermal cells that connect parental and rootlet endodermis originate as rootlet cortical cells.

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