A developmental framework for endodermal differentiation and polarity

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The endodermis is a root cell layer common to higher plants and of fundamental importance for root function and nutrient uptake. The endodermis separates outer (peripheral) from inner (central) cell layers by virtue of its Casparian strips, precisely aligned bands of specialized wall material. Here we reveal that the membrane at the Casparian strip is a diffusional barrier between the central and peripheral regions of the plasma membrane and that it mediates attachment to the extracellular matrix. This membrane region thus functions like a tight junction in animal epithelia, although plants lack the molecular modules that establish tight junction in animals. We have also identified a pair of influx and efflux transporters that mark both central and peripheral domains of the plasma membrane. These transporters show opposite polar distributions already in meristems, but their localization becomes refined and restricted upon differentiation. This “central–peripheral” polarity coexists with the apical–basal polarity defined by PIN proteins within the same cells, but utilizes different polarity determinants. Central–peripheral polarity can be already observed in early embryogenesis, where it reveals a cellular polarity within the quiescent center precursor cell. A strict diffusion block between polar domains is common in animals, but had never been described in plants. Yet, its relevance to endodermal function is evident, as central and peripheral membranes of the endodermis face fundamentally different root compartments. Further analysis of endodermal transporter polarity and manipulation of its barrier function will greatly promote our understanding of plant nutrition and stress tolerance in roots.

Casparian strips | cell polarity | root development | endodermis | Arabidopsis

The endodermis is one of the fundamental cell layers found in the roots of higher plants (1). It sets up a diffusion barrier between the extracellular space of the root cortex, connected to the soil, and that of the vascular tissue, connecting the root with the aboveground organs. The endodermis is thought to be crucial for the efficient and selective uptake and sequestering of nutrients from the soil, necessary for plant survival (1). Specification of endodermal cells is very well understood and thought to depend on the SHR transcription factor. SHR acts as an evolutionary conserved short-range signal that moves out from the inner tissues of the stele, thereby promoting asymmetric cell division and specification of the innermost cortical tissue layer into endodermis (2). It is entirely unknown, however, how and when this initial specification event is translated into differentiation of the endodermis. The best known feature of a differentiated endodermal cell is the “Casparian strip,” a highly localized cell wall deposition in the transversal and anticlinal walls of the cell, which surrounds the cell like a belt and is tightly coordinated with respect to neighboring cells (Fig. 1*A*). The Casparian strip thereby forms a supracellular network between cells within the layer, sealing the extracellular space (3). It has been known for a long time that this cell wall modification is associated with special features of the underlying plasma membrane (PM), which was shown to be very electron dense and to be tightly attached to the cell wall upon plasmolysis (4).

Because of the central position of the endodermis, its PM is exposed to two very different compartments. One domain faces the periphery of the root, the cortex, because it is peripheral to the Casparian strip barrier. The other domain faces the central part of the root, the stele, because it lies central to the barrier defined by the Casparian strip. Accordingly, transporters in the endodermis should localize either to the outer (peripheral) or the inner (central) domain of the endodermal PM, depending on their function in uptake or loading of nutrients. Indeed, it has recently been shown in rice that a pair of silicon transporters localize either to the outer or to the inner domain of the PM of differentiated exodermal and endodermal cells, in accordance with their function in influx or efflux (xylem loading) of silicon (5). Currently, most of our knowledge about endodermal structure and function comes from studies in organisms other than Arabidopsis and has mostly been concerned with a description of already differentiated endodermal cells through electron micrographs and histochemical staining procedures. Because of this limitation, we are ignorant about when and how an endodermal cell differentiates and we lack the marker lines and tools that would allow us to study this process in a mechanistic and developmental fashion.

Here we report the establishment and use of molecular markers for the analysis of endodermal polarity and Casparian strip (CS) formation in Arabidopsis. Using these markers in a quantitative analysis, we establish the developmental sequence of events that leads to a differentiated endodermal cell. We demonstrate establishment of a PM subdomain that coincides with the formation of the Casparian strip. This subdomain diffusively separates inner from outer PM domains and mediates tight adherence to the cell wall: the plant extracellular matrix. In addition, we show that endodermal polarity is defined independently of this subdomain, is present already in meristems, and becomes established early during embryogenesis. Inner and outer polar markers appear to have distinct trafficking requirements, but use common polar cues that are different from those establishing apical–basal PIN polarity. PIN proteins are efflux carriers for the plant hormone auxin, often localize to the apical or basal plasma membrane domains of cells, and define their orientation with respect to the organ or body axis (6).

Results and Discussion

Endodermal Differentiation Occurs in a Narrow Developmental Window.

We established a PM marker line with endodermis-specific expression to allow a precise visualization of exclusively endodermal cells in live imaging. We measured both the speed and the duration of elongation of cells after their exit from cell division, as well as their position with respect to their length (Fig. 1*A* and *B*,

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of stepwise maturation. The finding that the CSD causes diffusional separation between the inner and outer domains of the PM highlights a very distinct feature of the endodermis, which should have strictly separated polar domains, very unlike the situation found in the apical–basal polarity of PINs.

Endodermal Polarity Is Already Present in Meristems and Is Organized with Respect to the Central Stele. The only proteins shown to mark outer and inner domains of the endodermis are the silicon transporters Lsi1 and Lsi2 in rice (5). As in the case of our generic PM marker YFP-NPSN12, we used the cell-layer-specific *SCARECROW* (*SCR*) promoter that drives expression exclusively in the endodermis. This allows good image acquisition of this inner cell layer and endodermal signals are not confounded with signals from neighboring cells. We tested fluorescent protein fusions of a number of candidates that were reported to have a function in the endodermis or to localize to an outer membrane domain in other cell types. The rice silicon transporters did not display a polar localization in Arabidopsis. OsLsi1 accumulated in the ER, whereas OsLsi2 showed a weak, apolar localization (Fig. S2). Proteins reported to localize to the outer domain of epidermal cells in Arabidopsis, such as BOR4 (12), or that showed weak, apolar signals in the endodermis, such as other transporters (AHA4, NRT1;1, DSP/WBC11), did not accumulate and show also weak apolar signals (Fig. S2). Yet, two boron transporters, NIP5;1 and BOR1, were additional good candidates for polarity markers, on the basis of their reported function in uptake and xylem loading (efflux) of boron (13–15). Indeed, in these cases, a clear polar localization to the inner and outer domains of the PM was observed for BOR1 and NIP5;1, respectively (Fig. 2*A* and *B*). Thus, we have identified markers that define two complementary plasma membrane domains within the endodermis of Arabidopsis. These findings are also reported in an independent work on BOR1/NIP5;1 polarity in an accompanying paper by Takano et al. (16).

As expected from our analysis, signals of both proteins showed a sharp drop in intensity across the transversal PM at the position of the CSD (Fig. 1*G* and *I*). We then investigated whether the CSD is necessary for the establishment of this polarity. The *SCR* promoter used also drives expression in meristematic pro-endodermal cells, initials, and the quiescent center (QC) cells. To our surprise, both boron transporters also accumulated in a polar fashion in these undifferentiated cells (Fig. 2*C* and *D*). However, localization in these cases was less restricted and extended in a gradient across the entire transversal PM. This result suggests that the Casparian strip domain is not needed for establishment or maintenance of polarity per se, but rather is “built into” an already polarized setting and acts to refine and separate partially overlapping polar domains. To visualize this separation directly, we generated a line expressing the two proteins fused to spectrally distinct fluorophores. The two proteins indeed showed significant overlap in transversal cell sides in undifferentiated cells (Fig. 2*E*), but became completely separate at the point of CSD establishment (Fig. 2*F*).

In a median optical cut, BOR1 polarities in the cells to the left and the right of the stele are facing each other. The *SCR* promoter allows observation of what happens to polarity in the quiescent center cells, where cells of the left and right cell files meet and might show an opposite or apolar localization. Contrary to our expectations, polarity of BOR1 was maintained, but gradually changed to apical when approaching the quiescent center (Fig. 2*G*). The reverse happened in the case of NIP5;1, which gradually changed to a more basal localization (toward the base of the plant, the root tip) (Fig. 2*H*). These observations are strongly suggestive of a scenario in which polarity in the endodermis does not follow global coordinates, such as left and right, apical or basal, but always orients toward or away from the center of the root, the stele. We tested if orientation of polarity toward the stele is a special feature

Fig. 2. Endodermal polarity is different from PIN polarity and organizes with respect to the stele. (*A–D*) Signals of BOR-mCit and mCit-NIP5;1 at opposite cell sides in differentiated (*A* and *B*) and meristematic (*C* and *D*) cells. (*E* and *F*) Colocalization of BOR1-mCit (*Left*), mCherry-NIP5;1 (*Center*), and overlay (*Right*). Colocalization can be observed in meristematic (*E*), but not in differentiated cells (*F*). (*G* and *H*) Localization of BOR1-mCit and mCit-NIP5;1 in quiescent center and initials and proendodermal cells. BOR1 signals polarize toward NIP5;1 signals away from the stele. (*I* and *J*) Signals of BOR1 and NIP5;1 in nonendodermal cells, expressed from 35S or UBQ10 promoter, respectively. Signals from neighboring cells are confounded, and the same orientation of polarity is observed as in the endodermis. (*K*) Localization of PIN2-GFP is apolar in quiescent center and basal in initials and pro-endodermal cells. Localization switches to apical in elongating cells (*K'*) and to the central side in differentiated cells (*K''* and *K'''*). (*L*) PIN1-GFP shows basal localization in endodermis. Overview (*Upper*) and zoom in (*Lower*) are shown. ct, cortex; en, endodermis; ep, epidermis; st, stele. Open arrows indicate direction of polarity of individual cells. Arrowhead indicates position of the CSD (Scale bar: 10 μ m.)

of the endodermis, which is immediately bordering and enclosing the stele. We therefore used lines expressing BOR1 and NIP5;1 under constitutive promoters. Polarity of individual cells is more difficult to observe, because of signals from neighboring cells. However, careful inspection of cortical, epidermal, and root cap initial cells also revealed a polar accumulation toward or away from the stele (Fig. 2*I* and *J*). In our opinion, our observations are explained most readily in a scenario in which signals emanating from the stele act as polarizing cues for more peripheral tissue layers of the root.

The observed polar localization of the boron transporters in the quiescent center also illustrates that the polar cues and machinery used to orient boron carriers in the endodermis are different from those orienting PIN proteins, because PINs are

mostly apolar in these cells (17, 18). To further investigate this difference, we used a line expressing PIN2 from the SCR promoter (19). We observed a weak, but completely apolar signal of PIN2 in the quiescent center (Fig. 2*K*). Outside the quiescent center, it did not polarize like either of the boron transporters, but showed a basal localization, as reported for PIN2 in the cortex (Fig. 2*K*). This polarity then switched to a very shallow localization gradient toward the upper (apical) side of the cell (Fig. 2*K'*). A similar switch was reported previously for PIN2 localization in cortical cells (20). In a differentiated endodermis, PIN2 signals became weaker and the signal started to preferentially accumulate at inner sides (Fig. 2*K''* and *K'''*). Like YFP-NPSN12, PIN2-GFP was excluded from the CSD (Fig. 2*K''*, arrowhead). Thus, PIN2 expressed in the endodermis showed a very different localization pattern from that of either BOR1 or NIP5;1, in QC, meristematic, and elongating cells, showing that it follows different polarization cues in those cells. PIN2 retains a propensity to localize in a polar fashion along the apical–basal axis. Its eventual accumulation at the central side of cells might simply be due to a slightly delayed turnover of protein from the central side. We observed similar polar accumulation with DSO/WBC11 and BOR4 immediately before their complete disappearance (Fig. S2 *I* and *L*). We also investigated PIN1, a protein that shows a weak expression in the endodermis under its endogenous promoter. Using PIN1-GFP, we were able to observe a basal polarity of PIN1-GFP, as observed for PIN2 (Fig. 2*L*). On the basis of this observation, we conclude that endodermal cells in the meristem are able to define at least three different polar distributions of PM proteins: toward the center (stele), toward the periphery (cortex), and toward the base of the plant. PIN3 has been reported to localize differently from PIN1 and PIN2 in proendodermal cells, in a way that more resembles the center-oriented polarity of BOR1, suggesting that BOR1 and PIN3 might be able to use the same trafficking pathway in endodermal cells, distinct from that of other PINs (21).

The Polarity Realized in the Endodermis Is Established Early During Embryogenesis. Our finding that the “central–peripheral” polarity of endodermal cells is not a feature of differentiated endodermal cells, but preexists in the meristem, begged the question at which point endodermal polarity is initially established. We therefore investigated at which point polar localization of BOR1 and NIP5;1 can be observed during embryogenesis. To our surprise, we found that both BOR1 and NIP5;1 are already localized in a polar fashion in heart and triangular-stage embryos (Fig. 3*B, C, F*, and *G*). In globular-stage embryos we observed polar localization in the lens-shaped precursor cell of the quiescent center in most embryos (Fig. 3*A* and *E*). No signals were observed in earlier embryo stages for BOR1 and occasional NIP5;1 signals in the undivided hypophysis were apolar. Thus, polarity cues are apparently becoming established at the time when the formative divisions of the provasculature are taking place. Again, these findings suggest a clear difference between PIN polarity and the polarity visualized by the boron transporters. PIN1, PIN4, and PIN7 are expressed in the lens-shaped cell, but their localization appears mostly apolar, although some degree of polarization cannot be excluded (18, 22). Our markers now provide a clear indication of the presence of complementary polar domains within the lens-shaped cell. This cell is produced by an asymmetric division, gives rise to the stem cell organizing center, and faces a clonal boundary (23). Therefore, the ability to visualize its orientation and polarity might be of importance for understanding its function.

Polarity in the Endodermis Does Not Require a Polarized Cytoskeleton, but Needs Endocytic Trafficking. We then investigated the cellular basis of the endodermal polarity and subdomain establishment. We first asked whether there are any specific arrangements of the actin cytoskeleton associated with either the CSD or any of

Fig. 3. Central–peripheral polarity becomes established early in embryogenesis. (*A–C*) BOR1-mCit signals in quiescent center precursor cells, initials, and proendodermal cells display polarity oriented toward the center of the embryo. Arrows point to cell sides with visible polar accumulation. (*Left*) Transmitted light image. (*Right*) Confocal image. (*D*) Number of embryos observed with polar BOR1-mCit signals over total number of embryos inspected. (*E–G*) mCit-NIP5;1 signals in quiescent center precursor cells, initials, and proendodermal cells display polarity oriented toward the periphery of the embryo. Arrows point to cell sides with visible polar accumulation. (*Left*) Transmitted light image. (*Right*) Confocal image. (*H*) Number of embryos observed with polar mCit-NIP5;1 signals over total number of embryos inspected. (Scale bar: 10 μ m.)

the two polar (central vs. peripheral) domains. Polar actin accumulation is involved in polarity establishment in a number of different cellular systems (24). We again expressed an actin microfilament reporter (YFP-Fimbrin) specifically in the endodermis, to obtain images of sufficient quality. Fig. 4*A–C* shows sequences of images from equivalent stages in the epidermis and endodermis. No evident accumulation or special arrangements of microfilaments were observed in endodermal cells at any developmental stage (Fig. S3), either toward one or the other PM domain or at the site of CSD formation. This observation contrasts, for example, with the easily observable sites of root hair outgrowth in epidermal cells (Fig. 4*A*). Thus, in contrast to many cell types in other organisms, endodermal cells are apparently able to polarize and establish the CSD without using a polarized actin cytoskeleton for vesicle delivery.

Consequently, interfering with actin polymerization in the endodermis by Latrunculin B (LatB) treatment (Fig. 4*D*) had no effect on the polar localization of either BOR1 or NIP5;1 (Fig. 4*G* and *H*). The Casparian strip domain was also maintained in the presence of the actin depolymerizer (Fig. 4*E* Lower). To determine whether the actin cytoskeleton is required during the establishment of the CSD, we quantified the cellular distance from the meristem at which the depletion zone of the CSD appeared. A shift away from the meristem would indicate that nondifferentiated cells at the beginning of the treatment (Fig. 4*E* Upper) did not form a new CSD. No significant shift was observed after a 5-h treatment (Fig. 4*F*), which would suggest that the actin cytoskeleton is also not needed for establishment of the CSD. A similar analysis was done for the microtubule cytoskeleton, with similar results; i.e., we did not observe any specific microtubular structures in the endodermis or any effect of MT depolymerization (Fig. S4).

Fig. 4. Dependence of endodermal differentiation and polarity on actin and vesicle trafficking. (A) 35S::YFP-Fimbrin highlights actin during root hair formation in epidermis. (B and C) Surface (B) and median optical section (C) of SCR::YFP-Fimbrin. No localized accumulation of actin can be seen. (D) Signals after 1 h treatment with 10 μ M LatB. (E, G, and H) Localization of BOR1, NIP5;1, or NPSN12 before and after differentiation (top and bottom) at the PM is unaffected after LatB treatment (10 μ M, 1 h). (F) No shift in meristem distance is observed for the NPSN12 depletion zone after LatB treatment (10 μ M, 5 h). (I) PM localization of NPSN12 is unaffected after BFA treatment (50 μ M, 90 min) before (Left) and after (Right) differentiation. (J) No shift in meristem distance for the NPSN12 depletion zone is observed after BFA treatment (50 μ M, 5 h). (K and L) BOR1 and NIP5;1 polarity is unaffected by BFA treatment (50 μ M, 90 min). (M) WM treatment (50 μ M, 1 h) effects of YFP-RabF2b, a WM-sensitive compartment marker in endodermis and stele, as seen by the higher background, irregular dots, and ring-like structures (Lower), compared to control (Upper). (N) WM treatment (50 μ M, 1 h) leaves the NPSN12 depletion zone (CSD) intact (Left) and does not affect localization before differentiation (Left). (O) WM treatment (50 μ M, 1 h) does not affect BOR1 polarity in elongating cells. (P) WM treatment (50 μ M, 1 h) leads to complete depolarization of NIP5;1 in cells before differentiation (Left), but does not affect polarity in differentiated cells (Right). Arrowheads indicate position of the CSD. (Scale bars: 10 μ m and (E) 5 μ m.)

We then tested two widely used inhibitors of membrane trafficking in plants, the endosomal recycling inhibitor Brefeldin A (BFA) and the endocytic inhibitor Wortmannin (WM). BFA treatment led to accumulation of BOR1, NIP5;1, and NPSN12 in

endosomal aggregates (BFA compartments) in differentiating endodermal cells (Fig. 4 I, K, and L). This result showed that all three proteins are trafficked at least partially through BFA-sensitive endosomes and that BFA accumulated and was active in the endodermis. However, BOR1 and NIP5;1 still retained their polar accumulation at the PM, both before and after CSD establishment (Fig. S5). This result is unlike the depolarization observed for PIN1, for example, but resembles the BFA-resistant localization of apically localized PIN2 in the epidermis (25, 26). In contrast to our results, a BFA sensitivity of BOR1 polarity in meristematic epidermal cells was observed by Takano et al. (16) in an accompanying paper. It remains to be seen whether this observation points to some underlying difference in endodermal vs. epidermal transport pathways or reflects some subtle differences in the experimental setup. No shift was observed in the onset of the depletion zone after BFA treatment (Fig. 4J).

WM treatment, by contrast, led to strong depolarization of NIP5;1 at the PM before CSD establishment (Fig. 4P Left), whereas it left BOR1 polarity and the CSD unaffected (Fig. 4N and O). Intriguingly, NIP5;1 polarity became resistant to WM in cells that had established Casparian strips (Fig. 4P Right). Again, we could show that WM penetrates and acts in inner cell layers, using a marker line for a WM-sensitive compartment (Fig. 4M) (27). One straightforward explanation for this striking difference in WM sensitivity is based on our observation that the CSD suppresses lateral diffusion and separates the two polar domains. It has repeatedly been reported that WM affects internalization of proteins from the PM, which might be independent—or an indirect consequence—of its action on the endosomal PI-3 kinase (28–30). Lower WM concentrations expectedly induced accumulation of BOR1 and NIP5;1 in aberrant endosomes, revealing that a certain fraction of both proteins became endocytosed and trapped in these compartments. However, no accumulation was observed at the higher concentrations that led to depolarization of NIP5;1 (Fig. S5). This observation can only be explained by Wortmannin acting on an earlier step of endocytosis at higher concentrations, most probably inhibiting internalization from the PM. Indeed, inhibition of PM internalization would explain depolarization of NIP5;1 by allowing it to distribute within the entire PM by lateral diffusion, even if polar targeting would remain intact. Such a case has been observed for a polar PM SNARE in budding yeast, for example (31). The resistance to WM in differentiated cells would then simply be due to the suppressed lateral diffusion by the CSD, which confines NIP5;1 within its polar domain, even if endocytosis is blocked. The relevance of the CSD in maintaining polarized distributions of proteins is also highlighted by independent findings of Takano et al. (16) in the accompanying paper.

Conclusion and Perspectives. The hormonal and transcriptional networks that determine meristem size and progression into elongation have started to become unraveled in recent years (32). However, much less is known about the actual differentiation that is the end result of the patterning processes in the meristem. This lack is reflected in the vague term “elongation and differentiation zone” (EDZ) that is often employed for this region. We have now defined a set of differentiation readouts for the endodermis. This set, in combination with long-term *in vivo* tracking of endodermal cells, now places endodermal differentiation in a precise time window and at a defined cellular distance from the meristem. Our work provides the basis for identifying genuine endodermal differentiation genes and contributes to connecting the current gap between cell-fate specification and actual cellular differentiation (32). The orientation of polarity of peripheral cell layers with respect to the stele suggests a scenario whereby the stele provides polar cues to the peripheral root region. In our view, the terms central (facing the center of the root/plant) and peripheral (facing the periphery) are well suited to describe this polarity. They are generally applicable to all cell types and probably best encompass

the function underlying this polarity, i.e., vectorial transport of substances toward the center or their expulsion toward the periphery. Alternative terms in the literature are “proximal” and “distal” (16). We demonstrate that the CSD has features of both tight and adherens junctions. It therefore resembles polarized epithelia of animals. Yet, the endodermis lacks all of the central factors that establish polarized epithelia in animals, such as PAR proteins, Claudins, E-Cadherins, ZO proteins, or others (33–36). We are now identifying and characterizing the factors that make up the plant tight-junction equivalent, the CSD. It will be intriguing to understand how plants have independently evolved the capacity to organize a selective and polarized cellular barrier, one of the first and most fundamental features of multicellular organisms (33). Both polarity and CSD domain establishment are immediately relevant to the cellular barrier function of the endodermis. Other cell types also maintain a central–peripheral polarity, which will contribute to the vectorial transport of nutrients toward or away from the stele (12) (ref. 16 and our work). It is only the endodermis, however, where central and peripheral PM domains face different extracellular spaces, as is the case for polarized animal epithelia. We predict that understanding and manipulating endodermal CSD formation and polarity will provide us with a new level of understanding of how plants manage to selectively take up and reject substances from the soil and how they ascertain plant integrity and homeostasis in the face of various biotic and abiotic stresses.

Materials and Methods

Plasmid Construction and Transformation. Standard cloning procedures were used for plasmid construction. For information about constructs, see *SI Materials and Methods*.

Plant Material and Growth Conditions. Columbia background was used for all experiments. Plants were germinated after 2 days at 4 °C in the dark. Seedlings were grown in Percival chambers at 22 °C, under long days (16 h light/8 h dark), and were used at 4–6 days after germination. For growth conditions for live imaging see *SI Materials and Methods*.

Microscopy, Quantitative Analysis, and Image Processing. Confocal laser scanning microscopy was performed on an inverted Leica TCS SP2 AOB5 confocal microscope. Image processing was done with ImageJ from the National Institutes of Health using plug-ins from the MBF ImageJ bundle (<http://www.macbiophotonics.ca/imagej/>). “Onset of elongation” was defined as the point when an endodermal cell in a median optical section was clearly more than twice its width. From there, cells in the file were counted until the respective differentiation feature was observed. For live imaging experiments, different z-axis pictures (minimum of three) were collected to be able to follow cells through z-axis drift.

Detection of Casparian Strip Autofluorescence. Autofluorescence of Casparian strips was detected with standard GFP filter sets after clearing of roots according to ref. 9.

Embryo Analysis. Analysis was done as described in ref. 37.

Tracer, Drug, and Plasmolysis Assays. For incubation conditions and dye and inhibitor concentrations, see *SI Materials and Methods*.

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