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Root layers: complex regulation of developmental patterning

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SUMMARY OF RECENT ADVANCES

Developmental patterning events involve cell fate specification and maintenance processes in diverse, multicellular organisms. The simple arrangement of tissue layers in the *Arabidopsis thaliana* root provides a highly tractable system for the study of these processes. This review highlights recent work addressing the patterning of root tissues focusing on the factors involved and their complex regulation. In the last two years studies of root patterning have indicated that chromatin remodeling, protein movement, transcriptional networks, and an auxin gradient all contribute to the complexity inherent in developmental patterning events within the root. As a result, future research advances in this field will require tissue specific information at both the single gene and global level.

INTRODUCTION

Over the past two decades studies of primary root patterning in *Arabidopsis thaliana* initiated using classical genetic approaches were extended to the molecular level by the advent of molecular biology techniques, and then were propelled into the genomics era with the sequencing of the *Arabidopsis* genome. These approaches have led to substantial insights into cell fate specification and the positioning of the stem cell niche within the root. The simplicity and transparency of the *Arabidopsis* are the keys that have unlocked these discoveries. The *Arabidopsis* primary root is composed of concentric rings of tissue layers along the radial axis and morphologically distinguishable developmental zones along the longitudinal axis.

Specifically, from the root tip to the root-stem junction exist distinct zones that are visible as regions of small, actively dividing cells (called meristematic cells), elongated cells, and terminally differentiated cells marked by the root hairs of the epidermis (Figure 1, Figure 3). As such, the position of each cell along this axis can be used to infer its developmental age.

The root has an outer layer of epidermis and an inner core of vascular tissue that is spatially separated by a layer of ground tissue (Figure 2A). This elegant radial organization is derived from asymmetric cell divisions of stem cell initials and the daughter cells they produce. For instance, the ground tissue is generated from asymmetric division of the cortex-endodermal initial cell (CEI) to renew itself and produce a daughter cell (CED) that subsequently divides to generate the endodermal and cortex cell lineages [1•].

A small population of cells that rarely divides, called the quiescent center (QC) is surrounded by undifferentiated stem cells, such as the CEI, from which the different tissue layers arise. Analogous to animal systems, the QC and stem cells of the *Arabidopsis* root, together termed

the plant stem cell niche, possess the ability to renew themselves and are essentially ageless unlike the daughter cells they produce [2,3]. Since plants are immobile, this pattern is not achieved by cell migration as it is in many animals, but rather specified by positional information exchanged between cells [1•].

In the last two years, microarray profiling of these cell populations comprising the QC, root cell layers and longitudinal zones, termed the 'root map', has resulted in abundant gene expression information in both space and time with resolution unprecedented in any other multicellular organism [4•]. Modeling of the localization and directional activity of the PINFORMED1 (PIN) proteins that transport auxin has unveiled an auxin gradient robust to perturbations in auxin concentrations [5••]. Remarkably, the PLETHORA (PLT) transcription factors are expressed in graded patterns resembling this auxin gradient and when mutated, shift the boundaries between zones along the longitudinal axis [6•].

In contrast, along the radial axis the GRAS family transcription factor SHORT ROOT (SHR) has been demonstrated to specify a single layer of endodermis within the ground tissue via its movement from the central vascular tissue into neighboring cells where its interaction with a transcription factor of the same family, SCARECROW (SCR), leads to its sequestration into the nucleus. Once in the nucleus, SHR is restricted from moving to outer cell layers and specifies this single layer of endodermis by regulating a number of transcription factors, including SCR which in turn positively regulates itself [7••]. This is just one example of a tightly-controlled, complex regulatory process embedded in the beguilingly simple patterning of the root. A direct link was also recently demonstrated between chromatin remodeling of the upstream region of the HDZIP transcription factor GLABRA2 (GL2) by the GL2 modulator (GEM) and epidermal patterning [8••]. This review focuses on these landmarks in root patterning research in relation to other findings over the last two years in a framework of the root's tissue layers and their underlying complexity at the molecular level.

From the outside in: Epidermal patterning in the root

Specification of hair (H) versus non-hair (NH) cell fate in the epidermal tissue layer is known to involve an intricate network of transcription factors. Specifying NH cells are the GLABRA2 (GL), GLABRA3 (GL3), ENHANCER OF GLABRA3 (EGL3), TRANSPARENT TEST GLABRA (TTG), and WEREWOLF (WER) transcription factors, while the CAPRICE (CPC), TRIPTYCHON (TRY), and ENHANCER of TRIPTYCHON and CAPRICE (ETC) transcription factors specify H cells [9]. The exact model of NH versus H cell fate is an active subject of controversy in this field. However, most models include results from previous studies suggesting a TTG/GL3/EGL3/WER transcriptional complex binds to the GL2 promoter to repress root hair cell fate, while the same complex simultaneously induces CPC expression in NH cells [9]. CPC then moves into neighboring epidermal cells to repress GL2 expression, resulting in H cell specification [9].

A new player to this model, TRANSPARENT TESTA GLABRA2 (TTG2) was implicated in epidermal cell specification from experiments using transgenic plants expressing a chimeric protein of TTG2 fused to the EAR repression domain (TTG2:SRDX) [10]. In these transgenic roots, GL2 expression, as detected using a GL2:GUS promoter-reporter fusion construct, was repressed resulting in ectopic root hair formation. Further analysis of reporter lines in the TTG2:SRDX background showed that *TTG2* itself, *GL2*, and *CPC* expression was reduced, while *TTG1*, *WER*, *GL3*, and *EGL3* expression was relatively unchanged [10]. These data imply that in NH cells, TTG2 can activate expression of itself, *GL2*, and *CPC* independently of a *TTG1/WER/GL3/EGL3* transcriptional complex. Using various promoter-deletion constructs and one-hybrid analysis they also showed that WER most likely binds to a specific MYB regulatory element in the TTG2 promoter [10]. Thus while TTG2 can activate GL2

independently, it does not do so until it is activated by the *TTG1/WER/GL3/EGL3* complex. This suggests a new step exists in the regulatory cascade of epidermal patterning involving this WRKY family transcription factor in NH cells. However, as *ttg2* does not exhibit epidermal patterning defects in the root, it is unclear how critical this new step is in the cascade. This is also true of the newly proposed TRY lateral inhibition feedback loop [11]. The *try* mutants have a normal epidermal cell type pattern, even though the author's expression studies of *cpc try* and *gl2* mutants indicate that TRY, like CPC, is part of a regulatory loop in which GL2 promotes *TRY* expression and then TRY represses *GL2* expression in cells located in the H position [11]. Nevertheless, in the future these two loops may prove important in the timing and/or levels of protein movement and transcriptional complex action.

Elegant complementation and gel-shift experiments with chimeric proteins of the R3 MYB regions of WER and CPC revealed that the CPC R3 region cannot functionally substitute for the WER R3 region in H cell differentiation. They also showed that the CPC chimera protein containing the WER R3 motif binds to the GL2 promoter, but not the WER chimera protein containing the CPC R3 [12•]. These data support a model of competition between WER and CPC in transcriptional complexes regulating GL2 expression in epidermal patterning [12•]. The recent identification of yet another *CPC* gene in *Arabidopsis* [13] further illustrates the point that the number of transcription factor complexes and combinatorial and competitive interactions is dauntingly large. Mathematical and computational models will be needed to fully understand their dynamics and a comprehensive model of all the factors involved. To test these models, experimental techniques will need to be developed and used to visualize transcription factor complex dynamics and biochemically purify complexes from H or NH cells *in planta*.

These studies suggest that a delicate balance exists between the different transcription factor complexes converging on promoters of GL2, CPC, and perhaps others, to specify hair and non-hair cells in the appropriate position within the epidermis (Figure 2B). A recent report of the GL2 expression modulator (GEM), a protein identified as interacting with the *Arabidopsis* homologue of the eukaryotic licensing factor for DNA replication (CDT) [8••], supports a new layer of complexity in the specification of H and NH epidermal cells [8••, 14]. *Arabidopsis* plants over-expressing GEM1 have reduced GL2 messenger RNA levels that correlate with the increased root hair density of these plants. The authors tested three possibilities for the GEM effect on GL2: (1) Direct binding of the GEM protein to the GL2 promoter, (2) GEM regulates expression of GL2 transcriptional regulators, and (3) GEM is recruited specifically to the GL2 promoter via protein-protein interaction with the TTG-GL3-EGL3-WER/CPC transcriptional machinery. Their results eliminated the former two possibilities and showed by a combination of yeast two- and three-hybrid experiments and pull-down assays that the third possibility was most likely from observed interactions between GEM and TTG1 [8••]. Not stopping there, the authors tested the histone modification status of GL2 and CPC promoters by ChIP experiments. They found that both promoters contained histone H3K9acK14AC acetylation and H3K9 methylation in *gem-1* plants that was absent in GEM over-expression plants, suggesting a repressive role for GEM in GL2 and CPC expression by histone modification [8••]. This is exciting because GEM provides a direct connection between the TTG transcriptional complex and chromatin modification of GL2. It will be interesting to learn if this is a tightly controlled switch of cell fate as previously proposed for the role of chromatin remodeling in root epidermal specification [15•]. It is also possible that chromatin remodeling provides a graded response mechanism corresponding to the amount of histone modification. In this context, it will be interesting to see if the degree of chromatin modification correlates with the developmental competence of epidermal cells along the root's longitudinal axis and/or the duration and intensity of exposure to environmental stresses in the modulation of root epidermal cell specification.

These studies emphasize the importance of future study of transcriptional complex dynamics, protein movement, and histone modification as aspects of regulation in root epidermal patterning. Next steps in the field will also include connecting information from these studies with that from studies regarding root hair outgrowth/shape and Ca^{++} signaling [16], auxin regulation of root hair positioning within a root hair cell [17•], and the role of signaling in transcriptional control of epidermal patterning [18].

Ground tissue patterning in the root

In the ground tissue patterning field, the buzz was all about the long sought after elucidation of the downstream targets of the GRAS family transcription factor SHORTROOT and its mode of action. The longitudinal cell division of the cortex/endodermal initial daughter cell (CED) does not occur in *shr* mutants and due to this only a single layer with cortex features is present in these mutants [19–20].

Meta-analysis of the results from microarray experiments of *shr-2*, an inducible SHR line in the *shr-2* background, and cells sorted for SHR:GFP expression identified 8 direct targets of SHR [21•]. Four of these were confirmed *in vivo* by ChIP-qPCR including the predicted target, the GRAS transcription factor *SCARECROW* (*SCR*), and two closely related C2H2 zinc finger genes *NUTCRACKER* (*NUC*) and *MAGPIE* (*MGP*) [21•]. The recently identified *JACKDAW* (*JKD*) is also a member of this subfamily [22]. SCR expression is abated in *jkd* mutant roots and yeast-two hybrid and onion bombardment Bimolecular Fluorescent Complementation (BiFC) assays suggest JKD interacts with itself as well as with SCR and SHR [21]. Taken together, the studies in [21•] and [22] imply a regulatory network exists between the GRAS family transcription factors and this C2H2 zinc finger subfamily. It will be interesting to see if the protein interactions found in yeast and onion cells can be demonstrated in *Arabidopsis* and how the different complexes formed by these families act to effect transcriptional changes. Based on the sizes of these families, one can imagine the interactions may be as entangled and challenging to study as those of the proteins involved in epidermal patterning mentioned above.

SHR action has been a hot topic since the vascular tissue (stele)-specific gene expression of *SHR* was first reported, seemingly at odds with the loss of endodermis observed in the ground tissue of *shr-2* mutants [19]. The SHR protein was later shown to move from the stele into the adjacent ground tissue, where it became nuclear localized [23–24]. However, while there were hints that this movement was limited by SCR [24], the mechanism for it remained unknown. Confocal microscopy of RNAi lines knocking down SCR (SCRi) to different levels and expressing SHR-GFP and pSCR::GFP led to insights into this mechanism [7••]. SCRi lines displayed supernumerary layers inversely correlating with *SCR* transcript levels and both pSCR::GFP and SHR-GFP were expressed in these layers, SHR-GFP primarily being localized to the nucleus. SHR-GFP was present in both daughter cells of the CEI and after each additional division the level of SHR-GFP was reduced [7••]. These results, taken together with the authors' ChIP-qPCR data and demonstrated *in vivo* protein-protein interaction between SHR and SCR [7••], strongly support their hypothesis that SCR restricts SHR movement by sequestering it into the nucleus to create a SHR/SCR-dependent positive feedback loop for SCR transcription specifying endodermis (Figure 2C).

Studying the dynamics of SHR/SCR is an obvious next step. Another question is: are there other proteins that facilitate SHR movement?

In summary, this ground tissue patterning research points to exciting downstream transcriptional targets that now can be linked to later differentiation processes and an enlightening mechanism for how cell fate can be specified by a combination of protein movement, interaction, and transcription control. So far, the SCR/SHR pathway does not seem

to involve the plant hormone auxin. This is intriguing given that auxin seems to be implicated in most developmental processes of the root, including QC and stele function as discussed in the next sections.

Stele patterning in the root

Little is known about the factors involved in the specification of cell types in the stele [25]. The role of auxin is prominent in this tissue and the action of PINFORMED (PIN) auxin transporters affects vascular patterning in the stele [26]. This is perhaps due to the comparatively complex number and locations of different cell types within this tissue. Genes expressed in protophloem have recently been identified from enhancer trap screens [27], including the previously characterized transcription factor BREVIS RADIX (BRX) that mediates feedback between brassinosteroids and the plant hormone auxin [28•,29–30]. Regulation of the bilateral symmetry within the stele was recently shown to be eliminated in *lonesome highway* roots [31]. As this gene encodes a protein with similarity to bHLH transcription factors, it will be interesting to know if it interacts with SHR and/or BRX to control stele patterning as well as to know of the nature of any overlap between the targets of these transcription factors.

Patterning the root's stem cell niche

Auxin response and transport are central to recent work on the patterning of the root's stem cell niche. A maximum of auxin response visualized by reporter genes up-regulated by auxin corresponds to the position of the QC [32], suggesting the QC's position is defined by this maximum. Protonated auxin can move into cells by passive diffusion, while the PIN auxin transporters facilitate the movement of negatively ionized auxin out of cells because auxin is a weak acid and the extracellular pH is lower than cytoplasmic pH in root cells [33]. 'Inverted fountain models' have been proposed based on the asymmetric distribution of PIN proteins within single root cells to describe the direction and magnitude of auxin fluxes (Figure 3) [34].

Based largely on these models combined with experimental information about the spatial localization of the PIN family members in the root, and accounting for simple diffusion of auxin, a recent mathematical model correctly predicted the position of this auxin maximum, but also proposed an auxin gradient in the root [5••]. Although direct quantification of auxin levels in individual cells would definitively prove the existence of this gradient, this has not yet been achievable in plants. Moreover, while the authors base their model largely upon PIN localization, they do not describe simulations of auxin gradients expected in *pin* mutants. Despite this, the model has an impressive ability to simulate a variety of perturbations to the auxin maximum that correctly match experimental observations, including laser ablation of the QC, high levels of auxin applied to the root, and amazingly, decapitation of the plant removing the root's main source of auxin [5••]. Decapitated plants were able to survive for 10–30 days depending upon the amount of 'reflux', which depended upon PIN localization to the lateral face of cells to direct auxin flux back into the downward-directed flow within the vascular tissue [5••]. In this sense, the root possesses an auxin 'battery' that holds charge, but slowly loses it at a rate proportional to inefficient reflux.

Remarkably, the PLETHORA (PLT) transcription factors are expressed in a pattern resembling this gradient [6•,35]. Various *plt* double and triple mutants have reductions in *PIN* expression [6•,35], suggesting a connection between PLTs and an auxin gradient involving PINs. A direct relationship between PLTs and *PINs* has not yet been demonstrated, but one may not expect there to be one, because QC ablation experiments suggest there is a significant lag time between the appearance of the QC auxin maxima and PIN protein localization [36•].

A host of transcription factors involved in QC specification and maintenance may function during this lag time, including WUSCHEL-RELATED HOMEOBOX 5 (WOX5), SHR, and SCR, whose expression have already been shown to appear during it [36•]. Since SHR, SCR, and WOX5 have roles in the QC [19–20,23,37–40], they represent attractive candidates for connecting PLTs and PIN derived gradients. Knowledge of the PLT transcriptional targets will be pertinent to future evaluations of their role in the auxin gradient, but information about *PLT* upsteam regulators will be as well. Interestingly, double mutants in the recently identified *OBERON1* and *OBERON2* plant homeodomain finger proteins were shown to lack *PLT1*, *WOX5*, and *SCR* expression, suggesting the nuclear-localized OBE1 and OBE2 may be upstream regulators in QC identity and specification [41].

Although this information about PLT pathways will be valuable, knowledge of PIN localization, regulation, and dynamics is just as beneficial to understanding the nature of an auxin gradient guiding root patterning and growth. It is thus worthwhile for researchers to consider how transcriptional information impacts and is integrated with other proteins modulating PIN, such as P-glycoproteins, AUXIN RESPONSE FACTORS, VACUOLAR PROTEIN SORTING 29, protein phosphatases 2A/ROOT CURLING ON NPA, and PINOID kinases [42–49].

CONCLUSION

Study of the patterning mechanisms establishing and maintaining the patterning of the *Arabidopsis* root began over two decades ago. In the last two years, it has become increasingly clear that the deceivingly simple structure of this organ is specified and maintained by many, potentially redundant, factors. Recent work has uncovered complex layers of regulation controlling these root patterning factors, such as chromatin remodeling, protein movement, transcriptional complexes, and an auxin gradient. An additional regulator that was not mentioned here is the potential role of small RNAs in root patterning [50]. Genome-wide studies of protein abundance and interactions, small RNAs, and histone modification at the resolution level achieved in [4•] should be of considerable use in future research dissecting this complexity.

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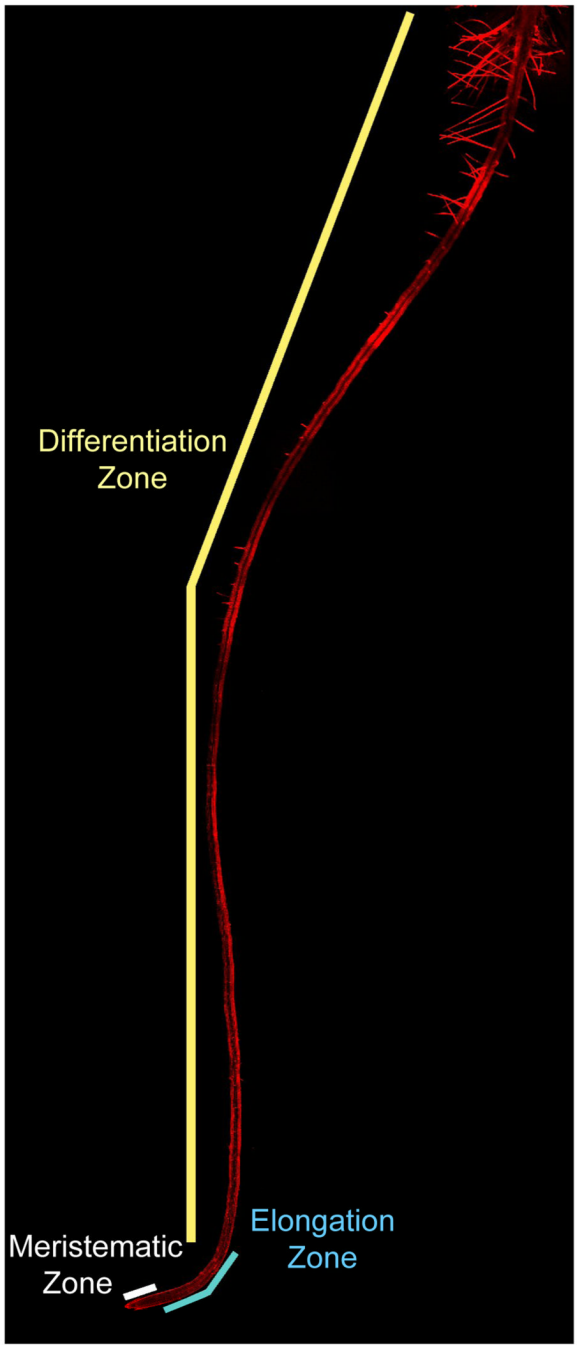

Figure 1.
The *Arabidopsis* primary root (6 days old) as viewed by laser confocal microscopy under 10X magnification. Three distinct zones mark the longitudinal axis of the root, namely, the meristematic (white bar), elongation (blue bar), and differentiation (yellow bar) zones. A close up of the meristematic and elongation zones is shown in Figure 3. Cell walls are visible in red from propidium iodide staining.

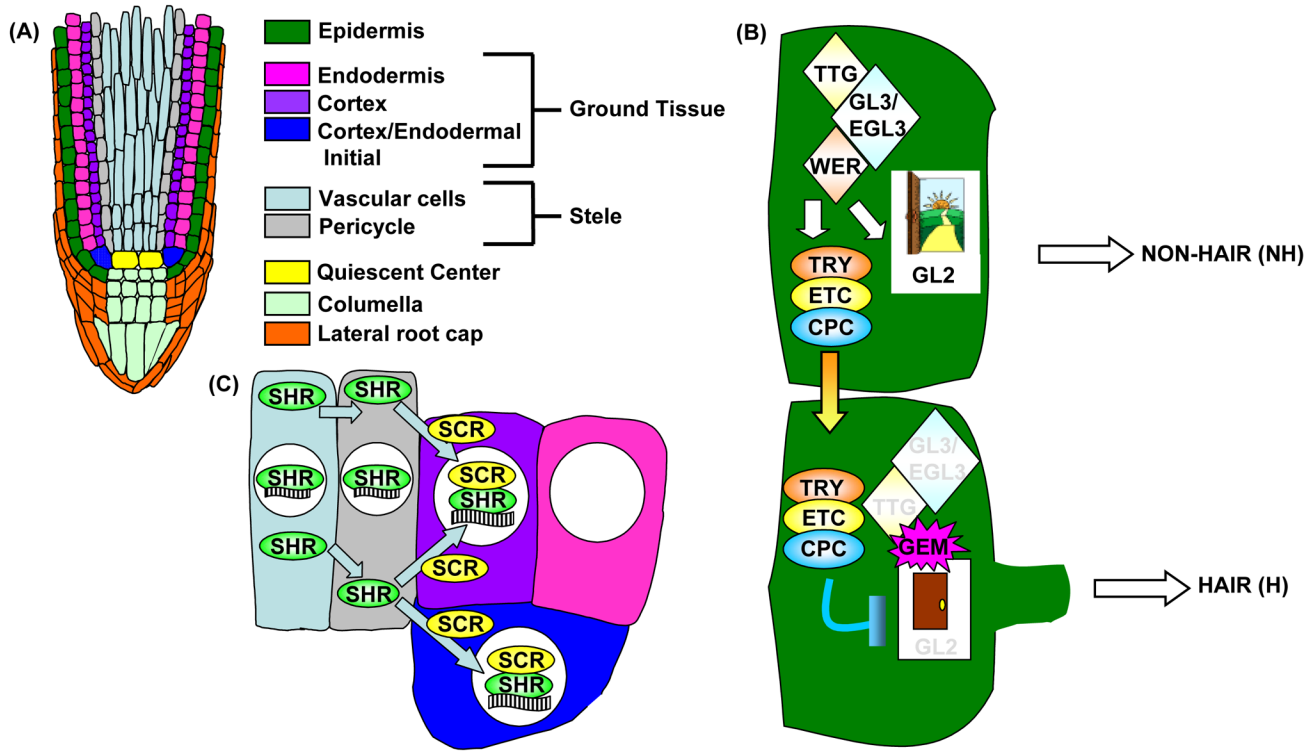

Figure 2.

The simple structure of the *Arabidopsis* primary root is specified by complex regulatory mechanisms. (A) Schematic drawing of a longitudinal slice of the primary root tip. (B) Non-hair epidermal cell fate is specified by high levels of *GL2*. *GL2* is activated by TTG/GL3/EGL3/WER transcriptional complexes that also activate *TRY*, *ETC*, and *CPC*. CPC (and presumably TRY and ETC) moves to neighboring epidermal cells to repress *GL2* expression, resulting in hair cell fate. GEM chromatin modifications regulate the 'open' or 'closed' configuration of the GL2 promoter. (C) SHR moves from the stele into adjacent cells where it is sequestered by SCR into the nucleus. Nuclear SHR-SCR complexes activate *SCR* expression, specifying the endodermis. White circles in (C) denote the nucleus, waved black/white-striped rectangle represents the *SCR* promoter DNA. Throughout the figure, cell colors indicate the associated cell-type shown in (A) and colored arrows depict protein movement.

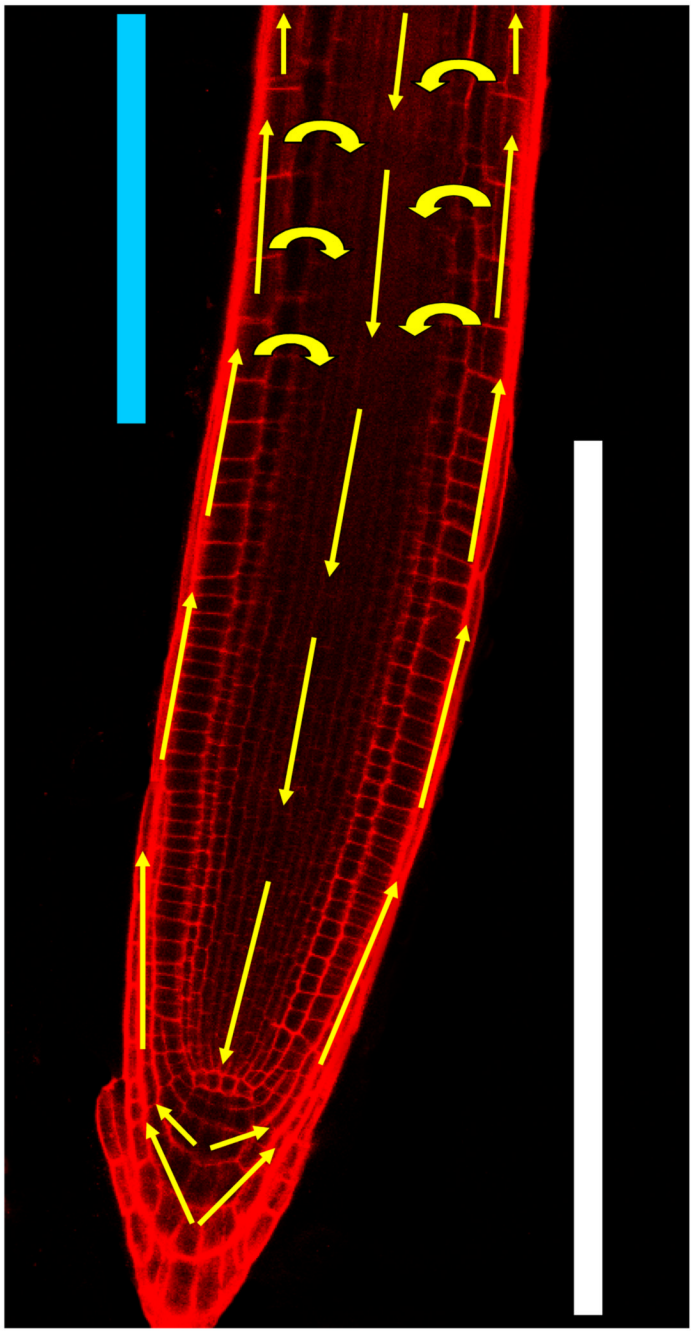

Figure 3.
Simplified schematic of the efflux routes of auxin resembling an inverted fountain in the *Arabidopsis* primary root (7 days old) as viewed by laser confocal microscopy under 25X magnification. Auxin is thought to be transported from the shoot downward to the root tip via PIN carriers in the central cell layers of the root. Once auxin reaches the tip it is believed to be directed outward to the outer cell layers by different PIN carriers. It is then thought to be transported upward toward the shoot by still other PIN carriers. Some of this auxin may also be recycled from the outer root layers back into the inner ones to provide a 'battery' like mechanism that maintains auxin levels within the root tip. The meristematic (white bar) and

elongation (light blue bar) zones are shown. Cell walls are visible in red from propidium iodide staining.