Review

New Phytologist

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Genes and networks regulating root anatomy and architecture

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Received: *3 March 2015*
Accepted: *20 April 2015*

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New Phytologist (2015) **208:** 26–38
doi: 10.1111/nph.13469

Key words: Arabidopsis, lateral root, networks, root, root architecture.

Summary

The root is an excellent model for studying developmental processes that underlie plant anatomy and architecture. Its modular structure, the lack of cell movement and relative accessibility to microscopic visualization facilitate research in a number of areas of plant biology. In this review, we describe several examples that demonstrate how cell type-specific developmental mechanisms determine cell fate and the formation of defined tissues with unique characteristics. In the last 10 yr, advances in genome-wide technologies have led to the sequencing of thousands of plant genomes, transcriptomes and proteomes. In parallel with the development of these high-throughput technologies, biologists have had to establish computational, statistical and bioinformatic tools that can deal with the wealth of data generated by them. These resources provide a foundation for posing more complex questions about molecular interactions, and have led to the discovery of new mechanisms that control phenotypic differences. Here we review several recent studies that shed new light on developmental processes, which are involved in establishing root anatomy and architecture. We highlight the power of combining large-scale experiments with classical techniques to uncover new pathways in root development.

I. Introduction

Uncovering the genetic regulation of root anatomy and architecture is a fundamental challenge in plant biology despite the remarkable benefits of the root as a model system. At the cellular level, two developmental axes characterize the morphology of vascular plant roots. Along the longitudinal axis, cells progress from a stem-cell state to differentiated cells that have specific functions (Fig. 1a,b). Radially, each concentric layer represents a unique tissue (in some plants, a tissue is made up of more than one layer), defined by morphological, biochemical and molecular markers that provide distinct functions such as defense or transport (Fig. 1b). The majority of studies on the development of root anatomy in the last 25 yr have utilized *Arabidopsis thaliana* (Arabidopsis) due to its relatively simple translucent structure, which makes cells readily accessible to microscopic examination. From an architectural perspective, root system complexity in vascular plants is derived from the formation of embryonic roots as well as post-embryonic

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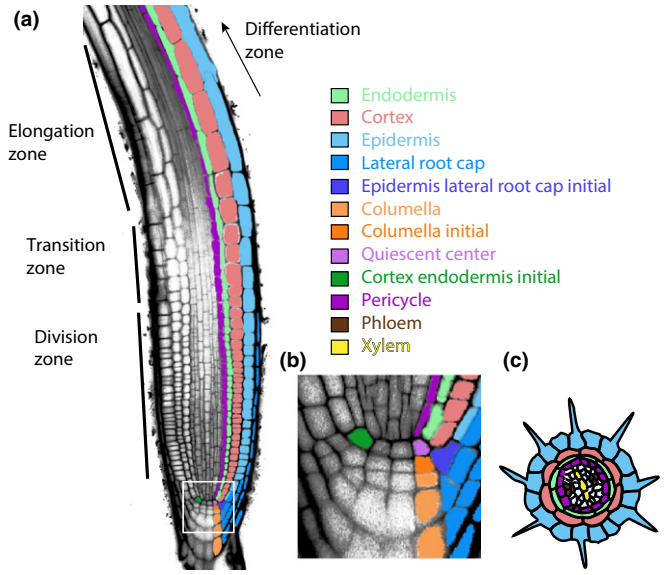

Fig. 1 Morphology of Arabidopsis root. (a) Longitudinal cross-section of confocal image along the meristematic, transition and elongation zones. The right side of each tissue (except for the vasculature) is color-coded according to the legend on the right. (b) Magnification of the stem cell region marked by white box in (a). (c) Schematic cross-section through a differentiated root region. Note the hair and nonhair cell types in the epidermis.

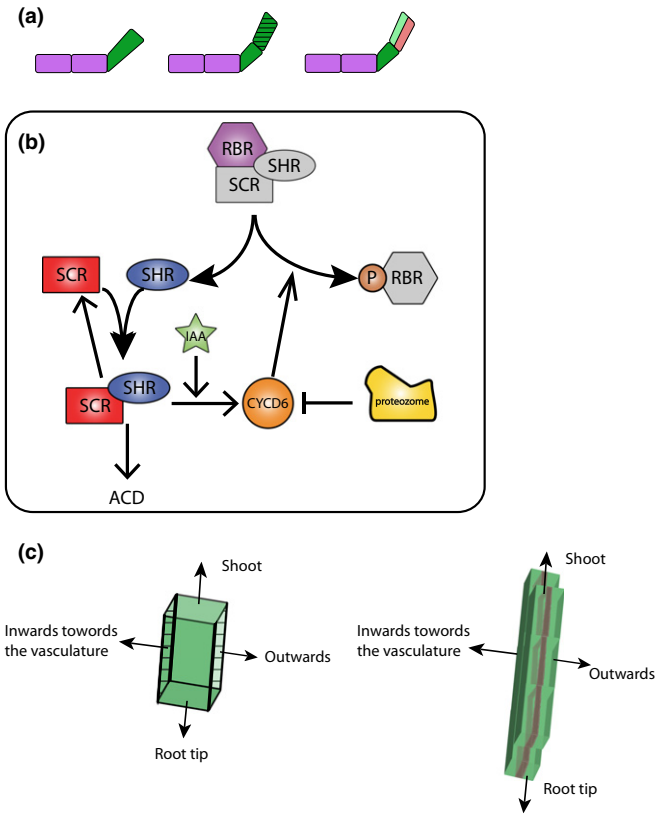

Fig. 2 Early and late events during endodermis specification in Arabidopsis. (a) The cortex endodermal initial (CEI; dark green) undergoes an anticlinal cell division to regenerate itself and generate the CEI daughter (CEID; striped dark green). The CEID then divides periclinally to form the first cells of the endodermis and cortex lineages (light green and red, respectively). (b) Schematic representation of the networks that control the asymmetric cell division in the CEID. Straight arrows represent activation or repression; curved arrows with closed arrowheads represent assembly or dissociation of complexes. (c) Left, a single cell in the proximal meristem showing the two periclinal faces (green striped, facing inner and outer regions) and the four anticlinal sides (green). Right, a section of two adjacent cell files from mature endodermis with developed Casparian strip (brown) along the median band of anticlinal cell walls. Note that this configuration facilitates the sealing of inner regions from intercellular diffusion. SCR, SCARECROW; RBR, RETINOBLASTOMA-RELATED; SHR, SHORTROOT; CYCD6, CYCLIN D6;1; ACD, asymmetric cell division; IAA, indole-3-acetic acid.

initiation of other root types. For dicots, a simple taproot architecture is mainly composed of an embryonically derived primary root and lateral roots that emerge from it post-embryonically. For the fibrous root system characteristic of monocots, root architectural complexity is enhanced by the presence of additional root types including various shoot-borne roots and different embryonic roots. In this review we highlight several of the latest studies that aim to uncover the genetic regulation of root anatomy, with a principal focus on Arabidopsis, and describe advances in our understanding of root architecture regulation both in monocots and dicots.

II. Signaling modules that regulate cell fate

Because plant cells, unlike animal cells, do not move during development, the overall organ and tissue patterning is largely determined by the orientation of cell division. In this section we will first discuss the developmental module controlling the initial cell fate decision to specify two root tissues, the cortex and endodermis. Many years of work have been devoted to understanding the regulation of a single asymmetric cell division in the cortex/endodermis stem cell daughter, which is critical for generating these two tissues (Dolan *et al.*, 1993). Second, we will discuss the latest advances in our understanding of how the endodermis proceeds to differentiate and becomes a functional tissue.

1. Early specification of the endodermis and cortex

In Arabidopsis, the cortex and endodermis are formed when the cortex/endodermis initial stem cell (CEI) undergoes an anticlinal division to regenerate itself and produce a proximal daughter cell (CEID) (Fig. 2a). The CEID then divides periclinally to generate the two separate and distinct cell layers for which it is named. These two cells then undergo several rounds of anticlinal divisions followed by differentiation to form functional tissues. Developmental time, from stem cell to differentiated cell, can be observed along the longitudinal axis of the root. In older plants, the periclinal asymmetric cell division takes place in the CEI rather than in the CEID and additional periclinal divisions in endodermal cells generate a third layer that acquires cortex characteristics (Baum *et al.*, 2002; Paquette & Benfey, 2005). Although the number of cortex cell layers can vary among different plant species, almost all possess a single endodermis layer, possibly due to its unique differentiated feature – the Casparian strip.

The stereotypical trajectory of stem cell to differentiated cell makes the endodermis and cortex an excellent system for studying

developmental processes. One of the better-characterized developmental networks underlies the asymmetric division of the CEID. The SHORTROOT (SHR) transcription factor is expressed in the vasculature and moves into the quiescent center (a group of approximately four cells located at the meeting point of all cell files), endodermis, CEI and CEID, where it localizes to the nucleus and interacts with a second transcription factor, SCARECROW (SCR). SCR localizes to the nuclei of the endodermis, CEI and CEID as well as the quiescent center cells (Fig. 1). Loss-of-function mutations in either SCR or SHR are sufficient to block the periclinal division of the CEID, resulting in a single mutant tissue layer (Benfey *et al.*, 1993; Di Laurenzio *et al.*, 1996). Recent studies (Sozzani *et al.*, 2010; Cruz-Ramirez *et al.*, 2012) have shown that a network consisting of components from the cell cycle machinery, the proteasome, SHR and SCR transcription factors and the phytohormone auxin mediates the asymmetric cell division in the CEID (Fig. 2b). Specifically, a D-type cyclin, CYCD6;1, is a key regulator of this division and its expression is predominantly confined to the CEID by proteasome-dependent degradation in other cell types, along with high amounts of auxin in the CEID region and activity of the SCR-SHR complex. The CYCD6;1-CDKB1 complex phosphorylates the cell cycle inhibitor RETINOBLASTOMA-RELATED (RBR), thereby inhibiting its action as a repressor of SCR, which positively regulates CYCD6;1 (Fig. 2b). A second, tangential loop is formed by activation of SCR by the SHR-SCR complex and sequestration of SHR from the cytoplasm to the nucleus by SCR. The RBR-free SHR-SCR complex is formed only upon phosphorylation of RBR by CYCD6;1-CDKB1. The topology of this network creates a bistable switch that allows the correct spatiotemporal turn-on (and off) of the asymmetric cell division.

This network can explain the dynamics of the asymmetric division in the CEID, but some questions remain. First, the role of SCR in SHR sequestration is still not entirely clear because nuclear localization of SHR occurs in the absence of SCR (Nakajima *et al.*, 2001). The mechanism that leads to periclinal division exclusively in the CEID (and later in the CEI) and its arrest after the formative CEID division in older seedlings is still unknown. SCR and SHR are required for SCR transcription in the quiescent center, CEI and CEID to promote the asymmetric cell division (Helariutta *et al.*, 2000; Heidstra *et al.*, 2004). In the absence of SHR or SCR, SCR transcription is lower in the CEID in comparison to its descendants (Helariutta *et al.*, 2000; Heidstra *et al.*, 2004). This suggests that the dynamics of the SCR-SHR module are different between these cell populations. This differential behavior might play an important role in distinguishing the periclinally dividing CEID from its endodermal descendants that divide only anticlinally.

The question of what regulates SHR movement from the vasculature into the endodermis and how this movement is facilitated has recently been addressed. One route for SHR movement is through pores that connect adjacent cells called plasmodesmata. Deposition of callose in plasmodesmata is sufficient to exclude SHR not only from the endodermis, but also from the pericycle – the cell layer between the vasculature and the endodermis – suggesting that plasmodesmata are the route through which SHR moves from the vasculature through the pericycle (where some of the protein is retained) to the endodermis. One candidate gene, *SHORTROOT INTERACTING EMBRYONIC LETHAL* (*SIEL*; Koizumi *et al.*, 2011) has been identified as a regulator of SHR movement. SIEL is a nuclear and endosome-associated protein that interacts with SHR and hypomorphic alleles show reduced SHR movement. Unfortunately, *siel* null alleles are embryonic lethal, thus it remains unknown if this is the only pathway for SHR movement.

The genetic network underlying cortex and endodermal identity has been extensively studied for more than two decades. Some outstanding questions still remain, such as which transcription factors regulate SHR expression and the low levels of SCR to initiate the feedback loop. However, this well-established network lays the foundation for future studies to address the dynamic nature of cortex and endodermis formation. This network has been established based on static observations. In the future, uncovering the dynamics and interplay between network components will be essential for understanding cell fate readout. The use of advanced microscopy, genetics and sequencing techniques should be able to further our understanding of network dynamics.

2. Differentiation processes during endodermis maturation

In the last few years our understanding of endodermal differentiation has significantly advanced, mainly as a result of several studies on Casparian strip formation. The hallmark of endodermal differentiation is the formation of the Casparian strip, originally described by Robert Caspary in 1865. The Casparian strip is a lignin-based cell wall deposition located along the equatorial region of anticlinal cell walls in mature endodermis cells (Fig. 2c; Naseer *et al.*, 2012). The Casparian strip has been proposed to function as a selective apoplastic barrier to both restrict movement of substances into the vascular tissue and prevent the backflow of water and ions from the vasculature (Enstone *et al.*, 2003; Pfister *et al.*, 2014). Surprisingly, the Casparian strip does not seem to be a general diffusion barrier because mutations in *SCHENGEN3* (*SGN3*) which lead to severe disruption in the Casparian strip, only affect potassium and magnesium homeostasis and do not cause any alteration in the concentration of most ions that were examined (Pfister *et al.*, 2014). These results suggest that an alternative mechanism is employed to maintain vasculature homeostasis in the absence of the Casparian strip or that the Casparian strip regulates only certain ions.

The first study to shed light on the restricted deposition of the Casparian strip describes three specific subdomains in the plasma membrane of differentiating endodermis cells: (1) the peripheral domain (facing the epidermis), (2) the central domain (facing the vascular tissue) and (3) the Casparian strip domain that coincides with the location of the Casparian strip (Alassimone *et al.*, 2010). In subsequent studies (Roppolo *et al.*, 2012; Lee *et al.*, 2013) the same group identified five Casparian strip membrane domain proteins (CASP1-5) that localized to the Casparian strip domain and are required for Casparian strip formation. Interestingly, these genes were identified by mining published datasets (Birnbaum *et al.*, 2003) for endodermis genes that are secreted or plasma-membrane enriched. These CASP proteins localize to the Casparian strip

domain before the formation of the Casparian strip and act as a scaffold for localization of a specific NADPH oxidase (RBOHF) and peroxidase (PER64), enzymes that are required for polymerization of monolignols into lignin (Lee *et al.*, 2013) to form the Casparian strip barrier.

In addition to being key to the function of the Casparian strip, a polarized endodermis can provide insights into other development processes. The peripheral domain and central domain are marked by an arsenate transporter, NIP5;1 and boron transporter, BOR1, respectively. In mature endodermis cells, each of these proteins is not only restricted to one domain, but also excluded from the Casparian strip domain. NIP5;1 and BOR1 are localized in the endodermis as well as in the quiescent center and in the CEI towards and away from the vasculature, respectively; this polarity coincides with the formation of the vasculature during embryogenesis. Together these findings imply that the vasculature provides mobile signals to establish polarity during development (Alassimone *et al.*, 2010). These findings might also explain how asymmetric cell divisions occur as a result of differential cues from the quiescent center and how directional signals are translated into pattern formation in the root meristem. It has been known for many years that the quiescent center transmits signals to the surrounding stem cells to keep them undifferentiated (van den Berg *et al.*, 1997). The asymmetric localization of BOR1 and NIP5;1 suggests that the quiescent center may be polarized; quiescent center polarity domains could feed into the SCR-SHR-RBR network and then be translated into the periclinal division of the CEID. It is possible that polarly localized proteins in the quiescent center might provide differential local cues to the surrounding stem cells.

The two networks described in this section, SHR-SCR and the genes involved in Casparian strip formation, have been primarily studied by standard molecular biology techniques that require detailed analysis of each gene – for example, its functions, expression and cellular localization. However, it is clear that the genetic network underlying root development is much more complex and comprises many additional players. Thus, in the next section we describe approaches recently used to understand the larger gene regulatory networks underlying root development.

III. Understanding global connections through gene regulatory networks

Many small-scale regulatory networks that underlie specific processes, such as the SHR-SCR and Casparian strip networks, have been identified. However, these networks do not function in isolation, and one of the current challenges is to place these smaller networks into the context of a system-wide gene regulatory network. These larger networks, generated through computational or experimental approaches, can describe protein–protein interactions, biochemical processes or protein–DNA interactions (Bassel *et al.*, 2012). Here, we highlight two approaches that have been used to generate protein–DNA interaction networks. One study used genome-wide transcriptomic data and a computational Bayesian network model to predict protein–DNA interactions during cell fate determination in the epidermis (Bruex *et al.*, 2012). The Bayesian approach uses probabilistic modeling to predict the likelihood of an interaction network given *a priori* knowledge (e.g. transcriptomic datasets). By contrast, we also highlight a second study that generated a protein–DNA network describing secondary cell wall formation in the xylem by directly testing interactions in yeast (Taylor-Teeples *et al.*, 2014).

A handful of different mathematical models have been used to computationally predict network connections (Le Novère, 2015). Although these approaches can encompass genome-scale data and predict emergent properties, they often suffer from a lack of robustness and the type of model highly influences network connections (Le Novère, 2015). Recently, combining multiple mathematical models has increased robustness, but the predictions from these models require experimental validation (Le Novère, 2015). By contrast, direct determination through experimental approaches tends to be more robust, but still suffers from high false-negative and false-positive rates, as well as being limited by available experimental resources. Ultimately, once gene regulatory networks are identified, the predicted connections can be perturbed as a way of testing hypotheses.

1. Gene regulatory networks identify new regulators of epidermal cell fate progression

The epidermis is the outermost tissue layer of the root and consists of two cell types: hair and nonhair cells (Fig. 3a). Numerous studies using both forward and reverse genetic approaches have defined a complex lateral inhibition mechanism mediated by eight transcription factors that define the early decision of hair vs nonhair cell fate. These include five nonhair cell fate regulators (*TRANSPARENT TESTA GLABRA* (*TTG*), *GLABRA3* (*GL3*), *ENHANCER OF GLABRA3* (*EGL3*), *WEREWOLF* (*WER*) and *MYB23*) and three hair cell fate regulators (CAPRICE (CPC), *TRIPTYCHON* (*TRY*) and *ENHANCER OF TRY AND CPC* (*ETC1*)) (Schiefelbein *et al.*, 2014) (Fig. 3a). Hair and nonhair cell fate is determined by a lateral inhibition mechanism. The nonhair transcription factors form a core complex that regulates nonhair fate, and also induces expression of the hair transcription factors, which then move into the hair cells and inhibit nonhair transcription factors (Schiefelbein *et al.*, 2014) (Fig. 3a). Genes downstream of these early factors include transcription factors, metabolic genes, signaling and structural components (Schiefelbein *et al.*, 2014). To identify the gene regulatory network underlying the cell fate decisions of immature epidermis, Bruex *et al.* (2012) generated epidermal-specific transcriptome data from 17 different mutant combinations of the aforementioned upstream and downstream regulators. This analysis identified seven additional bHLH transcription factors as probable epidermal fate regulators. Mutants of these transcription factors display defects in different aspects of epidermal development including changes to root hair length and morphology (Bruex *et al.*, 2012).

The authors combined the epidermal-specific mutant transcriptome data with epidermal-specific hormone treatment transcriptomics, developmental time microarray data (Brady *et al.*, 2007) and molecular genetic data to infer gene regulatory network connections with Bayesian modeling (Bruex *et al.*, 2012) (Fig. 3b).

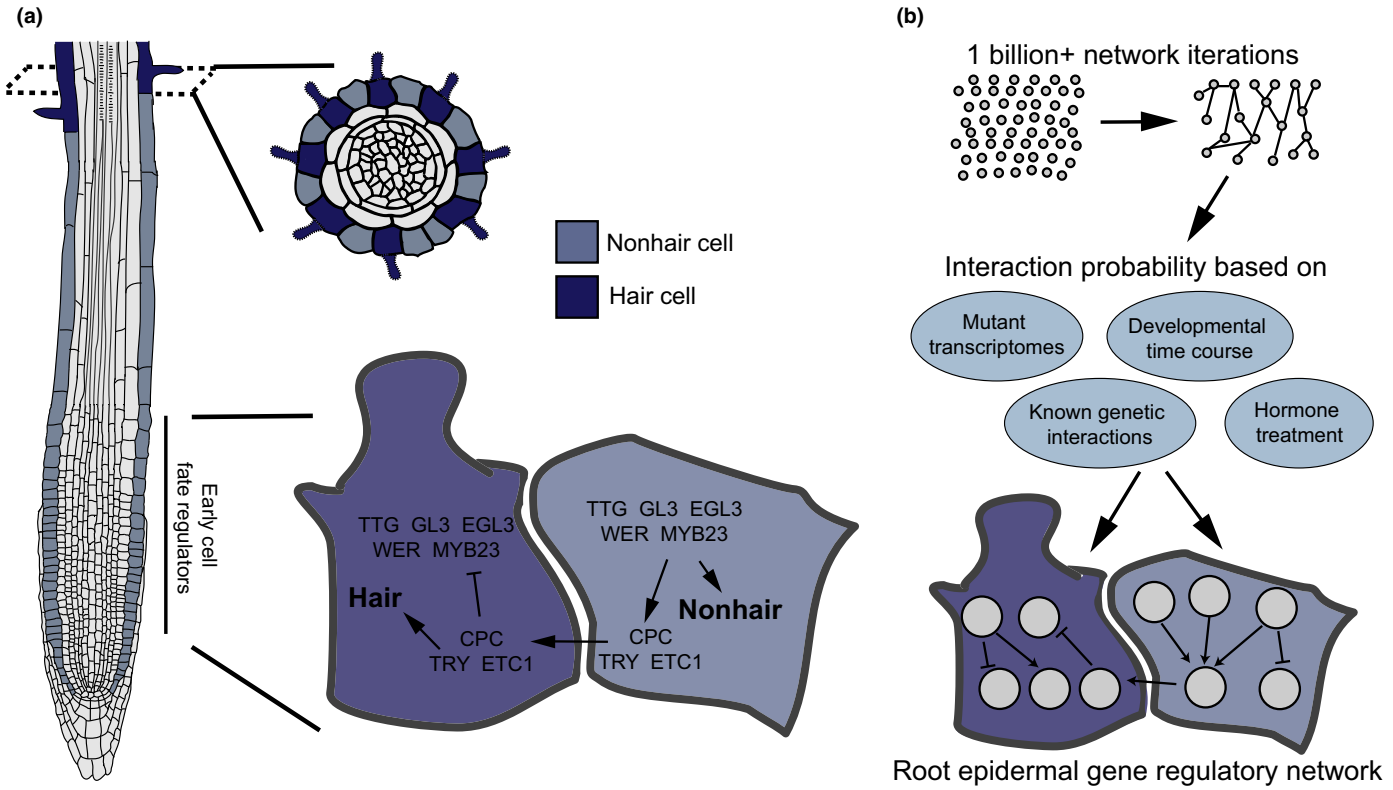

Fig. 3 Transcriptomic data used to infer the gene regulatory network underlying epidermal cell fate determination in Arabidopsis. (a) The Arabidopsis root epidermis is composed of two cell types: hair cells (dark blue) and nonhair cells (light blue). Eight transcription factors in immature epidermis generate a lateral inhibition to specify hair and nonhair cell fates. The transcription factors, TRANSPARENT TESTA GLABRA (TTG), GLABRA3 (GL3), ENHANCER OF GLABRA3 (EGL3), WEREWOLF (WER) and MYB23 promote the nonhair cell fate and activate three transcription factors, CAPRICE (CPC), TRIPTYCHON (TRY) and ENHANCER OF TRY AND CPC (ETC1). CPC, TRY and ETC1 move into the adjacent hair cell to inhibit nonhair regulators and promote the hair cell fate. (b) To generate an epidermal gene regulatory network, the authors utilized a Bayesian modeling approach. Over 1 billion network iterations were generated and compared to experimentally derived transcriptome data from mutant epidermis, a root developmental time course, hormone treated epidermis and known interactions. The consensus among the top scoring networks was used to infer a network regulating cell fate in the hair and nonhair cells.

Thousands of hours of computational time generated over 1 billion iterations of the network, which were compared to the input transcriptomic data with the final network being the result of a consensus among the top scoring iterations (Bruex *et al.*, 2012) (Fig. 3b). This network successfully predicts spatiotemporal interactions and validates several spatiotemporal patterns of gene expression regulating epidermal development. Building on a wealth of previous knowledge, the authors were able to infer network connections and identify additional components required for epidermal cell fate decisions. The epidermis proved to be the ideal tissue for this approach because the prior knowledge of many regulators at multiple stages of development facilitated formation of a high confidence gene regulatory network. One of the current challenges with this approach is to generate and utilize networks to perform *de novo* predictions when prior knowledge is more limited.

2. A secondary cell wall gene regulatory network identifies novel regulators and facilitates network prediction

In instances where fewer regulatory components are known, a more direct approach has proven informative. Specifically, Taylor-Teeples *et al.* (2014) utilized large-scale enhanced yeast-1-hybrid assays (Gaudinier *et al.*, 2011; Reece-Hoyes *et al.*, 2011) to identify protein–DNA interactions from which they generated a network that regulates the formation of secondary cell walls. Secondary cell walls are structural reinforcements laid down on specialized cell types, including the root xylem (Fig. 4a,b). They are composed of cellulose, hemicellulose and lignin. Several transcription factors including two *VASCULAR-RELATED NAC DOMAIN* (*VND*) family members and five HD-ZIPIII family members (including *REVOLUTA* (*REV*)), are known to regulate this process (Kubo *et al.*, 2005; Carlsbecker *et al.*, 2010). However, these transcription factor families have been shown to act redundantly in secondary cell wall formation, making forward and reverse genetic approaches unable to identify additional regulatory components. The mutant transcriptomic approach used for the epidermal network has reduced value when there is genetic redundancy and therefore has difficulty identifying new genes in a given regulatory network. By contrast, direct protein–DNA interaction assays, such as the enhanced yeast-1-hybrid assays used in this study, deal well with redundancy but are prone to high false-positive and false negative rates. In this example, the utilization of a directly determined gene regulatory network successfully identified interactions underlying the formation of secondary cell walls (Taylor-Teeples *et al.*, 2014). Specifically, the authors describe two feed-forward loops predicted to function in lignin biosynthesis. The first feed-forward loop

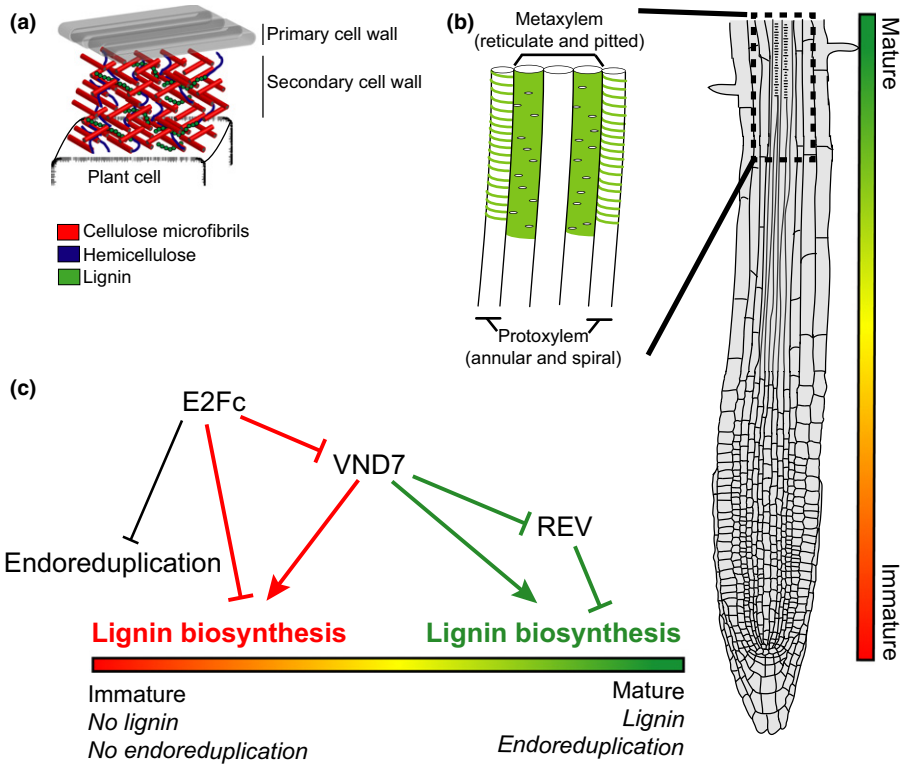

Fig. 4 A secondary cell wall gene regulatory network defines two feed-forward loops regulating xylem cell fate in Arabidopsis. (a) Secondary cell walls are deposited in specific cell types to provide structural support in mature cells. The secondary cell wall is composed of cellulose (red), hemicellulose (blue) and lignin (green), and is deposited between the primary cell wall and plasma membrane. (b) In the *Arabidopsis* root, secondary cell walls are deposited in mature xylem tissues. In protoxylem (the outer xylem vessels), secondary cell walls are deposited in ring-like annual or spiral patterns. In metaxylem (the inner xylem vessels), secondary cell walls are deposited in reticulate or pitted patterns. (c) Two feed-forward loops regulate lignin biosynthesis in the root xylem. In immature tissues, cells lack secondary cell walls and have not undergone endoreduplication. To coordinate these developmental processes, E2Fc, a negative regulator of endoreduplication is also a negative regulator of lignin biosynthesis. E2Fc directly inhibits lignin biosynthesis and inhibits an activator of lignin biosynthesis, VND7. By contrast, in mature tissues, lignin biosynthesis is promoted through a VND7 feed-forward loop. VND7 directly promotes lignin biosynthesis and also inhibits an inhibitor or lignin biosynthesis, REV. Together these feed-forward loops provide one mechanism by which secondary cell wall formation is restricted to mature xylem.

consists of E2Fc, a negative regulator of endoreduplication, which represses lignin biosynthesis and a NAC domain transcription factor, *VND7*, which promotes lignin biosynthesis (Taylor-Teeples *et al.*, 2014). The net result of this feed-forward loop is reduced lignin biosynthesis (Fig. 4c). The second feed-forward loop consists of *VND7* (an activator of lignin biosynthesis) repressing the HD-ZIPIII transcription factor *REV*, which represses lignin biosynthesis (Taylor-Teeples *et al.*, 2014). The net result of this second feed-forward loop is increased lignin biosynthesis (Fig. 4c). These regulatory modules likely function in different developmental zones to coordinate the onset of differentiated attributes such as endoreduplication and secondary cell wall formation. Interestingly, these feed-forward loops highlight the importance of negative regulation for tightly controlled developmental processes. Thus, the use of a direct approach such as yeast-1-hybrid was able to identify novel regulators of secondary cell wall development.

As mentioned above, one goal of generating gene regulatory networks is to predict gene expression changes upon perturbation. Using the secondary cell wall network, Taylor-Teeples *et al.* hypothesized that the response to abiotic stress is most likely to occur by co-opting a developmental process. They successfully predicted gene expression changes under different abiotic stress conditions (Taylor-Teeples *et al.*, 2014). For example, *REV* was predicted and confirmed to play a key role in the lignin biosynthesis response to iron deprivation (Taylor-Teeples *et al.*, 2014). These results are promising for the future of developing gene regulatory networks and using them to predict the outcome of perturbations.

The two network approaches outlined here demonstrate how network generation and analysis can further our understanding of root development and response to environment. Networks have begun to yield a deeper understanding of gene regulation and provide an approach to identify new regulators of developmental processes. However, genetic redundancy and buffering continues to prove a challenge in fully elucidating the role of individual genes. In Drosophila, *Caenorhabditis elegans*, bacteria and yeast this has been addressed by the synthetic lethality approach (Lucchesi, 1968; Tong *et al.*, 2001; Baugh *et al.*, 2005; Butland *et al.*, 2008). Double mutant combinations of nonlethal genes are screened for lethality (Tong *et al.*, 2001). When the double mutant results in lethality, it suggests that the two genes are part of parallel pathways generating the same output. Unfortunately, this approach to identify redundancies is more difficult in plants, especially when the output is

more subtle than lethality. An alternative approach to identify redundancy would be to look at double mutants of genes with the highest number of shared network connections given the likelihood that they function in redundant molecular pathways. Specifically for root development, this hypothesis could be tested by generating double mutants and phenotyping for root patterning defects.

IV. Signaling mechanism of organ formation: lateral root formation

The formation of lateral roots in Arabidopsis is initiated through a series of cell divisions in mature pericycle cells (Fig. 5; Malamy & Benfey, 1997; Lucas *et al.*, 2013). The first divisions occur in three pairs of adjacent files (Fig. 5a); however, most of the lateral root primordium is derived from cells in the middle file (Kurup *et al.*, 2005). The sequence of events that eventually leads to the formation of mature primordia can be divided into four stages. The first is marked by oscillatory expression of DR5 (a synthetic auxin reporter) and several other genes in the border of the meristem and the differentiation zone (oscillation zone; De Smet *et al.*, 2007; Moreno-Risueno *et al.*, 2010). Each oscillatory event develops into a pre-branch site for marking a region competent to become a lateral root primordium. Currently, there is no known mechanism that can explain this periodic behavior; however, > 1000 genes were found to oscillate in-phase with DR5 and some of them have reduced number of primordia when mutated (Moreno-Risueno *et al.*, 2010). The second step occurs when two longitudinally adjacent pericycle cells express the GATA23 transcription factor (De Rybel *et al.*, 2010) followed by nuclear migration towards their shared cell wall (Fig. 5a,b). This step takes place simultaneously in three neighboring pericycle cell files (Kurup *et al.*, 2005). Subsequently, the two cells (one pair in each file) divide asymmetrically leading to two small central cells flanked by two longer cells (Fig. 5d; De Smet *et al.*, 2007). This is the first event that shows distinct cytological and morphological features of the lateral root founder cell population. In the next phase, the cells continue dividing to form a dome-shaped structure and cells start to acquire their specific cell fates. Although the first few divisions seem to be stereotypical and similar in most primordia, the orientations of subsequent divisions show a high degree of variability (Lucas *et al.*, 2013) suggesting that the pattern of cell division has only a minor contribution to the overall dome-shaped morphology and emergence of the primordia. This conclusion is supported by two earlier findings: first, wheat plants treated with colchicine, a strong inhibitor of mitosis, still form lateral root primordia that bulge out from the main root (Foard *et al.*, 1965). This means that the formation of organ primordia can occur, to some extent, in the absence of any cell division. Second, primordia still form in the *aurora1-2;aurora2-2* (protein kinases that are key regulators of mitosis) double mutant, which shows severe aberrations in cell division planes (Van Damme *et al.*, 2011).

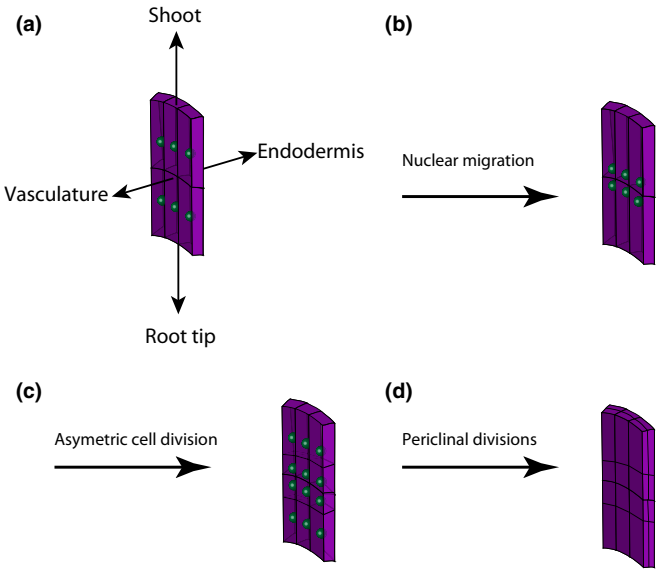

Fig. 5 Early events in lateral root primordia formation in Arabidopsis. (a) Three pericycle cell files with two cells in each are primed by an early oscillatory event (not shown). (b) Nuclear migration. (c) Asymmetric cell division forms two longer outer cells and two shorter inner cells in each of the files. (d) Periclinal divisions lead to thickening of the developing primordium. Nuclei are marked as green spheres and pericycle cells as purple boxes.

The development of lateral root primordia requires a high degree of intercellular communication between the site of initiation in the pericycle and the other cell layers (xylem, endodermis, cortex and epidermis). For example, SHY2/IAA3, an AUX/IAA protein that functions in auxin perception (reviewed in Calderon-Villalobos *et al.*, 2010) is expressed in the endodermis and acts as an inhibitor of lateral root initiation (Tian & Reed, 1999; Vermeer *et al.*, 2014). Ectopic expression of a *shy2* gain-of-function allele in the endodermis blocks the initiation of primordia as early as the first asymmetric cell division (Vermeer *et al.*, 2014). This is most likely due to structural changes such as swelling of the pericycle and flattening of the overlying endodermis, which require normal auxin-dependent SHY2 activity. Earlier studies showed that the LAX3 auxin influx carrier promotes the expression of cell wall degrading enzymes (Swarup *et al.*, 2008), thereby relaxing physical constraints and allowing the primordia to keep expanding and eventually compress the overlying cells, as the last stage of primordia development. LAX3 is expressed in the vascular tissue and in LRP overlying cortex and epidermis. Auxin responsiveness in endodermis cells adjacent to the LRP is mediated by SHY2/IAA3 expression. Thus, several different auxin responses in the three overlying layers are necessary for the emergence of LRP.

The formation of lateral roots in Arabidopsis and other angiosperms poses several interesting questions. First, why do lateral roots initiate from cells that are already differentiated, necessitating a dedifferentiation process. This is in contrast to lateral organs in aerial parts that are formed around the shoot meristem from an undifferentiated, dividing cell population. This difference suggests that despite similarities between the postembryonic processes of organogenesis in the shoot and root, some of the mechanisms are fundamentally different. In the fern, *Ceratopteris richardii* (*C. richardii*) lateral roots initiate from a single cell within the root meristem (Hou *et al.*, 2004) where cells are still in a 'dividing' state. Because ferns are the first plants with a true vascular system to occupy nonaquatic habitats, it is unclear why higher plants have evolved a dedifferentiating mechanism.

Another intriguing question is why lateral roots initiate in an inner cell layer (pericycle in higher plants and endodermis in ferns), a process that requires disrupting the overlying cells at the time of emergence. Last, auxin signaling plays an important role in almost every aspect of plant development and indeed, several auxin response modules are involved in primordia initiation. For example, *slr/iaa14* gain-of-function mutants, which have reduced sensitivity to auxin lack primordia and cannot initiate the first asymmetric cell division (Fukaki *et al.*, 2002, 2005). This inhibition likely acts though the SLR/IAA14 putative repressed targets ARF7/NPH4 and ARF19, whose double mutant phenocopies *slr/iaa14* (Fukaki *et al.*, 2005). Several other IAA-ARF modules, such as MSG/IAA19-NPH4/ARF7 (Tatematsu *et al.,* 2004) have been implicated in lateral root development, but the broad expression of almost all IAA-ARF proteins involved in lateral root initiation cannot explain how lateral roots are formed locally. Interestingly, auxin does not affect lateral root formation in *C. richardii* (Hou *et al.*, 2004), suggesting that auxin recruitment to this process was not a prerequisite during the transition to land ecosystems.

The formation of root and shoot architecture requires inputs from several hormonal pathways including auxin, cytokinin and strigolactones. For example, auxin inhibits lateral organ development in the shoot (Thimann & Skoog, 1933, 1934) whereas it induces lateral root emergence (Thimann & Koepfli, 1935). Cytokinins have the opposite effect in that they perturb auxin dependent lateral root initiation (Laplaze *et al.*, 2007) and antagonize the repressive growth effect auxin exerts on shoot axillary buds (Wickson & Thimann, 1958). By contrast, strigolactones repress the outgrowth of both shoot axillary buds and lateral roots. Therefore, two major hormonal mechanisms were adopted differently in the root and the shoot to regulate lateral organ outgrowth. Despite the difference in molecular mechanisms for the formation of aerial and underground organs, apical dominance is a key feature that controls both root and shoot branching. Decapitating the shoot or the root apical meristem (Thimann, 1936; Van Norman *et al.*, 2014) promotes branching indicating that the apical meristem has a general inhibitory effect on lateral organ outgrowth to control branching architecture.

V. Genetic regulation of global root architecture

The majority of work highlighted in this review has focused on understanding root form and function in Arabidopsis. Although Arabidopsis is a powerful model organism, its relatively simple dicotyledonous root system is composed of an embryo-derived taproot and associated lateral roots. By contrast, most food crops are monocots, which have a fibrous root system composed of both embryonic (seminal) and stem-borne (crown) roots (Fig. 6a). Monocot roots also have different root anatomy than dicot roots. However, for the purpose of this review we will focus on the global root architecture. For a detailed description of root types, we recommend a recently published comprehensive review by Atkinson *et al.* (2014). Understanding the genetic control of global root architecture in monocots is more of a challenge than in Arabidopsis, because the complexity and size of the root system presents several phenotyping challenges. An ideal root phenotyping platform provides highly accurate measurements in a high-throughput and environmentally relevant setting. To date, no single approach has mastered all of these requirements. Two recent reviews highlight different plant phenotyping approaches including those that have been developed to measure root architecture traits (Zhu *et al.*, 2011; Fabio & Schurr, 2013). However, no single phenotyping platform is ideal for every situation, and the choice largely depends on the questions being pursued. For example, a high-throughput, highly accurate gel-based imaging platform is ideal to uncover the genetic regulation of early root architecture establishment (Iyer-Pascuzzi *et al.*, 2010; Clark *et al.*, 2013; Topp *et al.*, 2013; Zurek *et al.*, 2015), whereas a high-throughput, albeit less accurate, rhizotron-based imaging platform is better for studying root architecture in response to different soil environments (Taylor *et al.*, 1990; Nagel *et al.*, 2012).

1. Genetic regulation of root architecture

As mentioned above, it is likely that root system response to the environment occurs through co-opting developmental mechanisms. Thus, determining the genetic control of root architecture under controlled conditions should facilitate our understanding of how roots respond to a changing environment. Although several genes have been identified that result in the presence or absence of different root types, these genes demonstrate Mendelian inheritance patterns and are unlikely to account for the overall variation observed in different germplasm. Instead, subtle shifts in root architecture resulting in, for example, deeper, wider or thicker roots are likely controlled by complex interactions among tens to hundreds of genes. Much like the genetic redundancy challenges encountered using network analysis, the identification of genes controlling root architecture faces a similar obstacle. Quantitative genetic approaches use the probability of association between a genotype and a phenotype to identify candidate genetic loci. Natural variation within a population as a result of evolutionary adaptation can be exploited to predict the contribution of genotype to phenotype through quantitative trait loci (QTL) identification.

To date, only one gene has been identified that regulates a relatively subtle alteration to root architecture in a monocot. *DEEPER ROOTING 1* (*DRO1*) was identified as a major QTL for deeper rooting in rice (Uga *et al.*, 2011) (Fig. 6b). Phenotypically, deeper rooting is achieved by a combination of root growth angle and increased root length. Positional cloning of the *DRO1* QTL identified an uncharacterized membrane-associated gene, called *DRO1* (Uga *et al.*, 2013). DRO1 regulates root angle by modulating the response to gravity. Under standard growth conditions *DRO1* is expressed in the root tip and crown root primordia. However, in response to gravitropic stimuli (i.e. rotation), *DRO1* becomes enriched at the outer edge of the distal elongation zone, where it is proposed to promote cell elongation (Uga *et al.*, 2013) (Fig. 6c). This enrichment of *DRO1* is facilitated by the phytohormone auxin, which inhibits *DRO1* expression on the inner edge of the root (Fig. 6c) (Uga *et al.*, 2013). Thus, *DRO1* is the first gene to be described that controls a global root architecture trait in monocots.

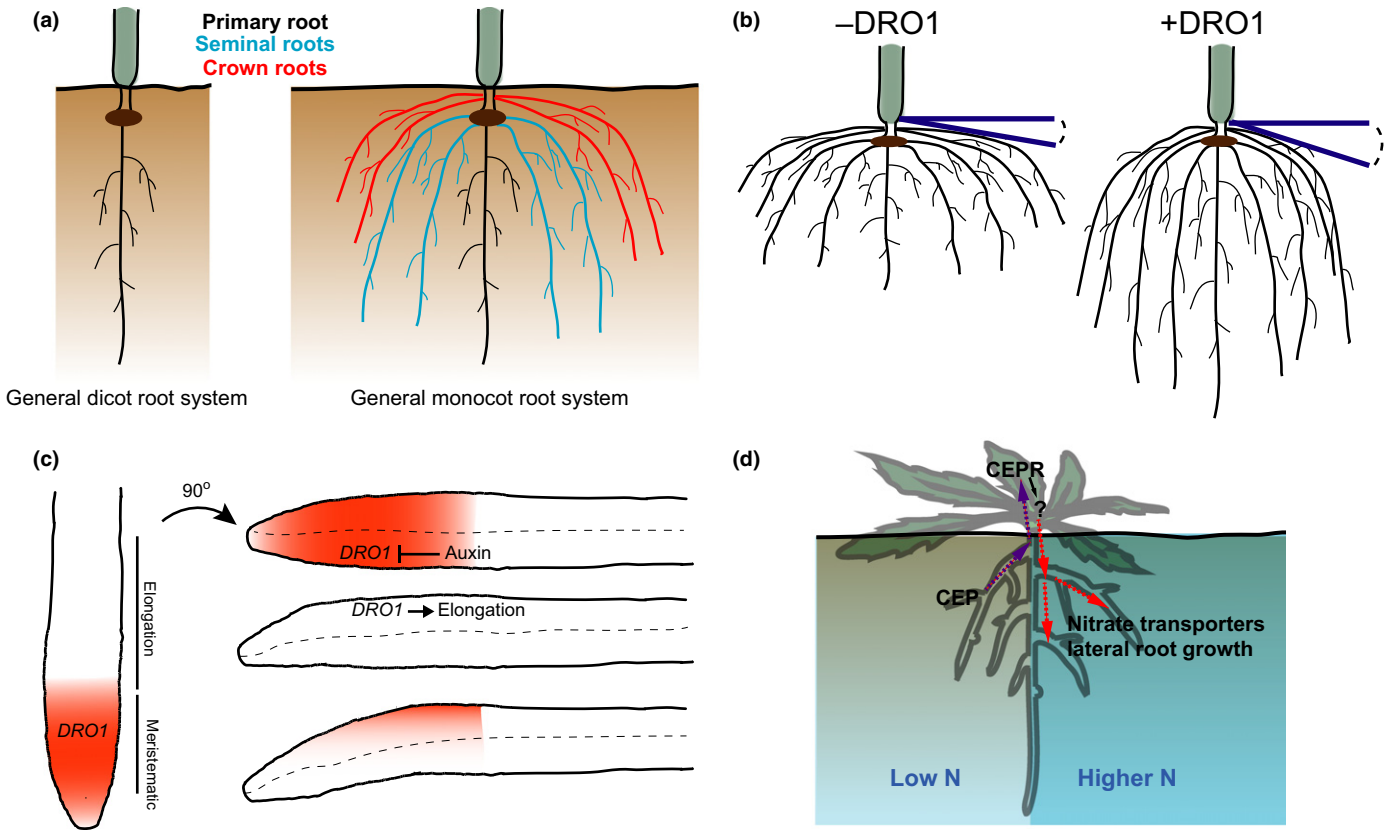

Fig. 6 Genetic control of root architecture and response to the environment. (a) Root system architecture in dicots is generally composed of a single primary root and associated lateral roots (black). By contrast, monocot root architecture consists of a fibrous root system. Fibrous root systems are composed of the primary root (black), as well as seed-borne seminal roots (blue) and shoot-borne crown roots (red). (b) The DRO1 (DRO1-DEEPER-ROOTING 1) quantitative trait locus (QTL) and associated gene regulate root angle in rice. The presence of the DRO1 allele results in a larger root angle and thus deeper roots. (c) DRO1 regulates root angle by enabling a more rapid response to gravity. Under standard gravitropic conditions, DRO1 is expressed in root meristems. In response to gravistimulation (i.e. 90° rotation), DRO1 becomes enriched at the outer edge of the root. This enrichment is promoted through inhibition of DRO1 by the phytohormone auxin on the inner edge. DRO1 then promotes cell elongation at the outer edge, facilitating root turning in response to gravistimulation. (d) A root-to-shoot-to-root signal relay regulates the global response to local nutrient deprivation. Roots in contact with low concentrations of nitrogen (N) (light blue) produce CEPs (C-TERMINALLY ENCODED PEPTIDES). CEPs then move through the xylem into the shoot, where the peptides interact with their receptors, CEP receptors (CEPRs). The shoot CEPRs then transmit an unknown signal back to the roots to promote lateral root outgrowth and the expression of nitrate transporters in regions of higher N (dark blue).

The positional cloning and characterization of *DRO1* highlight one approach to uncover the genetic control of root development. However, QTL cloning is quite laborious and the influence of a single gene on a complex phenotype is highly dependent on the percentage of variation of the trait that a QTL accounts for. The *DRO1* QTL accounts for 66.6% of the phenotypic variation observed in deeper rooting between two rice cultivars (Uga *et al.*, 2011), suggesting that its impact is quite significant; other root architecture QTLs that have been identified in rice have an impact that ranges from 3.6% to 51.8% (Kamoshita *et al.*, 2002; Price *et al.*, 2002; Zheng *et al.*, 2003; Yue *et al.*, 2006; Topp *et al.*, 2013). QTLs that account for a low percentage of the phenotypic variation can either indicate that many genes control a given trait or that the phenotypic descriptors for root architecture are not optimum. Although both explanations may well contribute to low effect sizes, it is important to consider that a QTL is unlikely to regulate a single trait without also influencing other aspects of root development. The use of multivariate composite traits that cover several root architecture parameters resulted in QTLs that account for a much higher percentage of variation (24–37%) than individual traits alone (7–15.7%) (Topp *et al.*, 2013). Thus, as we move forward with the identification of genes that regulate root development, it is important to consider alternative computational approaches to interpret root phenotypes, such as multivariate QTL analysis.

2. Root architecture response to the environment

One of the goals in elucidating the genetic regulation of root architecture is to facilitate our ability to predict how a genotype will respond to a given environment. Understanding the genotype by environment (G × E) interaction is at the forefront of agricultural improvement initiatives, including root system improvement. Because roots are the first point of contact and uptake for soil nutrients and pathogens, it is reasonable to assume that changes in root architecture can lead to improved plant performance under different biotic or abiotic stress conditions. Here we highlight recent advances in our understanding of root responses to two abiotic stress conditions, low phosphorus (P) and low nitrogen (N).

Phosphate is a finite, nonrenewable resource, whose stocks are being rapidly depleted. Soil P is relatively immobile and therefore availability is generally greatest in the topsoil (Shen *et al.*, 2011). One way to overcome these increasingly limited resources is to generate plants that can thrive under P-limited conditions. Over a decade ago, a major QTL for P-deficiency tolerance in rice, *PUP1*, was identified, for which the underlying gene has only recently been cloned (Wissuwa *et al.*, 2002; Gamuyao *et al.*, 2013). The delay in gene identification can be attributed, in part, to the absence of this gene model from the rice reference genome. The responsible gene, *PHOSPHORUS-STARVATION TOLERANCE 1* (*PSTOL1*) is a putative receptor-like cytoplasmic kinase (Gamuyao *et al.*, 2013). Similar to *DRO1*, *PSTOL1* expression is localized to crown root primordia; however, the exact mechanism of how PSTOL1 promotes fitness under P-deficiency remains to be determined (Gamuyao *et al.*, 2013). Surprisingly, known P-starvation genes were unchanged in response to PSTOL1 expression. Instead there were constitutive changes in genes related to general root growth and stress response (Gamuyao *et al.*, 2013). Thus, PSTOL1 is proposed to promote plant productivity under low P by increasing early root growth (Gamuyao *et al.*, 2013). Although PSTOL1 was shown to increase yield by 60% under low P conditions, high amounts of PSTOL1 also promote the uptake of other nutrients, including N and potassium (K) (Gamuyao *et al.*, 2013). This suggests that increased early root growth may be beneficial under other nutrient limited conditions as well. However, it is important to remember that there is no universal root architecture that will benefit every plant under every condition. Instead, it has been suggested that there are tradeoffs between different aspects of root architecture to enable the acquisition of multiple resources that may reside in different soil layers (Ho *et al.*, 2005).

Although PSTOL1 was identified based on plant performance under low P conditions, it regulates root growth independent of the nutrient environment (Gamuyao *et al.*, 2013). Consistent with this observation, root architectures that promote topsoil foraging have been shown to have a dominant influence on P acquisition independent of a nutrient response (Lynch, 2011). However, other changes in root anatomy and architecture (e.g. increased root hair length and density) occur in response to low P (Lynch, 2011) and we still know very little about the signals induced in response to nutrient deficiency and their mechanism of action. Recent work from Tabata *et al.* (2014) identified a root-to-shoot-to-root signaling mechanism through a peptide-receptor relay that facilitates the perception and response to low N in *Arabidopsis*. In roots, local N-deprivation induces the distal upregulation of nitrate transporters and increased lateral roots in regions of sufficient N (Forde, 2002). Until recently, it was unknown how local nutrient deprivation could elicit a long-distance signal and influence distal root function. Tabata *et al.* (2014) identified a ligand-receptor relay in which a local root-derived peptide signal is transmitted through the xylem to shoot-localized receptors and then an unknown shoot-derived signal is transmitted to distal roots to elicit local changes in N-sufficient environments (Fig. 6d). Specifically, a family of *C-TERMINALLY ENCODED PEPTIDES* (*CEP*) are locally upregulated in response to N-deficiency and lead to local root growth inhibition (Tabata *et al.*, 2014). CEPs act as an ascending signal by moving from the root through the xylem into the shoot, where they interact with their receptor-like kinases, CEPRs, and transmit an unknown descending signal to increase the expression of nitrate transporters and lateral root growth in roots with higher local N concentration (Tabata *et al.*, 2014) (Fig. 6d). An open question is if this type of systemic response is a general mechanism that plants use to integrate local abiotic or biotic stresses into global responses. Support for a general mechanism comes from the identification of a similar signaling relay for the symbiotic relationship between legumes and N-fixing rhizobia. Rhizobia associate with legumes and form root nodules for N-fixation, which generally benefits the plant. However, too much nodulation can be detrimental. To control the amount of nodulation, rhizobia induce the *CLAVATA3/EMBRYO SURROUNDING REGION-RELATED* (*CLE*) family of small peptides (Okamoto *et al.*, 2009). The root-derived CLE peptides translocate to the shoot and interact with a shoot receptor-like kinase, *HYPERNODULATION ABERRANT ROOT FORMATION 1* (*HAR1*) (Okamoto *et al.*, 2013). The HAR1 receptor then transmits an unknown signal back to the root to limit nodulation (Krusell *et al.*, 2002; Nishimura *et al.*, 2002). Together these results suggest that a systemic relay in which local peptide signals are transmitted globally to elicit distal responses may be a common approach facilitating environmental adaptation.

These intriguing results open many new questions, the most obvious being what is the descending signal. Whereas phytohormones can directly influence transcription, receptor-like kinases transmit signals by phosphorylation of target proteins (De Smet *et al.*, 2009). In plants, very few receptor-like kinase substrates have been identified, but the few that have include phospho-relays which lead to changes in gene transcription (Gendron & Wang, 2007; Casson & Gray, 2008; Tang *et al.*, 2008; Kim *et al.*, 2009). Although the transcriptional changes downstream of CEP-CEPR remain to be identified, the types of mobile signals that plants utilize include transcription factors, peptides and phytohormones (Sparks *et al.*, 2013). Phytohormones are not directly regulated by transcription, but transporters or other regulators could be altered. It will be interesting to see which of these mechanisms is required for the second half of these signaling relays.

VI. Conclusions

Historically, efforts to improve crop yield have focused on aboveground, shoot-related phenotypes. In recent years, roots have been seen as an under-utilized resource for yield improvement and have become the focus of numerous investigations. Often difficult to visualize and analyze, the so-called 'hidden half' of plants requires innovative approaches to understand its developmental processes and responses to external cues. The past several years have seen great strides in advancing our understanding of the genetic regulation of root formation at the cellular, organ and structural levels. We have highlighted a few of these significant advances. Among the outstanding challenges, genetic redundancy is near the top. As sessile organisms, plants have incredible regulatory control

mechanisms to recruit developmental processes and cope with external conditions. This is advantageous from a plant evolutionary perspective, but can be challenging from a research perspective. Several initiatives are working to develop double mutant collections of closely related genes in Arabidopsis (e.g. GABI-DUPLO; Bolle *et al.*, 2013) and the recent rise of genome editing technologies (e.g. CRISPR-Cas9; Bortesi & Fischer, 2015) promises a bright future in uncovering more of the genetic control mechanisms involved in root development.

Another challenge is reducing the time from QTL identification to mapping and identifying the responsible gene(s). Integration of gene expression data and gene regulatory network connections should speed the time to gene identification. This is not useful in some situations when the gene is absent from the reference genome, as for *PSTOL1*. However, as DNA sequencing becomes more and more affordable we predict the release of thousands of cultivar-specific genomes in the next 10 yr. For rice, 3000 genomes have already been sequenced (3,000 rice genomes project, 2014) and other species are not far behind. Lastly, we want to highlight the recent appreciation for peptide-receptor signaling in plant development and response to the environment. Although the importance of phytohormones and transcription factors in root development is well-known, small peptides and receptor-like kinases have been largely ignored until recently. There is a growing understanding of the importance of signal perception at the cell membrane through receptor-like kinases and the transmission of global response signals through peptide movement in root development. In this review we have presented recent advances and perspectives for future root research and predict that our understanding of how root anatomy and architecture contribute to plant development and response to a changing environment will significantly improve over the next 5–10 yr.

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