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To branch or not to branch: the role of pre-patterning in lateral root formation

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Summary

The establishment of a pre-pattern or competence to form new organs is a key feature of the postembryonic plasticity of plant development, and the elaboration of such pre-patterns leads to remarkable heterogeneity in plant form. In root systems, many of the differences in architecture can be directly attributed to the outgrowth of lateral roots. In recent years, efforts have focused on understanding how the pattern of lateral roots is established. Here, we review recent findings that point to a periodic mechanism for establishing this pattern, as well as roles for plant hormones, particularly auxin, in the earliest steps leading up to lateral root primordium development. In addition, we compare the development of lateral root primordia with *in vitro* plant regeneration and discuss possible common molecular mechanisms.

Key words: Auxin, Callus formation, Lateral root development, Periodic gene expression, Pre-patterning

Introduction

The postembryonic formation of lateral organs in plants occurs when cells acquire a new fate, generally based on positional cues, and then undergo a coordinated program of cell division and differentiation to produce an organ primordium. In the root, lateral branches are formed primarily from cells of the pericycle (see Glossary, Box 1), which is an internal tissue surrounding the central vascular cylinder (Fig. 1). On a regular basis, subsets of pericycle cells become competent to form lateral roots (LRs, see Glossary, Box 1) and, depending on the species, this occurs in the proximity of phloem (e.g. in maize) or protoxylem (e.g. in *Arabidopsis thaliana*) strands (Casero et al., 1995; Dubrovsky et al., 2000; Hochholdinger and Zimmermann, 2008). The frequency of these events establishes the number of sites competent to form LRs over time and is, therefore, crucial in shaping the final root system architecture, which is a major determinant of agronomic productivity. After competence is established, the development of a lateral root primordium (LRP, see Glossary, Box 1) occurs either strictly through division of cells derived from the pericycle (e.g. in *Arabidopsis*) or through division of pericycle-derived cells and recruitment of cells in the adjacent endodermis (e.g. in maize) (Bell, 1970; Hochholdinger and Zimmermann, 2008).

The development of LRP can be induced or repressed in response to environmental conditions and thus provides a mechanism for the plant to cope with changing edaphic conditions (Malamy, 2005). A great number of environmental variables have been shown to influence LRP development. For example, osmotic stress (drought) inhibits the developmental progression of early stage LRP (Deak and Malamy, 2005), and activation of the meristem in emerged LRP is blocked by exogenous abscisic acid, a plant hormone involved in stress responses (De Smet et al., 2003). LRP development is also sensitive to the availability of nutrients, including growth-limiting nutrients such as nitrogen and phosphorous (reviewed by Jones and Ljung, 2012; Lavenus et al., 2013; Péret et al., 2011). Although some environmental stimuli have a clear involvement in late stage LRP development, nitrogen and phosphorous can also act earlier in LRP development (Lima et al., 2010). It is unclear whether environmental stimuli can only influence the developmental progression of sites that are already established as competent to form LRP, or if lateral root pre-patterning (see Glossary, Box 1), which has, to date, been shown to be primarily dependent on time (Moreno-Risueno et al., 2010), can also be impacted by environmental cues. Although the final outcome would be similar – a change in LR number – the distinction between environmental impact on later developmental stages or pre-patterning would reflect a difference in the plant's strategy to achieve developmental plasticity under variable conditions. Therefore, understanding the regulation of LR pre-patterning and subsequent primordia development has captured the interest of many plant biologists.

The molecular and cellular mechanisms of lateral root formation (see Glossary, Box 1) have been most extensively studied in the model plant *Arabidopsis thaliana*. In this species, relatively regular spacing of LRs was reported, with LR placement coinciding with the outside edge of curves along the primary root, particularly when roots show a bending or wavy growth pattern. To understand the basis for this regular branching pattern, it is crucial to understand the earliest developmental events occurring during LR formation. The *Arabidopsis* primary root tip is classically divided into three main developmental zones (Fig. 1A) (Dolan et al., 1993). The rootward-most portion of the root tip, the meristematic zone, contains the stem cell niche and cells that are undergoing active proliferation with relatively little expansion. The meristematic zone is occasionally described as having two parts: the basal and apical meristem. The basal meristem is the shootward-most region of the meristem and is also referred to as the transition zone, as cell division rates slow and cells begin to increase in size within this zone (Fig. 1A). This is followed by the elongation zone: a region in which proliferative cell divisions cease and cells undergo rapid and extensive cell elongation, increasing in length by 300% within 3 hours (Verbelen et al., 2006). Finally, cells enter the differentiation zone where they cease growth and the vast majority attain their final size and begin to differentiate, acquiring their specialized cellular features and functions (Fig. 1A). Additionally, the development of LRP begins in the differentiation zone.

A developing LRP becomes detectable microscopically when a primordium consisting of a single cell layer is generated through asymmetric cell division in the differentiation zone of the root

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Box 1. Glossary

Founder cells (FCs). The initial cells specified to become a new organ or tissue. Founder cells are typically histologically similar to related/nearby cells and can only be identified following other developmental events, such as the activation of cell division.

Lateral root (LR). A root that is branching from a parent root and has activated its apical meristem. In most plants, these organs are formed postembryonically.

Lateral root development. A term without a clear and accepted definition. This term can be used to encompass all the developmental stages of a lateral root primordium (stages I-VII) and is more clearly stated as 'lateral root primordium development'. The progression of any one lateral root primordium through the developmental stages is impinged upon by environmental cues.

Lateral root formation. A term that encompasses all of the events leading to the production of an actively growing lateral root.

Lateral root founder cells (LRFCs). A set of two longitudinally abutted cells in each of the two to three cell files of the xylem pole pericycle at one side of the root. These cells will undergo asymmetric cell divisions (also called formative divisions) to initiate a lateral root primordium. The first morphological indicator that these cells have a distinct fate is the migration of their nuclei towards the common cell wall. Additionally, expression of *DR5:GFP* and *genomicLBD16:GFP* is induced in these cells prior to asymmetric cell division.

Lateral root pre-patterning. The specification of a spatiotemporal region of the root that is competent to give rise to a lateral root primordium. The lateral root pre-pattern is predicted to be established by periodic gene expression in the oscillation zone and the formation of prebranch sites. Establishment of the pre-pattern is stable under various environmental conditions.

Lateral root primordia/primordium (LRP). A group of cells originating from the asymmetric division of lateral root founder cells that progress through a stereotypical set of developmental stages to produce a root *de novo*.

Oscillation zone (OZ). The region in which periodic oscillation in the expression of the *DR5:LUC* reporter and certain endogenous genes occurs. This region encompasses the shootward-most portion of the meristematic zone, as well as the elongation zone (see Fig. 1A).

Pericycle. A cell layer located between the vascular cylinder and the ground tissue (Fig. 1B). Like the vascular tissues, the pericycle has a bilaterally symmetric organization.

Prebranch site. Static points of *DR5:LUC* expression that occur following the oscillation of *DR5:LUC* in the oscillation zone. Prebranch sites are competent to form lateral roots in the future. Because these sites occur earlier than expected for lateral root founder cells and it has not been determined whether expression is cell type-specific, the relationship between prebranch sites and lateral root founder cells is unclear.

Priming. A process that occurs in select xylem pole pericycle cells that is proposed to coincide with the oscillation of gene expression. Priming is predicted to condition these cells for subsequent prebranch site and lateral root founder cell specification.

Stage I lateral root primordium. A lateral root primordium comprising a single cell layer and the first stage of lateral root primordium development. Initially, this structure is composed of two small cells resulting from asymmetric division of two to three files of lateral root founder cells; however, successive divisions result in a group of four to ten small, longitudinally abutted cells per file.

Stage II lateral root primordium. Following radial expansion, the cells of the stage I primordium reorient their division plane, dividing periclinally to the root longitudinal axis, resulting in a primordium comprising two cell layers.

Xylem pole pericycle (XPP) cells. Cells of the pericycle that flank the protoxylem cells (Fig. 1B). Xylem pole pericycle cells have distinct cellular morphology and gene expression profiles and the unique capacity within the differentiation zone to re-enter the cell cycle and undergo cell division. Xylem pole pericycle cell division is required for lateral root initiation, as well as for regeneration via callus.

(Fig. 1C) (Malamy and Benfey, 1997). The adjacent pairs of xylem pole pericycle (XPP) cells (see Glossary, Box 1) that undergo this cell division, also called LR initiation, are designated as lateral root founder cells (LRFCs, see Glossary, Box 1). Prior to cell division, LRFCs cannot be distinguished microscopically from the other pericycle cells without the use of specific reporter lines. These founder cells first undergo anticlinal cell divisions to generate a single-cell layered primordium containing up to ten small cells (a stage I lateral root primordium, see Glossary, Box 1). This is followed by periclinal cell divisions in the most central cells, giving rise to a two-cell layered primordium (a stage II lateral root primordium, see Glossary, Box 1). Several rounds of division in the central cells lead to an ellipsoid-shaped primordium that eventually grows through the outer cell layers of the parent root and finally emerges from the root surface (Fig. 1C) (Lucas et al., 2013).

Molecular evidence suggests that early events establishing the regular pattern of LRs, prior to LRFC identity and LR initiation, occur at a more rootward position in the root tip where recurrent expression of reporter constructs driven by the synthetic promoter element *DR5* (*DIRECT REPEAT5*) are observed (De Smet et al., 2007; Moreno-Risueno et al., 2010). *DR5* promoter activity, which is used to assay the transcriptional response to auxin, is correlated with subsequent LR initiation, suggesting that an oscillating transcriptional mechanism operates as an upstream driving force for the regular pattern of LRs. Indeed, a large number of genes were identified that oscillate both in phase and in antiphase with the *DR5* reporter, although the oscillatory system appears to function independently of local auxin levels (Moreno-Risueno et al., 2010). Furthermore, the 6-hour period of the transcriptional oscillation appears to be shorter than the frequency at which LRs initiate, suggesting that the establishment of competence to form an LR and the initiation of an LRP are distinct developmental events.

The oscillation in gene expression occurs over a region of the root termed the oscillation zone (OZ, see Glossary, Box 1) (Fig. 1A) (Moreno-Risueno et al., 2010). During the period of the oscillation, as many as 12 pericycle cells may exit the OZ (Verbelen et al., 2006), suggesting that several cells experience the oscillation in gene expression. Yet, generally only pairs of abutted pericycle cells are specified as LRFCs, suggesting that a mechanism exists to refine or restrict the number of pericycle cells that will adopt this fate. At the tissue-specific level, *DR5* reporter expression suggests that the oscillatory maximum occurs in the protoxylem cells adjacent to the pericycle (Fig. 1B). It might therefore be that XPP cells receive signals during the oscillation to prepare them for LR initiation, a process that has been termed priming (see Glossary, Box 1). After the oscillation, a static point of *DR5* expression marks prebranch sites (see Glossary, Box 1), which are defined as positions competent to produce LRs in the future. Subsequently, auxin signaling-dependent nuclear migration in LRFCs precedes the asymmetric cell divisions that generate stage I primordia.

Hence, the events leading up to and including the specification of LRFCs and LR initiation are crucial for LR organogenesis, but many questions surrounding the molecular mechanisms that underlie the earliest stages of LR formation and development (see Glossary, Box 1) remain unanswered. Here, we focus on these early developmental steps and reflect on the potential mechanisms that contribute to the establishment of the LR distribution pattern, which forms the basis of root system architecture.

Is there a mechanical mechanism involved in establishing the pattern of LRs?

Under experimental conditions, *Arabidopsis* roots grow in a serpentine manner, bending from side to side as they traverse the

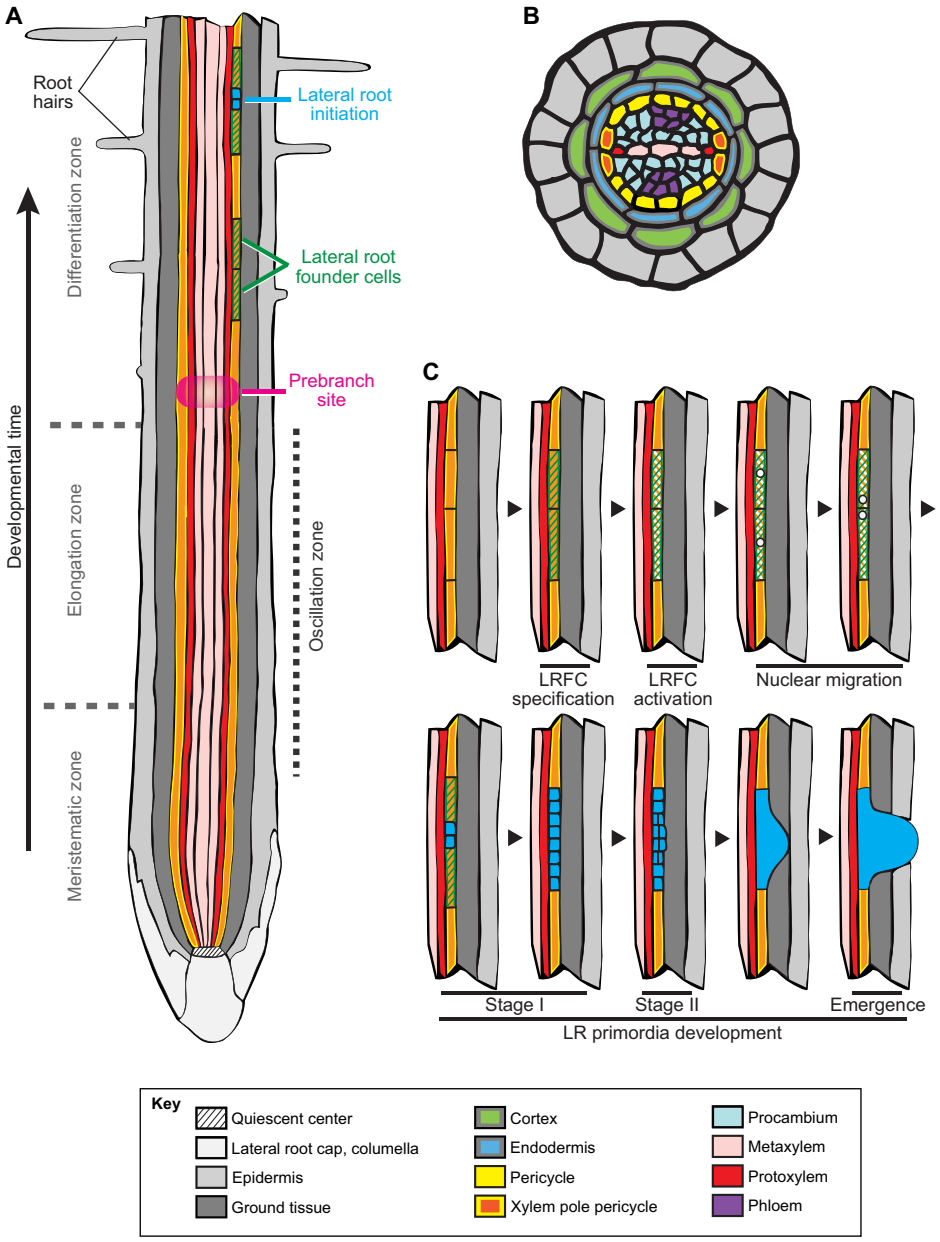

Fig. 1. Structure and development of the *Arabidopsis* root. (**A**) Median longitudinal section depicting developmental time (black arrow) in the longitudinal axis. A prebranch site (magenta) forms after an oscillation of gene expression within the oscillation zone (dotted line). Prebranch sites indicate competence to form a lateral root primordium (LRP) in the future. After competence is established, it is predicted that xylem pole pericycle (XPP) cells within a prebranch site can be specified as lateral root founder cells (LRFCs, green hatching). LRP initiate in the differentiation zone through asymmetric cell division of LRFCs, which gives rise to smaller cells (blue). (**B**) Transverse section. Periodic expression of *DR5:GUS* occurs in the protoxylem; however, because lateral root (LR) initiation occurs in the adjacent XPP cells, signaling between these cell types might be required for LRFC specification. Note that the ground tissue comprises two cell layers: the outermost cortex and the endodermis, which is immediately exterior to the pericycle. (**C**) Cut-away portion of the median longitudinal section focused on a region where an LR will form. XPP cells are predicted to be sequentially specified as LRFCs (green hatching), then activated to undergo cell division (green/white hatching). LRFC activation results in the coordinated migration of nuclei (white circles) towards the common cell wall in a pair of longitudinally abutted cells. These cells then undergo asymmetric division, giving rise to smaller cells (blue), to generate a stage I LRP. The primordium grows through the outer cell layers of the primary root until it emerges from the epidermis. Drawing is not to scale.

culture medium. Root waving has been described as the consequence of differential growth due to reorientation of growth in the direction of the gravity vector combined with thigmotropic growth [reorientation based on the touch response (reviewed by Oliva and Dunand, 2007)]. These root growth behaviors are hypothesized to be an evolutionary strategy to facilitate obstacle avoidance under rhizospheric conditions. Accompanying root waving, the development of LRP and the emergence of LRs coincides with the outside edge of these curves (Fortin et al., 1989), suggesting a relationship between the pattern of LRs and root waving.

As root waving results from alternating left and right turns by the root tip, the number of outside edges facing left and right is roughly equal. Coincident with the sidedness of the curves, the presence of LRs and LRP is also equal on each side of the root (Fig. 2A). Furthermore, an agravitropic auxin transport mutant, *aux1*, which turns in only one direction, shows a shift in LR distribution, with more LRs emerging on the outside edge of the coiled root (De Smet et al., 2007). These results suggest that the distribution pattern of LRs is linked with root waving and the gravity response via auxin transport. The co-occurrence of these processes was further investigated by inducing root bending by gravistimulation and mechanical methods (Ditengou et al., 2008; Laskowski et al., 2008; Lucas et al., 2008; Richter et al., 2009). Gravistimulated bends occur when plants are reoriented with respect to the gravity vector, resulting in a sharp bend as the root tip reorients growth to realign with gravity. Mechanical bending can be induced through manual manipulation of root or seedling position, growth of the root into a barrier, or through gel sliding assays (Fig. 2B-E). Similar to root waving, induction of sharper bends in the root by any method resulted in emergence of LRs at the outside edge of the bends. Intriguingly, LRP develop at the outside edge of a bend even when a root is only transiently bent; however, LRP and mechanically induced bends only coincide when bending occurs a short distance from the root tip (Ditengou et al., 2008; Laskowski et al., 2008; Lucas et al., 2008; Richter et al., 2009).

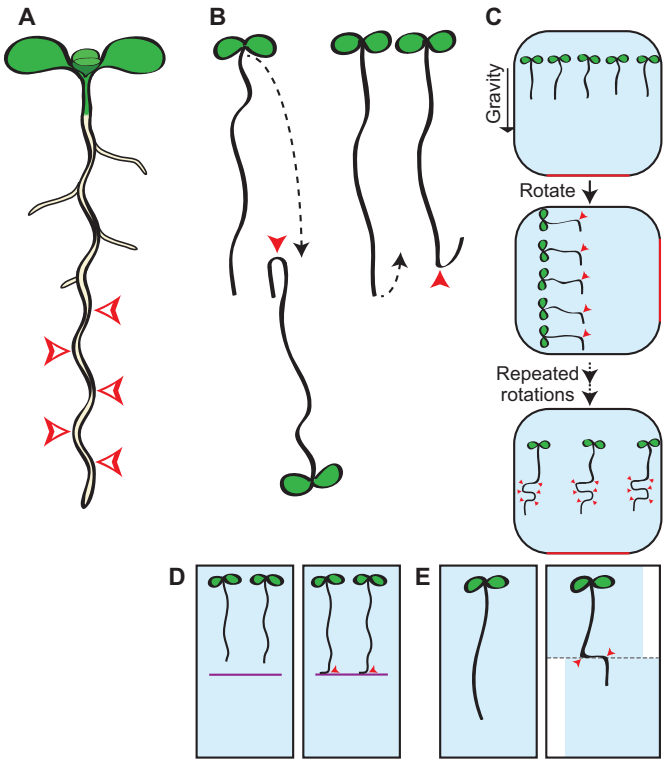

Fig. 2. LRs emerge from the outside of curves in the primary root. Schematics of root bends formed under various experimental conditions. (**A**) Root waving occurs as roots grow along the surface of agar plates. LRP develop and eventually emerge from the outside of the curves. The arrowheads indicate positions of incipient LRP. (**B**) Bends can be induced to form in the root through manual manipulation of the seedling either by pulling the shoot downward (left) or by pushing the root tip upward (right). (**C**) Gravistimulation-induced bends. If seedlings are reoriented with respect to the gravity vector, a bend will form as the root tip responds to realign the tip to gravity through differential growth. (**D**) In the absence of gravitropic response in either the root or shoot, a bend can be induced by root growth into a barrier (purple bar). (**E**) Bends can also be induced by cutting the agar on either side of a growing root (gray dotted line) and sliding the agar to one side, thereby creating two bends in the root. In these gel-sliding assays, neither the root tip nor shoot is exposed to manual contact or reorientation. Arrowheads (B-E) indicate the position of LRP emergence in response to induced bends.

The molecular link between gravitropism/root waving and LRP development is predicted to be auxin. It was proposed that altered auxin distribution upon root reorientation is sufficient to establish the pattern of LRs along the root. However, roots that are agravitropic due to defects in auxin signaling or transport, or due to removal of gravity-sensing tissues, still form LRs on the outside of curves, suggesting that the gravity response is not specifically required (Ditengou et al., 2008; Lucas et al., 2008; Richter et al., 2009). Recent observations of roots grown during spaceflight further indicate that the pattern of LRs and gravitropic responses of the primary root are separable; in the micro-***g*** environment, roots grow more slowly than those of control plants on Earth (at 1 ***g***), but root waving persists and LRs are observed on the outside of curves (Paul et al., 2012). Thus, root waving and the coincidence of LRP with curves occur independently of gravity. These results do not preclude the hypothesis that asymmetric auxin distribution at curves in the root, regardless of its cause, is linked to promoting the development of an LRP.

Indeed, the expression and/or localization of reporters for auxin signaling and transport show rapid changes (observed within 3-7 hours) after the induction of bends, suggesting that mechanical strain on the cells induces changes in auxin distribution and signaling (Ditengou et al., 2008; Laskowski et al., 2008). A computational model was developed whereby the physical deformation of cells upon bending leads to auxin accumulation on the outside of curves, which was suggested to trigger local competence of XPP cells, and then promote the development and emergence of LRP (Laskowski et al., 2008). However, mutants with defects in auxin signaling and/or transport and reduced LR production consistently form LRP or LRs when roots are manually bent (Ditengou et al., 2008; Richter et al., 2009). These results suggest that, although the development of LRP might be defective in these mutants, sites competent to form LRP are present. Furthermore, bends induced for very short durations (~20 seconds) are sufficient to increase the number of LRs observed at the outside of these transient bends. Following these bends, similarly rapid changes in cytosolic Ca^{2+} levels are observed, and treatment with calcium channel blockers inhibits both changes in cytosolic Ca^{2+} and the production of LRP after bending, indicating that Ca^{2+} signaling is required for bend-induced LRP development (Richter et al., 2009). These results suggest that rapid cellular signaling upon bending triggers events that lead to LRP development, prior to changes in cell shape and differential auxin distribution. This implies that events upstream of auxin signaling can promote LRP development and might indicate that the competence to form an LRP is already present at positions of mechanical bending. Alternatively, the pattern of LRs might be less dependent on developmental pre-patterning and is instead a consequence of root growth behaviors.

Nevertheless, evidence for an endogenous pre-patterning mechanism is observed in studies of bend-induced LRP development. Roots subjected to gravistimuli at regular intervals showed a maximum number of LRs when gravistimulation occurred at 6-hour intervals. However, LRP formed between the gravity-induced bends when the intervals between gravistimulation were extended to 12 and 24 hours (Lucas et al., 2008). Additionally, removal of the root tip prior to manual bending results in the formation of more LRs between the cut edge and the bent region in both wild type and auxin signaling mutants (Ditengou et al., 2008). These results suggest that the pattern of LRP is established independently of induced bends and indicates that, although a single LR typically emerges at an induced bend, additional nearby sites are competent to develop into LRP. These competent sites might be developmentally stalled by signals from the root tip, by the emerging primordia, or both.

Evidence for an endogenous mechanism in LR pre-patterning

An endogenous mechanism for establishing the pattern of LRs was proposed based on a temporal fluctuation in expression of the *DR5* reporter. At 15-hour intervals, expression of the *DR5* promoter fused to the *β-glucuronidase* (*GUS*) reporter gene was observed in the shootward-most portion of the meristematic zone, specifically in the two protoxylem cell files but not in the adjacent XPP cells (Fig. 1B). The longitudinal position of the sites of *DR5:GUS* expression in the meristem could be correlated with the subsequent development of an LRP (De Smet et al., 2007). Thus, it was suggested that *DR5*-expressing protoxylem cells signal to adjacent XPP cells to condition them for LRFC identity (i.e. priming). If the temporal changes in *DR5* expression are hypothesized to direct the

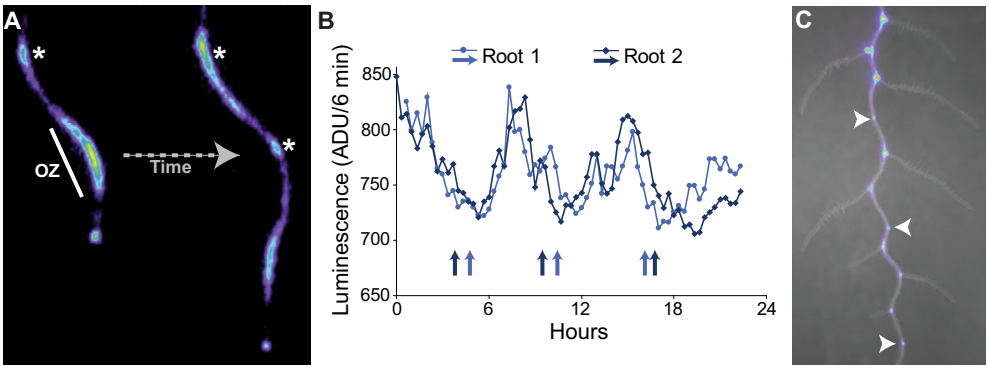

Fig. 3. Prebranch sites mark the positions at which LRP will subsequently develop and emerge. (**A**) An oscillation in *DR5:LUC* expression (chemiluminescence signal imaged at 5-6 minute exposure times) in the oscillation zone (OZ) leads to the formation of a prebranch site (asterisk). (**B**) Quantification of the oscillation of *DR5:LUC* expression in two individual roots. The oscillation has a period of ~6 hours and appears to precede the changes in growth direction of the root tip during root waving. Blue/dark blue arrows indicate the time points at which bends were formed in each of the primary roots. ADU, analog-digital units. (**C**) Overlay of a luciferase and brightfield image (taken 5 days after the luciferase image) to show emerged lateral roots. Arrowheads indicate positions at which LRP have yet to emerge. (B,C) Adapted with permission (Moreno-Risueno et al., 2010).

formation of an LRP, this recurrent process could explain the regular spacing between LRs under controlled growth conditions. However, as *DR5* expression occurs in both sets of protoxylem cells this cannot explain the alternating distribution of LRs on the sides of the root, suggesting that a subsequent mechanism determines LR sidedness (De Smet et al., 2007). For example, the mechanical strain and asymmetric distribution of Ca^{2+} and auxin that is described in cells upon bending occur in more differentiated regions of the root; therefore, it is possible that the sidedness of LR initiation is determined later in response to signals produced as a consequence of changes in cell shape.

Expression conferred by the *DR5* promoter was further examined by fusing it to the *luciferase* (*LUC*) gene, allowing visualization of its behavior *in vivo* (Moreno-Risueno et al., 2010). Expression of *DR5:LUC* in the root tip revealed oscillatory activity with a period of 6 hours. This dynamic expression pattern occurred over a larger region of the root tip than previously described and this region was, therefore, termed the OZ (Fig. 1A; Fig. 3A,B). Following each peak of the *DR5* oscillation, a static point of expression was observed that exhibited a similar longitudinal distribution as LRP and LRs. Indeed, subsequent examination of these points revealed them to be the future sites of LRP and LRs, and they were therefore designated as prebranch sites (Fig. 3C) (Moreno-Risueno et al., 2010).

DR5 expression is frequently utilized as a proxy for the distribution of auxin; however, an exogenously stimulated peak in auxin levels in the OZ was not able to trigger the formation of a prebranch site. Additionally, a reporter gene with similar response dynamics to exogenous auxin as *DR5:LUC* and expressed in the OZ did not exhibit periodic expression (Moreno-Risueno et al., 2010). These results suggested that oscillatory peaks in auxin itself are not sufficient to account for the dynamic behavior of *DR5* and the subsequent formation of prebranch sites. In an effort to determine the underlying cause of the oscillation, microarray analysis of gene expression identified more than 3400 genes with expression that oscillates either in phase or in antiphase with the *DR5* reporter. Several candidate transcriptional regulators were found to both exhibit oscillatory expression and be functionally important for LR formation (Moreno-Risueno et al., 2010). Although auxin-responsive genes do not necessarily show oscillatory expression in the OZ, some oscillating genes have established roles in LR formation and are involved in, or are downstream of, auxin signaling, including *LATERAL ORGAN BOUNDARIES DOMAIN 16* (*LBD16*) and *AUXIN RESPONSIVE FACTOR 7* (*ARF7*) (Okushima et al., 2007; Okushima et al., 2005). Unexpectedly, *ARF7* was found to oscillate in antiphase to *DR5:LUC*, and in *arf7* mutants the oscillatory expression of *DR5:LUC* is abnormal and prebranch sites form at irregular intervals, suggesting that ARF7 function is important for periodic gene expression in the OZ (Moreno-Risueno et al., 2010). Together, these results led to a model describing a ‘lateral root clock’, in which a complex periodic transcriptional mechanism specifies sites that are competent to form LRs, thus establishing an LR pre-pattern along the root axis.

Like the LRs that follow them, prebranch sites are found at curves that are produced during root waving. Although root waving shows a similar periodicity as prebranch site formation, the oscillation of *DR5* expression is observed prior to the reorientation of root growth direction (Moreno-Risueno et al., 2010). This suggests that, despite their occurrence at a similar position along the root, these events are separated by time. The link between bending and prebranch site formation was examined by exposing roots to gravistimuli and manual bending. Roots responded to gravistimulation asynchronously, with individual roots completing the last bend due to root waving prior to reorienting growth in the direction of the gravity vector, which is consistent with these being distinct growth behaviors. In manual bending assays, prebranch sites were observed at the bend and nearer to the root tip than bends could be made without disrupting the position of the root tip. Manual bending did not result in *de novo* prebranch sites and no LRs emerged from sites not previously marked by a prebranch site; yet, as observed previously, LRP emerged at the outside edge of the bends (Moreno-Risueno et al., 2010). These results are consistent with a hypothesis in which an endogenous patterning mechanism establishes sites competent to form an LR, but LRP development and perhaps sidedness of LRFC specification are subsequent developmental decisions, which integrate multiple cues.

The priming of XPP cells during the oscillation of gene expression in the OZ conceptually links *DR5* expression in the protoxylem with later LRP development in the adjacent pericycle. Although priming is thought to be XPP specific, prebranch sites cannot yet be examined at a cellular level for technical reasons (see

below). Priming of XPP cells would not be predicted to occur only on one side of the root as *DR5* expression is observed at both xylem poles. Additionally, the molecular character of primed XPP cells and the priming signal remain elusive. An alternative, and not mutually exclusive, hypothesis is that genes oscillating in the pericycle itself have important roles in establishing the LR pre-pattern. For example, *LBD16* is observed to oscillate and was recently reported to have XPP-specific expression and a key role in LR initiation (Goh et al., 2012; Moreno-Risueno et al., 2010). Because the root tissue examined for oscillating transcriptional profiles was specific to longitudinal regions but encompassed all root tissues, the tissue-specific nature of any oscillating transcripts was not captured (Moreno-Risueno et al., 2010). The necessity for vascular continuity between primary and lateral roots might be a crucial reason for coordination between vascular patterning and LR pre-patterning, and this is supported by additional molecular connections between vascular patterning and the development of LRP (Bonke et al., 2003; Ohashi-Ito and Bergmann, 2007; Parizot et al., 2008). However, the role of cell-to-cell signaling between protoxylem and XPP cells remains an intriguing question requiring further investigation.

LRFCs and prebranch sites

Organogenesis is generally thought to begin with the specification of founder cells (FCs, see Glossary, Box 1). This specification could involve cells acquiring competence to respond to an activation signal. Activation of FCs typically leads to cell division, which is the first morphological indication that a change in cell fate has occurred. However, prior to the activation of cell division, the identification of FCs is difficult as they are histologically indistinguishable from the surrounding cells. Another difficulty is that there are few molecular reporters for FCs, and for those markers that are available the function of the associated molecules in FC specification, activation or cell division is not entirely clear (Beveridge et al., 2007; Chandler, 2011). These general FC features are also true for LRFCs.

LRFCs are the specific XPP cells that will undergo asymmetric cell division (LR initiation) to produce a stage I LRP. The specification and activation of LRFCs are thought to occur within the differentiation zone of the root, where other cells have ceased division and growth and have differentiated. However, it is unclear if XPP cells dedifferentiate then redifferentiate into LRFCs, or if they are maintained in an undifferentiated state (Dubrovsky et al., 2000; Laskowski et al., 1995; Malamy and Benfey, 1997). Expression of the *DR5:GFP* reporter is observed in select XPP cells and precedes LR initiation. Therefore, activation of *DR5* expression is considered the first indication that specific XPP cells have acquired LRFC identity (Dubrovsky et al., 2008). Additionally, *aberrant lateral root formation 4* (*alf4*) mutants show *DR5:GFP* expression in select XPP cells, yet LRP are not produced as a result of defects in cell division (DiDonato et al., 2004; Dubrovsky et al., 2008). This suggests that *alf4* LRFC are either specified but not activated or are both specified and activated but cannot undergo cell division to produce a stage I LRP. Because *DR5:GFP* expression precedes LRFC division, and pericycle cells appear to be uniformly sensitive to exogenous auxin, it was proposed that local auxin accumulation, rather than increased auxin sensitivity, triggers LRFC specification (Dubrovsky et al., 2008). In addition, one of the first anatomical signs that XPP cells have taken on LRFC fate is the coordinated migration of the nuclei towards the common wall in a pair of cells; however, by this point, LRFC specification and activation have already occurred, as cell division is imminent (De Rybel et al., 2010; Dubrovsky et al., 2011).

Recent evidence shows that the developmental progression of LRFCs to stage I LRP requires activity of the auxin transporter PIN3 in endodermal cells, which are adjacent to the pericycle cells (Fig. 1B). However, LRFCs exhibit *DR5:GFP* expression prior to PIN3 accumulation in endodermal cells, suggesting that LRFC fate has already been specified (Marhavý et al., 2013). Accumulation of auxin in specific cells requires either directed transport or intracellular biosynthesis, with cellular retention of auxin. Either scenario requires that these select XPP cells attain higher auxin levels, suggesting that they might already be distinct from other XPP cells prior to the detection of *DR5:GFP* reporter expression. Thus, in contrast to the proposed role for auxin as a signal in LRFC specification, it might be that auxin acts as an activation signal of LRFC division. Based on this hypothesis it is possible that, in *alf4* mutants, LRFCs are specified and receive the activation signal (as visualized by *DR5:GFP* expression) but, due to mitotic defects, are unable to undergo coordinated cell division. Additionally, *ALF4* expression and protein localization appear to be independent of auxin signaling (DiDonato et al., 2004), suggesting that additional activation signals exist.

Prebranch sites are the static points of *DR5:LUC* expression that form at the position of the peak in the periodic oscillation of *DR5* after the oscillation is complete (Moreno-Risueno et al., 2010). Expression of *DR5*, as reported by GFP, is observed in XPP cells at one side of the xylem pole prior to the asymmetric division that gives rise to an LRP, identifying these cells as LRFCs (Dubrovsky et al., 2008). Because the expression of *DR5* is used to define both of these sites, they might be considered synonymous. However, it is important to keep in mind the difference between the reporter genes *LUC* and *GFP*. The LUC enzyme cleaves its substrate (luciferin), thereby producing light, and it then becomes inactive. Thus, although monitoring LUC activity is a highly dynamic and sensitive method to assay the *in vivo* activity of a promoter (de Ruijter et al., 2003), it is difficult to obtain cell type-specific resolution as light spreads outward in all directions from the source. GFP expression, however, can be localized in a cell type-specific manner using confocal microscopy, although the drawbacks of GFP are long maturation and stability times, higher thresholds for detectability, and a relatively high background fluorescence in plants (de Ruijter et al., 2003). Because the static points of *DR5:LUC* expression are visible earlier than expected for LRFCs, and because it is not yet possible to determine which cell type the LUC activity originates from or whether it is localized to one side of the xylem pole, it is not appropriate to describe these points of *DR5:LUC* expression as LRFCs (Moreno-Risueno et al., 2010). Prebranch sites might indeed be LRFCs that are visible at an earlier time due to the higher sensitivity of LUC. Alternatively, they might indicate a broader, competent site from which the specification of a restricted number of XPP cells into LRFCs will subsequently occur specifically at one side of the root.

A developmental window for FC identity and the first formative division to produce LRP

LRFC identity has been associated with an increase in the transcriptional response to auxin in select XPP cells shortly before they undergo asymmetric cell division (Benková et al., 2003; Dubrovsky et al., 2008). The time lag between the DR5:GFP expression in LRFCs and LR initiation is extremely brief and, consequently, both events are observed in the same region of the root, namely the early differentiation zone (Fig. 1A) (Dubrovsky et

al., 2011). Monitoring auxin response and distribution along the entire *Arabidopsis* primary root revealed a region with low auxin response and levels that was positioned between two distinct auxin maxima: one at the very tip of the root, including the quiescent center and meristematic zone, and a second in the vascular bundle of mature tissue in the shootward-most regions of the root. The region of 'auxin minimum' was, somewhat paradoxically, found to overlap with that in which increased auxin response (as assayed by induction of *DR5:GFP* expression) in LRFCs and LR initiation occurs. Therefore, this region has been proposed as the developmental window for LR initiation.

The developmental window is somewhat dynamic, shifting in the direction of the root apex as the root grows, thereby guaranteeing a rootward sequence of LR production under controlled growth conditions (Dubrovsky et al., 2006). In this region of lower auxin levels and response, cell- and tissue-specific auxin distribution and TIR1/AFB-dependent auxin signaling modules result in the induction of auxin-responsive genes, such as *GATA23* and *LBD16*, and the subsequent activation of LRFCs to undergo nuclear migration and asymmetric cell division (De Rybel et al., 2010; Goh et al., 2012). Downstream of the TIR1/AFB auxin receptor proteins, a family of transcriptional repressors comprising the AUXIN/INDOLE-3-ACETIC ACID INDUCIBLE (AUX/IAA) proteins are degraded upon auxin perception, leading to auxin-induced gene expression (reviewed by Chapman and Estelle, 2009). In *iaa28*, a gain-of-function mutant in which the IAA28 protein is stabilized, thus suppressing the auxin response, nuclear migration is interrupted, leading to inhibition of LRFC activation and a substantial decrease in LR formation (De Rybel et al., 2010). Similarly, upon repression of *LBD16*, a downstream target of auxin signaling of as yet unknown function in LR formation, nuclear migration in LRFCs is disrupted, thereby blocking the subsequent initiation of LRs (Goh et al., 2012). Likewise, disrupting polar auxin transport genetically or chemically alters auxin distribution in this region and inhibits LR initiation (Dubrovsky et al., 2011; Marhavý et al., 2013). The occurrence of these auxin response maximum-driven processes within a region of generally low auxin levels is intriguing and suggests that cells in this region might have enhanced responses to minor fluctuations in endogenous auxin availability. In such an environment, a subset of XPP cells could register local changes in auxin levels, providing a signal for developmental progression towards LR initiation, a situation that might not be possible in conditions of high auxin levels.

In contrast to auxin, cytokinins were identified as endogenous suppressors of LR formation. Their inhibitory mode of action was attributed to the hindrance of polar auxin transport, which could disturb local auxin distribution patterns and auxin signaling pathways (Benková et al., 2003; Laplaze et al., 2007; Li et al., 2006). More recently, however, cytokinin response, as monitored by a cytokinin-sensitive sensor (the *TCS* reporter), in the developmental window was shown to be minimal, although no decrease in active cytokinin levels could be measured within this region of the root (Bielach et al., 2012). Furthermore, exogenous cytokinin failed to induce expression of the *TCS* reporter (which measures cytokine pathway activity), indicating that strong repression of cytokinin signaling is at play in the developmental window and might be an important component for LR initiation. Categorizing the effects of increased cytokinin levels on LR formation either by endogenous expression of cytokinin biosynthesis genes or by exogenous cytokinin treatment demonstrated that the early phases of LR formation, including the pre-mitotic stages, are more sensitive to cytokinin than the later stages of LRP development. It was suggested that, in the developmental window in which auxin levels are low, ectopic cytokinin levels are more disruptive to early stage LRP, whereas in more developed primordia the auxin levels are more robust, thus diminishing the impact of cytokinins (Bielach et al., 2012).

LR formation: parallels with callus formation

Plants are well known for their ability to regenerate, either in response to wounding (i.e. cuttings) or in culture. The classic view that plant regeneration begins with the dedifferentiation of cells, followed by the formation of callus tissue (classically defined as an undifferentiated, disorganized mass of tissue), then redifferentiation into specialized tissues or organs (Skoog and Miller, 1957) has begun to be challenged. Recent investigations into regeneration following callus formation in culture indicate not only that callus is both organized and differentiated, but also that the capacity to generate callus resides primarily in a single specialized tissue. This tissue is the XPP in roots, whereas in shoots it is represented by xylem-associated pericycle-like cells (Atta et al., 2009; Che et al., 2007; Sugimoto et al., 2010).

In a remarkable parallel to stages I and II in LRP development, the XPP cells from root explants exposed to callus-inducing media (CIM) undergo a series of cell divisions producing smaller cells, which subsequently divide in the opposite plane to form a layered structure (Atta et al., 2009; Che et al., 2007). Additional similarities to LRP include transcriptional activation of the cell cycle and auxin signaling and transport reporters, and the presence of external-most cells that have features of differentiated root cell types (Atta et al., 2009). Unlike endogenously formed LRs, these so-called 'callus foci' or 'lateral root meristem-like protuberances' are derived from a larger number of XPP cells, appear closer together, are less stereotypical in shape and size, and show less refined reporter gene expression patterns (Atta et al., 2009; Che et al., 2007). Furthermore, increasing the concentration of the synthetic auxin analog 2,4-dichlorophenoxyacetic acid (2,4-D) in the CIM results in activation of cell division along the entire length of the root and in both sets of XPP cells (Atta et al., 2009), similar to what is observed in the lateral root inducible system (LRIS, see Box 2).

Calli were found to be similar to LRP not only with regard to their cells of origin and overall appearance, but also in their cellular organization and gene expression patterns (Sugimoto et al., 2010). Importantly, LRP-like features are also observed in calli induced from aerial organs. Calli produced from cotyledons (seed leaves) and floral petals originate from divisions in a xylem-associated pericycle-like cell layer and show similar tissue organization and reporter gene expression to callus from root tissue. Additionally, global gene expression profiles of callus tissue support similarity at the molecular level between roots and callus, even when the callus is derived from aerial tissues. Specifically, genes expressed at the root tip are enriched in the callus regardless of the tissue from which it is derived (Sugimoto et al., 2010). These data indicate that, in contrast to the classical definition, callus is a specialized, differentiated tissue with strong morphological and molecular similarity to LRP.

The molecular mechanisms underlying LR and callus formation, particularly the links between auxin signaling and cell cycle regulation, further connect these processes. In *slr/iaa14*, a mutant in another AUX/IAA transcriptional repressor involved in auxin signaling, the effects of experimental manipulation of both cell cycle progression and auxin levels suggest that these processes are sufficient to promote LR formation (De Smet et al., 2010; Vanneste et al., 2005). Similarly, *slr/iaa14* root explants could be induced to

Box 2. The lateral root inducible system

As initiation of an LRP involves few cells and is not coordinated in space or time between seedlings, the use of genome-wide approaches has been challenging. To address this, a method termed the lateral root inducible system (LRIS) was developed that involves sequential treatment of seedlings with an auxin transport inhibitor and then the synthetic auxin analog 1-naphthalene acetic acid (NAA) (Himanen et al., 2002). This treatment rapidly induces synchronous cell divisions throughout the XPP. The resulting small cells, which are similar to a stage I LRP, then divide parallel to the root axis similar to a stage II LRP. Finally, extended NAA treatment results in proliferative LRP development along the length of the root at both XPP axes (Himanen et al., 2002).

The LRIS was proposed to override the endogenous pre-patterning mechanism and stimulate LRP initiation *en masse*. This allowed application of transcriptional profiling techniques to begin to address the underlying molecular mechanisms. These analyses led to the characterization of novel proteins involved in the early steps of LR formation, including ARABIDOPSIS CRINKLY 4 (ACR4) and GATA23 (De Rybel et al., 2010; De Smet et al., 2008), and indicated sequential links between auxin signaling and cell cycle regulation (Himanen et al., 2002; Himanen et al., 2004). In brief, auxin signaling via SOLITARY ROOT (SLR/IAA14) is required for LR initiation under both standard conditions and in the LRIS (Fukaki et al., 2002; Vanneste et al., 2005). Whereas ectopic induction of XPP cell division in *slr/iaa14* mutants did not promote LR formation (Vanneste et al., 2005), LRs formed proliferatively when induction of XPP cell division was combined with NAA treatment (De Smet et al., 2010). Although endogenous and LRIS-induced LRs have common features, such as tissue of origin and links between auxin signaling and the cell cycle, differences in the pattern/distribution of lateral organs suggest that it is less clear how the LRIS informs endogenous LR pre-patterning. Although the LRIS might simply shift the endogenous LR patterning program into overdrive, these fundamental patterning differences might indicate that hormonal manipulation elicits a distinct response program in the XPP.

form shoots upon sequential treatment with CIM and shoot-inducing media, indicating that callus formation could also be promoted through manipulation of cell division and hormone levels (Atta et al., 2009). LBD16, LBD18 and LBD29, the functions of which have not been specifically defined but are known to occur downstream of auxin signaling, are also implicated in both LR initiation and callus formation (Fan et al., 2012; Feng et al., 2012). Additionally, ALF4, which is required for division of LRFCs and appears to function independently of auxin signaling (Celenza et al., 1995; DiDonato et al., 2004), is also required for callus formation as *alf4* mutants cannot be induced to form callus tissue from either root or shoot explants (Sugimoto et al., 2010). The phenotypes of these mutants indicate that the mechanisms regulating XPP cell division to initiate both LRP and callus development share common molecular features. Are other genes that function prior to or at LR initiation, such as *GATA23* and *ACR4*, also required for callus formation? Investigation into the roles of additional genes in each of these processes should help to clarify the relationship between them.

Conclusions

In recent years, and thanks to the development of novel reporter lines in *Arabidopsis*, insight has been gained into the ‘invisible phase’ of LR formation, namely the events that precede the first asymmetric cell divisions in LRFCs. The uncovering of previously unknown developmental steps has pushed researchers to formulate new concepts so that results obtained by different research groups working on LRs can be compared. In this Review, we aimed to provide a solid foundation for the coming years during which exciting new insights are expected to surface. We have summarized recent published work on pre-patterning mechanisms in the root, which consist of two important developmental steps: (1) a periodic oscillation of gene expression that triggers competence for LR formation; and (2) the perception of an auxin signal in FCs to set up LR initiation in the developmental window, a region of the root in which the integration of auxin and cytokinin signaling occurs. However, many questions remain unanswered.

We still lack cellular resolution of the oscillatory gene expression process. The current cellular information from the *DR5:GUS* reporter implies that signaling from the adjacent vasculature to the XPP cells is important for LRFC specification. However, the identity of such a signal remains unclear, as does the timing (in the OZ or later) of its transmission to the XPP. Because there does not appear to be a sidedness to the oscillation in *DR5* or endogenous gene expression, how LR sidedness occurs remains to be determined, but signals from the cells exterior to the pericycle upon cellular deformation might be involved. Finally, whether so-called priming signals and the cues that determine sidedness are distinct and sequential remains to be established. Once LRFCs become observable by reporter expression or nuclear migration, asymmetric cell division quickly follows. However, as the positional information transmitted by the oscillation of gene expression is likely to occur earlier, XPP cells might undergo a change in state that we are, as yet, unable to detect. A delay between competence and LRFC specification and activation would further increase the developmental plasticity of the root system by providing another ‘check-point’ for the developmental progression of organogenesis in the root.

Pre-patterning for LR formation is likely to be an example of the trade-off between resource investment and response time during plant development. Unlike animals, plants continually produce new organs in response to environmental cues. One option for a plant would be to wait for the cue and then begin the process of organ formation *de novo*. The obvious downside to this strategy is that the conditions that triggered the response might be short lived. To reduce response time, plants have instead adopted a strategy of commencing organ formation, then arresting it at various stages of development. An example is apical branch formation, in which branch points are positioned through phyllotaxis and primordia are initiated then arrested until the appropriate signal is received. The oscillatory gene expression process that establishes an LR pre-pattern of prebranch sites can be thought of as the equivalent of phyllotaxis, leading to the priming of select XPP cells, which then await a signal to form an LRP.

The presence of pre-patterning mechanisms implies the continuous production of organogenesis-competent cells during root growth. In contrast to this idea, organogenesis during plant regeneration from callus was thought to rely on *de novo* dedifferentiation of mature cells. However, recent comparative analyses of LR and callus formation have revealed clear and striking similarities. One important similarity is the requirement of high hormone levels for induction. In the LRIS (see Box 2), the transportable synthetic auxin analog NAA is applied to seedlings at about four times the concentration at which the non-transportable analog 2,4-D is applied to explants in the CIM (Atta et al., 2009; Himanen et al., 2002; Valvekens et al., 1988). However, treatment of root explants with the amount of 2,4-D used in CIM or of NAA used in the LRIS results in comparable gene expression patterns, indicating that these two treatments induce a similar response

(Sugimoto et al., 2010). Unexpectedly, root explants treated with high cytokinin levels or whole seedlings sequentially treated with NAA and cytokinin-enriched media are able to form shoot tissue at early stage LRP, suggesting flexibility in the developmental potential of LRP (Atta et al., 2009; Chatfield et al., 2013). These results suggest that, whereas the program for callus formation and its initial steps are similarly executed under various hormonal conditions, the formation of root or shoot tissue from callus or early LRP depends on hormonal context.

The comparison between the induction of LR and callus development also reveals that the XPP cells in the root and XPP-like cells in the shoot are unique among cell types in their ability to divide and form new structures/organs in differentiated tissues. Root pericycle cells at the poles of either xylem or phloem are further delineated in that they have distinct cellular morphology and transcriptional profiles and are the cells of origin for LRP (Brady et al., 2007; Jansen et al., 2012; Laskowski et al., 1995; Malamy and Benfey, 1997). In the *Arabidopsis* shoot, the XPP-like callus-forming cells are similar to root XPP cells in that they share marker gene expression and are associated with the vasculature, although presently this shoot tissue has not been specifically defined as XPP (Sugimoto et al., 2010). Given that they are the cells of origin for callus formation from both root and shoot tissues, the meristematic potential and properties of XPP and XPP-like cells have been greatly expanded. Perhaps the structural and molecular similarities, and the notion of a common cell of origin, between LRP and callus development indicate a common evolutionary origin. Given that hormonal context is a key aspect of determining which type of organ is formed by callus or early LRP, the possibility that LR development is an evolutionary offshoot of regeneration might be a viable hypothesis. In this context, the establishment of an LR pre-pattern might function to confine the meristematic potential of the XPP to specific sites.

What was once considered a largely random event primarily refined by lateral inhibition, LR formation is now revealed as a complex developmental process underpinned by a dynamic spatiotemporal pre-patterning mechanism. Advances in methods to interrogate cellular gene expression at finer resolution and the development of dynamic, cell type-specific reporter proteins will be key tools in future studies.

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