Dissecting *Arabidopsis* lateral root development

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Recent studies in the model plant *Arabidopsis* provide new insight into the regulation of root architecture, a key determinant of nutrient- and water-use efficiency in crops. Lateral root (LR) primordia originate from a subset of pericycle founder cells. Sophisticated mass-spectroscopy-based techniques have been used to map the sites of biosynthesis of auxin and its distribution in *Arabidopsis* seedlings, highlighting the importance of the phytohormone during LR initiation and emergence. Key components of the cell cycle and signal-transduction pathway(s) that promote and attenuate auxin-dependent LR initiation have recently been identified. Additional signals, such as abscisic acid and nitrate, also regulate LR emergence, raising intriguing questions about the cross-talk between their transduction pathways.

Root branching is a major determinant of plant architecture, albeit the 'hidden half'. As well as providing anchorage, lateral roots (LRs) contribute to water-use efficiency and facilitate the extraction of micro- and macronutrients from the soil. Investigating the factors that determine root architecture is of obvious agronomic importance. This article highlights the significant advances made using the model plant *Arabidopsis* to dissect the genetic, hormonal and nutritional control of LR initiation and emergence since this area was last reviewed, in 1997 [1].

Morphological changes associated with lateral root initiation in *Arabidopsis*

The *Arabidopsis* root has a simple anatomy composed of single layers of epidermal, cortical and endodermal cells surrounding the vascular tissues [2]. Lateral roots in *Arabidopsis* are derived from a subset of pericycle cells termed pericycle founder cells, which are adjacent to the two xylem poles [3] (Box 1). Dubrovsky *et al.* [3] defined pericycle founder cells as 'cells that acquire a developmental fate different from that of their mother and, as a consequence, play a principal role during the first stages of lateral root initiation.'

The first morphological event related to LR initiation occurs in two pericycle founder cells within the same cell file, adjacent to one of the xylem poles. Both founder cells undergo almost simultaneous polarized asymmetric transverse divisions, creating two short cells flanked by two longer cells [4,5] (Box 1). Daughter cells continue to divide symmetrically and asymmetrically, from the centre upwards and downwards, creating groups with a maximum of ten short cells that are similar in length [4,6]. Considerable plasticity in the precise order of these divisions is often observed. An identical series of mitotic divisions also occurs in both flanking pericycle cell files (Box 2). Following a period of radial expansion, the central short daughter cells divide periclinally, giving rise to a primordium composed of inner and outer cell layers, defined as stage II [6]. Figure 1 summarizes the subsequent divisions within the LR primordium (termed stages III–VII [6]) that

Box 1. Variations in the site of lateral root development in higher plants

In many plants, including *Arabidopsis*, *Allium cepa*, *Raphanus sativus* and *Helianthus annuus*, initiation of lateral root (LR) primordia only occurs in the pericycle adjacent to the xylem poles [5,55,56]. However, in *Zea mays* and *Daucus carota*, in which LRs appear close to the phloem poles, the asymmetric transverse divisions originate from pericycle cells located next to the phloem poles [56]. Although variations in the radial positioning of pericycle founder cells are observed, the formation of short derivatives by means of asymmetric transverse divisions represents a universal proliferative pattern during LR initiation (Fig. I).

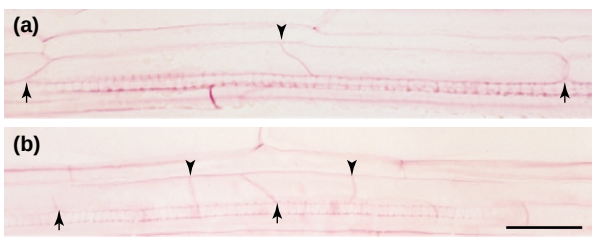

Fig. I. Transverse section through a stage 1 lateral root primordium in *Arabidopsis*. (a) A pericycle founder cell (PFC) has undergone a symmetrical transverse division. (b) A pair of short cells formed by asymmetric transverse divisions following one symmetric division. Arrows indicate the end cell walls of PFC. Arrowheads indicate the newly formed cell walls. Scale bar = 25 μm.

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Box 2. How many pericycle founder cells are required to form a lateral root primordium?

Following the first asymmetric pair of divisions, cells located in adjacent pericycle files are also activated. A median cross-section through a stage-II primordium highlights periclinal divisions in three adjacent pericycle cell files (Fig. Ia). Cells in adjacent files undergo an identical pattern of asymmetric divisions, forming a series of short cells (Fig. Ib). From these observations, a single file of pericycle cells is estimated to contribute two founder cells, although the minimum number of pericycle files involved in lateral root initiation is three. An estimate of six pericycle founder cells is in close agreement with experimental observations [7]. Cells involved in lateral root initiation have an average length about twice that of most of the pericycle cells and divide symmetrically before the first asymmetric transverse divisions [3], meaning that only three pericycle founder cells are required in this case. However, these conclusions contrast with another experimental estimate of 11 founder cells [5].

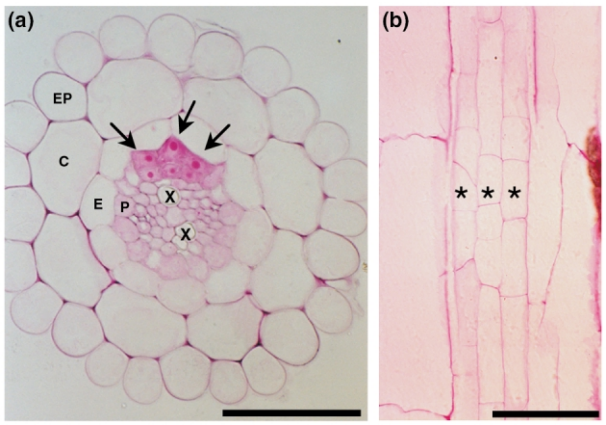

Fig. I. (a) Transverse section of the *Arabidopsis* primary root showing three radially expanded pericycle cells that that have undergone divisions (arrows). Abbreviations: C, cortex; E, endodermis; EP, epidermis; P, pericycle; X, xylem. (b) Longitudinal section showing groups of short cells in three adjacent files of pericycle cells (asterisks) located at the same transversal level. Scale bars = 25 μm.

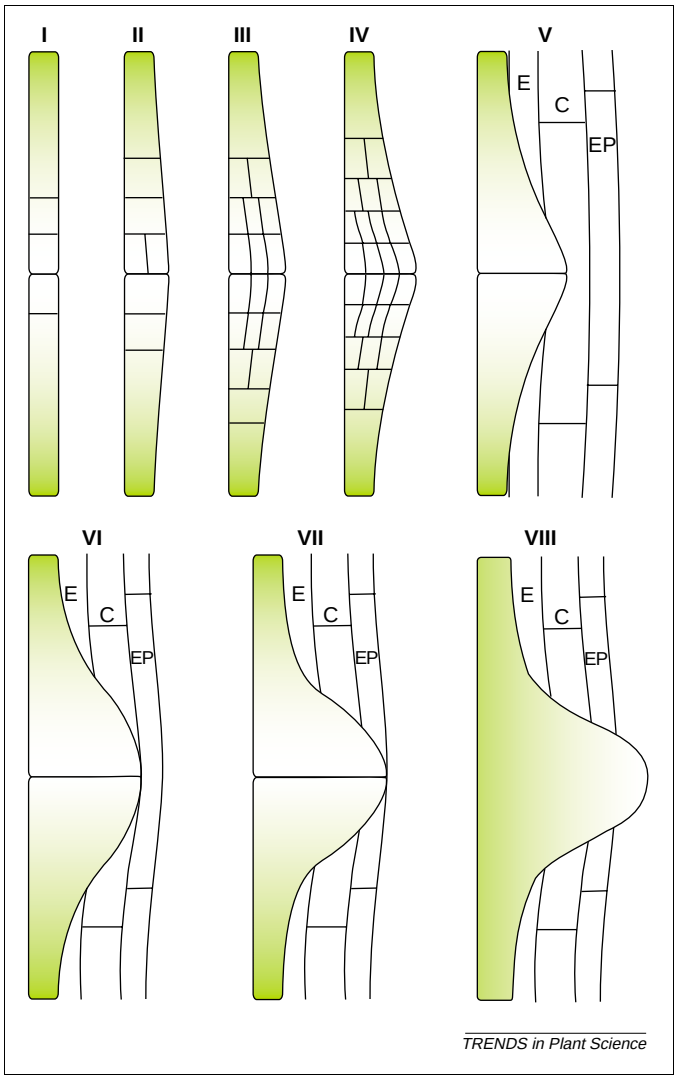

Fig. 1. Developmental stages during lateral root (LR) formation in *Arabidopsis*. The figure shows a series of longitudinal sections through LR primordia at specific developmental stages. The stage-I LR primordia contains a pair of short pericycle cells lying end to end and flanked by two longer cells. At stage II, cells undergo transverse asymmetric divisions, forming an inner layer (IL) and outer layer (OL). In stage-III LR primordium, OL cells undergo periclinal divisions to create a three-layered LR primordium. At stage IV, the LR primordium forms four layers because of periclinal divisions in the IL. By stage V, LR primordia are midway through the parent cortex, finally emerging at stage VIII. Abbreviations: C, cortex; E, endodermis; EP, epidermis.

ultimately lead to emergence (stage VIII). The LR primordium undergoes a noticeable expansion as it emerges from the parent root (stage VIII). Once emerged, the number of cells near the LR apex increases.

Cell-cycle events associated with *Arabidopsis* lateral root initiation

LRs originate from a zone distal to the *Arabidopsis* primary-root elongation zone [7]. Consequently, cell proliferation during LR development does not overlap the cell proliferation of the apical meristem. Hence, it is widely held that pericycle cells must de-differentiate and then re-enter the cell cycle. Furthermore, pericycle cells have been regarded as a population of cells that have left the cell cycle at G2 phase [8], implying re-entry at the G2–M control point in the course of LR initiation. Nevertheless, the 'de-differentiation' and simultaneous re-entry to the cell cycle of G2-arrested pericycle cells in the course of LR initiation has never been clearly demonstrated.

Two recent studies in *Arabidopsis* clearly emphasize the mitotic competency of the pericycle and counter the G2-re-entry hypothesis. Most of the pericycle remains in the G1 phase, with only the xylem-pole pericycle cells progressing from G1 to G2 phase [9]. Correspondingly, xylem-pole pericycle cells continue to cycle without interruption after leaving the root apical meristem [7]. Taken together, these results question the differentiated nature of pericycle cells and argue for the concept of a monolayered extended meristem. However, we must caution that both studies were exclusively focused on the regular acropetal LR formation that occurs in the young apical region of the root just above the elongation zone. New LRs can also initiate in more mature parts of the root, between earlier ones, which necessitates a de-differentiation and cell-cycle re-entry for pericycle cells.

The *Arabidopsis* pericycle constitutively expresses transcripts of many core cell-cycle genes (reviewed in Ref. [10]), which illustrates the special nature of its cell-cycle regulation. Several core cell-cycle genes have

Table 1. Summary of *Arabidopsis* genes that regulate lateral root development

Gene	Original mutant screen	LR mutant phenotype	Function	Refs
ABA1	Suppressor of *ga1* non-germinating response	Reduced sensitivity to high-nitrate inhibition of LR development and reduced drought rhizogenesis response	Zeaxanthin epoxidase	[29,34]
ABI1	Germination in the presence of inhibitory levels of ABA for wild type	Reduced drought rhizogenesis	Protein phosphatase 2C	[34]
ABI4	Germination in the presence of inhibitory levels of ABA for wild type	Reduced sensitivity to high-nitrate inhibition of LR development	?	[29]
ABI5	Germination in the presence of inhibitory levels of ABA for wild type	Reduced sensitivity to high-nitrate inhibition of LR development	?	[29]
ALF1 (allelic to *SUR1* and *RTY1*)	Visual screen for LR defect and adventitious roots on exogenous IBA	Increased root number	Aminotransferase-like sequence	[30,35,36]
ALF3	Visual screen for LR defect	Arrested LR development	?	[30]
ALF4	Visual screen for LR defect	Absence of LR development	?	[30]
ANR1	Antisense phenotype	Do not show the nitrate-induced stimulatory effect	MADS-box transcription factor	[25]
AUX1	Reduced auxin-sensitive root elongation	50% reduction in number of LR primordia	Putative auxin-influx carrier	[21]
At*MRP5*	Reverse genetic screen	Increased LR number	ATP-binding-cassette transporter	[37]
AXR1	Reduced auxin-sensitive root elongation	Reduced LR number	Related to ubiquitin-activating enzyme E1	[38]
AXR4	Reduced auxin-sensitive root elongation	Reduced LR number	?	[39]
AXR6	Reduced auxin-sensitive root elongation	Reduced LR number	?	[40]
DFL1	Altered hypocotyl length in light	Reduced LR number	Member of *GH3* family	[41]
HY5	Altered hypocotyl length in light	Increased LR number	bZIP protein	[42]
IAA28	Resistant to IAA–alanine	Reduced LR number	Putative transcription factor	[43]
LIN1	Insensitive lateral root development to high-sucrose, low-nitrogen medium	Overcomes LR repression on high-sucrose, low-nitrogen medium	?	[28]
NAC1	Antisense phenotype	Reduced LR number	Putative transcription factor	[17]
PXA1	Reduced IBA-sensitive root elongation	Reduced LR number	Peroxisomal ATP-binding-cassette transporter	[44]
PAS1	Altered cotyledon and leaf growth responses to cytokinin	Reduced LR number	FKBP-like protein	[45,46]
PAS2	Altered cotyledon and leaf growth responses to cytokinin	Increased LR number	Tyrosine-phosphatase-like protein	[45,47]
PAS3	Altered cotyledon and leaf growth responses to cytokinin	Reduced LR number	?	[45]
SBR	Ectopic expression of an IAA inducible reporter gene	Reduced LR initiation	?	[48]
SINAT5	Overexpression phenotype	Reduced LR number	Ubiquitin E3 ligase	[18]
SLR/IAA14	Root gravitropism	Absence of LR development	Putative transcription factor	[49]
SUR2	Adventitious root formation	Increased LR number	Cytochrome P450	[50]
TIR1	Reduced auxin-transport-inhibitor-sensitive root elongation	Reduced LR number	F-box protein	[51]
TIR3 (*BIG*)	Reduced auxin-transport-inhibitor-sensitive root elongation	Reduced LR number	Calossin/Pushover-related protein	[52–54]

Abbreviations: IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; FKBP, FK509 binding protein; LR, lateral root; ?, function unknown.

been proposed as triggering factors for LR initiation. For instance, the constitutive expression of the cyclin-dependent kinase (CDK) gene *CDKA;1* and the complete absence of transcripts encoding its potential regulatory subunit, the mitotic cyclin CYCB1;1, led to the hypothesis that this cyclin was the missing link needed for LR initiation. However, ectopically expressing *CYCB1;1* in the pericycle using the *CDKA;1* promoter did not result in an increased number of LRs [11], indicating that *CYCB1;1* expression is only a consequence of, not the trigger for, LR initiation. Furthermore, this result did not support the importance of the G2–M checkpoint, which is mediated by B-type cyclins. A D-type-cyclin gene (*CYCD4;1*) is expressed during LR initiation [12]. D-type cyclins play a prominent role in the G1–S-phase transition, which is the major control point at which cells can enter the cell cycle [13,14], suggesting that the trigger for LR initiation could be much earlier in the cell cycle than previously thought.

Auxin is a key signal during lateral root initiation

Many lines of experimental evidence strongly support a role for auxin during LR formation. For example, a survey of *Arabidopsis* LR mutants highlights the fact that many mutations also exhibit auxin-related defects (Table 1).

Several recent papers provide new insight into the mode of action and attenuation of the auxin signal during lateral root initiation. Roots deprived of endogenous auxin by growing them in the presence of the auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA) fail to initiate mitosis [4]. Deprivation of auxin keeps pericycle cells in G1 phase and re-addition of auxin promotes the G1–S transition [14]. The expression level of the *KRP2* gene, one of the recently identified CDK inhibitors, was high in roots treated with NPA and dramatically declined upon auxin administration [14]. *In situ* hybridization revealed that *KRP2* transcripts accumulated in pericycle cells not implicated in LR initiation and overexpression of *KRP2* reduced the number of LRs by more than 60%. Taken together, these data suggest a role for the CDK inhibitor KRP2 in controlling the branching pattern of a root. By binding to and thus inhibiting the permanently present CDK complexes, KRP2 could finely control the division activity of the meristematically competent pericycle.

Auxin levels in the root apex might control LR initiation via the transcriptional regulation of *KRP2*. Figure 2 illustrates the potential spatial relationship between auxin, *KRP2* expression and the longitudinal position of protoxylem-pole pericycle cells along the seedling root axis. The model is based on the analysis of the *KRP2* expression pattern by *in situ* hybridization on sections through root segments from different developmental stages in both *Arabidopsis* and radish [15]. Following germination, protoxylem-pole pericycle cells in the seedling root remain in G1 phase and *KRP2* expression is high (Fig. 2a). Subsequently, a subset of protoxylem-pole pericycle cells progress through the G1–S transition (Fig. 2b). This important cell-cycle event is characterized by a downregulation of *KRP2* transcription by auxin [15]. In Fig. 2c, some of the G2-phase pericycle cells enter mitosis at the point where metaxylem differentiation starts [16]. Following this event, *KRP2* expression reappears in those pericycle cells that are not involved in LR primordium formation.

How is the auxin signal attenuated in those pericycle cells that are not involved in LR primordium formation? It was recently reported that the product of *SINAT5* is a key element in the attenuation of the auxin signal during *Arabidopsis* LR development [17]. *SINAT5* was identified in a two-hybrid screen using the transcription activator NAC1 as bait. NAC1 transduces the auxin signal that promotes LR development [17]. Although the *SINAT5* loss-of-function data have not been presented, its overexpression leads to reduced LR formation [18]. SINAT5 has ubiquitin E3 ligase activity and can ubiquitinate NAC1 [18]. It has recently been suggested [18] that SINAT5-dependent proteolysis of NAC1 is a key step in the attenuation of the auxin signal that promotes LR formation.

Lateral root primordia and auxin

Genetic and physiological evidence suggests that auxin is required at several specific developmental stages to facilitate LR formation. For example, young LR primordia are unable to continue to divide when excised from the primary root unless supplemented with exogenous indole-3-acetic acid (IAA) [3]. However, between stages III and V, LR primordia become independent of externally applied auxin, indicating that they contain cell types that can act as an internal auxin source. Although it is clear that auxin is required at specific stages of LR formation, several important questions remained unanswered. First, what is the source(s) of auxin that drives primordium formation before stages III–V? Second, what is the role of auxin after stage V? Third, what is the source of this auxin after stage V? Recently, several papers have addressed such questions using a combination of auxin-responsive reporter genes and sophisticated mass-spectrometry techniques to perform auxin concentration mapping with high tissue resolution in *Arabidopsis* roots [4,19–21].

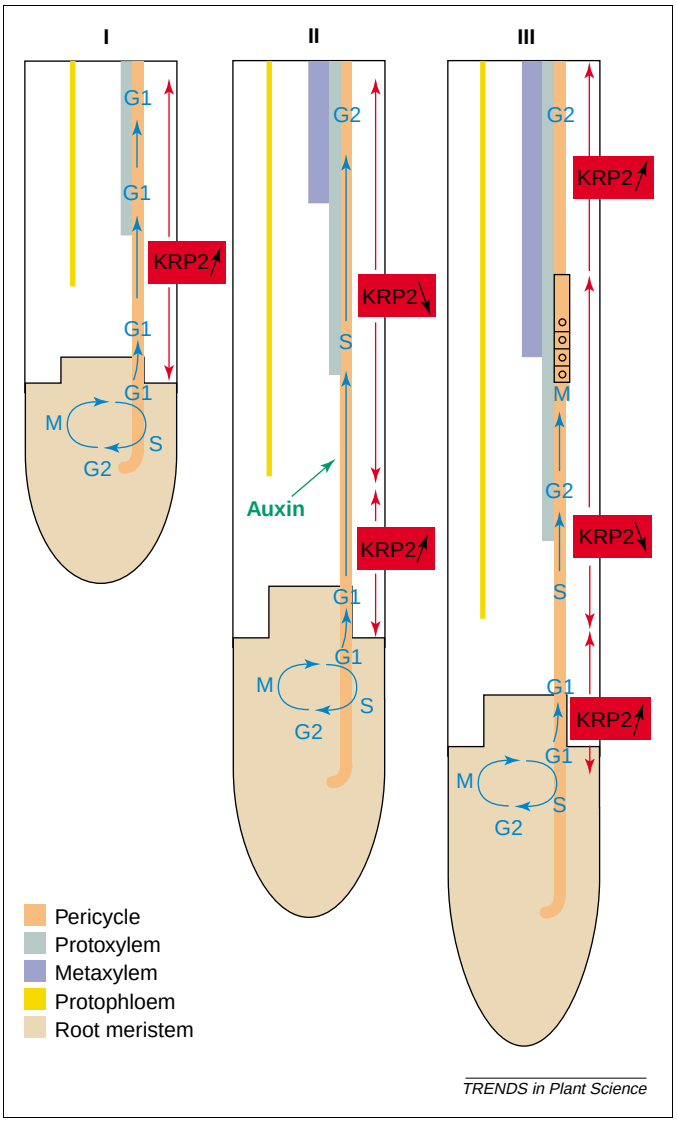

Fig. 2. Spatial relationships between the longitudinal position of protoxylem-pole pericycle cells along the seedling root axis, their cell-cycle phase and the hormone auxin. Three developmental phases (I, II and III) of the *Arabidopsis* seedling root are shown here. During phase I, protoxylem-pole pericycle cells remain in the G1 phase of the cell cycle and *KRP2* expression is high. During phase II, selected pericycle cells progress through the G1–S-phase transition, resulting in a distal zone of the root containing G2-phase pericycle cells that is characterized by a transcriptional downregulation of the cyclin-kinase-dependent-inhibitor gene *KRP2*. Given that *KRP2* downregulation is mediated by auxin [14], we postulate that an important auxin flux reaches the pericycle. During phase III, selected G2-phase pericycle cells enter mitosis and *KRP2* expression reappears in pericycle cells that are not involved in lateral root formation.

It has recently been reported that IAA accumulates at the root apex shortly after seedling germination [20]. Roots lacking the auxin-influx-carrier component AUX1 fail to accumulate IAA at the apex [19] and exhibit a 50% reduction in the number of LR primordia that initiate [21]. The AUX1 protein has been localized to the basal plasma membrane face of root protophloem cells [19], where the presumptive auxin permease facilitates post-phloem acropetal transport of IAA. Interestingly, the most apical protophloem element identified coincides with the position of the G1–S transition for a subset of proto-xylem-pole pericycle cells [16], suggesting that AUX1 facilitates an important auxin flux reaching the pericycle at this position.

Mass-spectroscopy-based auxin measurements have also revealed a pulse of auxin in the root system that promoted the emergence of the LR primordia [20]. Removal of shoot apical tissues abolished the IAA pulse, blocking emergence of laterals [20]. Is the root system dependent on shoot-derived auxin throughout its development? Measurement of auxin biosynthesis and auxin levels in the developing seedling's root system indicated that both auxin content and the biosynthetic capacity of root system increased substantially [22]. Hence, the root system might eventually become independent of auxin transported from the shoot. Nevertheless, the observed initial dependence of LR development on a shoot apical source of auxin [4,20,21] provides a way to coordinate LR emergence with the status of primary leaf development, allowing the young seedling to connect and balance carbon and nitrogen metabolism with their respective source organs, namely leaves (carbon) and roots (nitrogen).

Nutritional regulation of lateral root development

Nutrients such as nitrate also have an important impact on LR development. In soils or media with patchy nutrient distributions, LRs preferentially proliferate in a nutrient-rich zone. This phenomenon has been observed in many plant species and with several different nutrients [23,24]. Recent studies investigating the effect of nitrogen nutrition in *Arabidopsis* have revealed three different nitrogen-related regulatory mechanisms operating during LR development:

- A nitrate-induced localized stimulatory effect, which is most evident when plants grown on low nitrate are treated with a localized nitrate supply [25].
- A high-nitrate-induced inhibitory effect, which is most apparent when plants are grown on a medium with a uniformly high nitrate supply [26,27].
- Inhibition by a high sucrose-to-nitrogen (C:N) ratio, causing a dramatic repression of LR development [28].

The three nitrogen-related regulatory mechanisms appear to act at different developmental stages. The inhibition caused by a high C:N ratio occurs at the initiation step because of an impediment in auxin movement from the shoot to the initiation sites in the roots [28]. By contrast, the high-nitrate-induced LR inhibition occurs immediately after emergence, when the meristem would become activated [26,29]. Seedlings on high-nitrate media produced similar numbers of LR primordia to those with a low nitrate supply, yet they fail to elongate [26]. By contrast, localized nitrate application only increases the growth rate of LRs in the nitrate-rich zone, but has no effect on their number [27].

Experimental observations suggest that the three nitrogen effects might reflect different nitrogen-monitoring mechanisms. For example, the signal for the localized stimulatory effect is thought to be nitrate because it does not require nitrate metabolism and only occurs in LRs that are in direct contact with nitrate [26]. The signal for the high-nitrate-induced inhibition is believed to derive from nitrogen metabolites, because the inhibitory effect is systemic [26,27]. It is less clear what molecules act as the signalling cues in the high-C:N-induced LR repression. Although seedlings grown on a low sucrose, low nitrogen medium produce more LRs, it is likely that the presence of high sucrose in the high-C:N medium could increase the consumption of the limited nitrogen resource to form secondary metabolites and therefore aggravate nitrogen starvation.

Important advances have been made in understanding the mechanisms underlying the nitrogen-related LR regulation. For instance, a mutant termed *lateral root initiation 1* (*lin*1) can overcome LR repression on the high-sucrose, low-nitrogen medium. The *lin1* mutant produces a highly branched root system on media with high C:N ratios [28]. The cloning and characterization of *LIN1* will provide novel insights into the mechanisms that coordinate LR initiation with nutritional cues. An important component of the signalling pathway responsible for the nitrate-induced stimulatory effect has already been identified. *ANR1* encodes a nitrate-inducible MADS-box transcription factor. Plants in which *ANR1* is down-regulated do not show the nitrate-induced stimulatory effect but show a normal inhibitory effect [25], suggesting that there are different signalling mechanisms involved in the stimulatory and inhibitory responses to nitrate.

The plant hormone abscisic acid (ABA) plays an important role in mediating the inhibitory effect of high nitrate [29]. ABA-deficient mutants are much less sensitive to high-nitrate-induced LR growth inhibition, as are two ABA-insensitive mutants, *abi4* and *abi5*. Furthermore, exogenous ABA mimics the effect of high nitrate [16]. Interestingly, ABA acts through a different mechanism to inhibit LR development versus seed germination. For example, the high-nitrate LR-growth inhibition requires at least ten times less ABA than is needed to block seedling germination. In addition, mutants that are known to be ABA insensitive in respect of seed germination are ABA sensitive with regard to high-nitrate LR-growth inhibition [16]. Morphological analysis indicates that ABA inhibition occurs at a specific developmental stage, before the activation of the LR meristem. ABA-arrested LRs have the characteristic cellular pattern of a pre-activated LR primordium [16]. Current evidence suggests that an auxin-independent pathway mediates ABA-induced LR inhibition. First, the inhibition could not be rescued by either exogenous auxin application or elevated auxin synthesis. Second, a mutation in *ALF3*, which encodes an important component of the auxin-dependent regulatory pathway for post-emergence LR development [30], does

not affect LR sensitivity to ABA. Third, ABA and the *alf3-1* mutation do not act at the same developmental point [16].

Perspectives

Our understanding of *Arabidopsis* LR development is rapidly advancing at the cellular [4–6] and genetic levels (Table 1). The comprehensive characterization of the morphological events associated with wild-type *Arabidopsis* LR induction [4–6] (Fig. 1) has provided a valuable developmental framework to characterize the stage-specific effects of phytohormones such as IAA and ABA [4,29], and LR mutant phenotypes [21]. Nevertheless, there have been few detailed morphological studies that pinpoint the cellular, rather than molecular, basis for a mutant's LR defect, in spite of the cellular information being as enlightening as the gene sequence to explain the basis for the observed developmental defect.

The many *Arabidopsis* LR mutants isolated to date belie the fact that most of their altered phenotypes were originally selected on the basis of related traits such as reduced auxin sensitivity (Table 1). In the coming decade, a combined genetic–cell-biological approach offers real opportunities to develop mutant screens to select novel LR mutations that affect processes such as the spacing of LR primordia or nutrient regulation. Much progress is likely to be made using forward genetic screens that exploit the availability of stage-specific reporter genes [4,6] and high-throughput imaging techniques. Reverse genetic screens also have much to offer. However, the small number of cells involved has hampered the identification of genes expressed during LR initiation. An inducible system has recently been described in which the entire population of xylem-pole pericycle cells is synchronized for LR development [15]. This important development now makes it possible to perform genome-wide expression analyses and identify potential regulatory genes.

Although determining the sequence of transcriptional events that leads to the induction of LRs continues apace [7,9–16], our knowledge of the changes at the protein level is strictly limited. Investigating how levels of regulatory proteins such as KRP2 are controlled will be particularly important. For example, the pericycle cell cycle is likely to be regulated by the auxin-dependent degradation of KRP2 via SCFTIR1 [31] by a mechanism analogous to the ubiquitin-dependent proteolysis of the orthologous mammalian CDK inhibitor $p27^{Kip1}$ [32,33]. Protein localization studies are also likely to provide important insight into the mechanisms that regulate LR development. For example, localization of the auxin-influx-carrier component AUX1 to the basal plasma membrane face of protophloem cells [18] suggested that the *aux1* mutant's reduced ability to initiate LR primordia resulted from an impaired ability to mobilize IAA via the phloem [18,20].

Much progress has been made mapping auxin biosynthesis and distribution in wild-type and mutant *Arabidopsis* seedlings using a combination of reporter genes and sophisticated mass-spectroscopy-based techniques [4,19–22]. Although these studies have highlighted the primary importance of IAA during both LR initiation and emergence phases, little is known about how auxin regulates LR spacing. Could the attenuating effect of auxin-regulated proteins such as SINAT5 [18] limit the mitotic activity of auxin to just a few pericycle cells? The regulation of LR spacing will undoubtedly involve other developmental signals. Determining the nature of these signals and understanding how they interact to regulate LR development remains a major challenge. Adopting an integrated experimental approach that combines molecular genetics, cell biology, analytical chemistry and physiology is an effective strategy that has already produced dividends in our understanding of auxin [4,19–21], ABA and nitrate [16,26,27,29] regulated LR development.

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