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Hormone interactions during lateral root formation

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Abstract Lateral root (LR) formation, the production of new roots from parent roots, is a hormone- and environmentally-regulated developmental process in higher plants. Physiological and genetic studies using *Arabidopsis thaliana* and other plant species have revealed the roles of several plant hormones in LR formation, particularly the role of auxin in LR initiation and primordium development, resulting in much progress toward understanding the mechanisms of auxin-mediated LR formation. However, hormone interactions during LR formation have been relatively underexamined. Recent studies have shown that the plant hormones, cytokinin and abscisic acid negatively regulate LR formation whereas brassinosteroids positively regulate LR formation. On the other hand, ethylene has positive and negative roles during LR formation. This review summarizes recent findings on hormone-regulated LR formation in higher plants, focusing on auxin as a trigger and on the other hormones in LR formation, and discusses the possible interactions among plant hormones in this developmental process.

Keywords *Arabidopsis* · Lateral root initiation · Lateral root primordium development · Auxin

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Introduction

In higher plants, lateral root (LR) formation is critical for the development of root architecture (Charlton 1996). In most dicots, LRs are formed from root pericycle cells adjacent to the protoxylem poles of the parent root (reviewed in Charlton 1996; Barlow et al. 2004). As in most other plant species, *Arabidopsis* LR initiation starts from asymmetric, anticlinal cell divisions of two adjoining protoxylem pericycle cells of the same file, resulting in shorter and longer daughter cells (Dubrovsky et al. 2000; Beeckman et al. 2001; Casimiro et al. 2001; Dubrovsky et al. 2001; reviewed in De Smet et al. 2006a) (Fig. 1). In *Arabidopsis*, the single pericycle cell can also participate in these divisions (Dubrovsky et al. 2001, 2008). The shorter daughter cells expand radially over several rounds of cell division, and these cells divide periclinally to make outer layer and inner layer (Malamy and Benfey 1997; Casimiro et al. 2001). A mature LR primordium is formed through several developmental stages with well-ordered cell divisions and cell differentiation (Malamy and Benfey 1997). It has been shown that most of the cells in *Arabidopsis* LR primordia are derived from the central of the three axially adjacent cell files of the protoxylem pericycle (Kurup et al. 2005). LR initiation sites along the root are not predetermined, but LRs initiate in an acropetal sequence (Dubrovsky et al. 2006).

Plant hormones and environmental signals coordinately regulate LR formation (reviewed in Casimiro et al. 2003; Lopez-Bucio et al. 2003; Malamy 2005), with auxin as key stimulatory hormone in many plant species (Fig. 1) (Blakely et al. 1988; Laskowski et al. 1995; reviewed in Woodward and Bartel 2005). Recent studies using *Arabidopsis thaliana* and other plant species have shown that proper auxin transport and signaling are necessary for LR

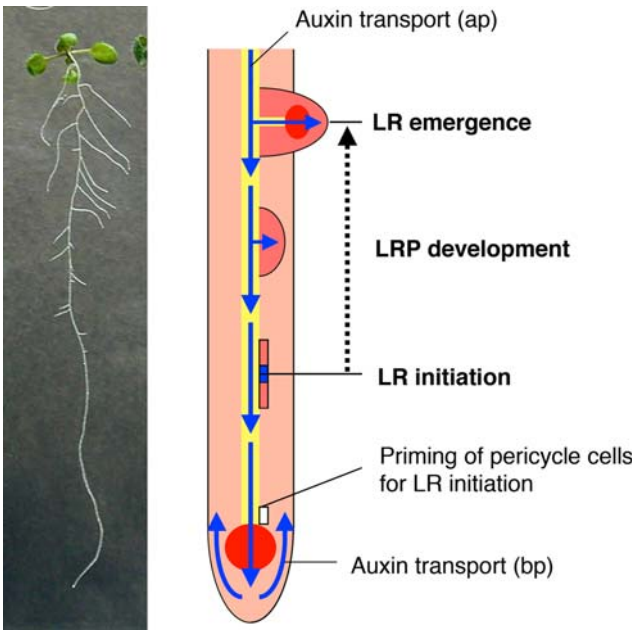

Fig. 1 Developmental events during LR formation. (1) Pericycle cells are primed for the future LR initiation [LRI] at the basal meristem (De Smet et al. 2007), (2) LR initiation (anticlinal cell divisions to produce shorter and longer cells), (3) LR primordium [LRP] development, and (4) LR emergence. Auxin is transported toward the young root tip (acropetal transport; ap), and then, at the root tip, it is re-directed to the basal part of the root (basipetal transport; bp) (Morris et al. 2004). Both acropetal and basipetal auxin transport are required for LR formation (Reed et al. 1998; Casimiro et al. 2001; Marchant et al. 2002). The apical meristems of primary and lateral roots are indicated by red ovals. A left photograph shows a 10-day-old wild-type *Arabidopsis* seedling (Columbia accession). After growth on standard MS medium (Fukaki et al. 2002), a seedling was transferred to fresh medium for the photograph

initiation and LR primordium development (reviewed in Fukaki et al. 2007). Whereas auxin is a key hormone for LR formation, the other hormones are also involved in LR formation either as positive or negative regulators. For example, cytokinin (CK), which is thought to act antagonistically to auxin, negatively regulates LR formation (Li et al. 2006; Laplaze et al. 2007; Kuderová et al. 2008). In contrast, brassinosteroid (BR) and auxin synergistically promote LR formation (Bao et al. 2004). However, it is not known how these hormones interact with each other in LR formation; i.e., how one hormone affects the biosynthesis, transport, or signaling of the others. A number of reviews on hormone- and environmentally-controlled LR formation have been published (Malamy 2005; Aloni et al. 2006; De Smet et al. 2006a, b; Fukaki et al. 2007; Osmont et al. 2007; Nibau et al. 2008). This review summarizes and updates the recent findings on hormone-regulated LR formation, focusing mainly on what is known about the developmental and regulatory roles of each hormone and their interactions.

Auxin promotes LR initiation and primordium development

Auxin-mediated cell cycle activation in LR initiation

Many physiological and genetic studies have shown that auxin plays important roles for LR formation, particularly during LR initiation and primordium development (Laskowski et al. 1995; Casimiro et al. 2001; Benková et al. 2003; reviewed in Fukaki et al. 2007). The application of exogenous auxin increases the number of LRs whereas inhibition of auxin transport decreases the number of LRs, indicating a positive role for auxin in LR formation (Reed et al. 1998; Casimiro et al. 2001; Marchant et al. 2002). In a cellular level, exogenous auxin promotes pericycle cell divisions (Laskowski et al. 1995), whereas auxin transport inhibitors (e.g. NPA) block de novo pericycle cell division during LR initiation (Casimiro et al. 2001), indicating that auxin positively regulates pericycle cell divisions in *Arabidopsis*. When *Arabidopsis* seedlings grown on NPA-containing medium for 72 h after germination are transferred onto NAA-containing medium, the pericycle cells of these seedlings start synchronously to divide (Himanen et al. 2002). This experimental system (Lateral Root Inducible System; LRIS) allows changes in the gene expression during auxin-induced LR initiation to be monitored (Himanen et al. 2002, 2004). In the LRIS, the expression of many kinds of cell cycle-related genes such as *Cyclins* and *Cyclin-Dependent Kinases* (*CDK*s) are activated by NAA treatment within 4 h (Himanen et al. 2002). In contrast, the expression of *KRP1* and *KRP2* genes, encoding the CDK inhibitors that inhibit the G1-to-S phase transition, are rapidly down-regulated upon NAA treatment within 4 h. *KRP2* is strongly expressed in non-dividing pericycle cells but is not expressed in dividing protoxylem pericycle cells (Himanen et al. 2002). Overexpression of *KRP2* decreases the number of LRs, indicating that KPR2 negatively regulates cell cycle progression during pericycle reactivation. These studies suggest that the G1-to-S checkpoint is one of the targets for auxin-mediated LR initiation (Himanen et al. 2002, 2004; Vanneste et al. 2005).

Auxin transport for LR initiation and subsequent LR primordium development

In the root, IAA is transported toward the root tip through the root vascular tissues (acropetal transport), and then IAA transported to the root tip is redirected toward the base of the root through the outer cell layers (basipetal transport) (Fig. 1) (according to Morris et al. 2004). From physiological analyses using auxin transport inhibitors (e.g. NPA), it has been proposed that both acrotpetal and basipetal auxin transport systems are important for LR

formation (Casimiro et al. 2001; Reed et al. 1998). In addition, genetic studies using *Arabidopsis* mutants demonstrate that an auxin transport system for influx and efflux is necessary for LR initiation and subsequent LR primordium development. Influx is mediated by AUX1 and LAX (Like AUX1), and efflux is mediated by PIN proteins (Casimiro et al. 2001; Benková et al. 2003; reviewed in De Smet et al. 2006a). The *aux1* mutant has a decreased number of LRs and other auxin-related phenotypes. The observation that the *aux1* mutation reduces accumulation of IAA in young seedling roots indicates that AUX1 acts as an auxin influx carrier for promoting LR formation by facilitating IAA distribution between shoot (source) and root (sink) tissues in the developing seedling (Marchant et al. 2002). It is also shown that AUX1-dependent basipetal auxin transport (from the root tip toward the base of the root) also regulates LR initiation (De Smet et al. 2007). In addition to AUX1, Swarup et al. (2008) reported that LAX3, an AUX1-like auxin influx carrier, regulates LR emergence. Interestingly, auxin induces the expression of LAX3 in cortical and epidermal cells directly overlaying new LR primordia. In the *lax3* mutant seedlings, the number of emerged LRs is reduced but LR initiation *per se* is not affected. The *lax3* mutation reduces the expression of several classes of cell-wall-remodelling enzymes in cells directly overlaying new LR primordia. These observations strongly suggest that LAX3-dependent auxin uptake in cortical and epidermal cells causes the induction of several classes of cell-wall-remodelling enzymes, thereby allowing LR emergence.

The *axr4* (*auxin resistant4*) mutant has *aux1*-like phenotypes, including reduced LR formation and reduced root gravitropism (Hobbie and Estelle 1995). While exogenous IAA and 2,4-D, which are transported into the cell by influx carriers, could not rescue the *axr4* defects, exogenous NAA, a diffusible synthetic auxin could rescue, strongly suggesting that AXR4 is involved in auxin influx (Yamamoto and Yamamoto 1999). In fact, the mutation in the *AXR4*, encoding a previously unidentified accessory protein of the endoplasmic reticulum (ER), resulted in abnormal accumulation of AUX1 in the ER of root epidermal cells, indicating that AXR4 is required for localization of AUX1 (Dharmasiri et al. 2006).

Auxin efflux regulated by PIN proteins, acting as auxin efflux facilitators at the plasma membrane, is also crucial for LR formation (Benková et al. 2003). Multiple *pin* mutations cause dramatic defects in root patterning, including LR primordium development (Benková et al. 2003; Blilou et al. 2005). The *pin1 pin4 pin7* or *pin1 pin3 pin7* triple mutants develop less well-defined LR primordia with massive divisions of pericycle cells in response to exogenous auxin, indicating that PIN-dependent polar auxin efflux is required for LR primordium development.

Furthermore, mutants impaired in the other components affecting the cellular localization of PIN proteins also have the decreased numbers of LRs or produce abnormal LR primordia (*tir3/doc1/big*, Ruegger et al. 1997; Gil et al. 2001; Paciorek et al. 2005; *gnom*, Geldner et al. 2004; *vps29,* Jaillais et al. 2007). For example, the GNOM/EMB30 protein, an Arf-GEF that regulates the Arf GTPase acting in vesicle transport, is necessary for the proper localization of PIN proteins at the plasma membrane (Steinmann et al. 1999). Loss of GNOM/EMB30 disturbs the proper cellular localization of PIN1, resulting in an embryo-lethal phenotype. However, a weak *gnom* mutant allele, which can grow after germination, also has severe defects in PIN-dependent developmental processes, including LR primordium development, presumably due to disorganized PIN1 localization (Geldner et al. 2004). These indicate that regulation of the cellular localization of PIN proteins is also important for LR initiation and LR primordium development.

It has been shown that members of the PGP/MDR (P-glycoprotein/multidrug resistance) subfamily of the ATP binding cassette transporter family have been shown to function in auxin transport (Geisler and Murphy 2005). PGP4 functions in the basipetal transport of auxin from the root tip toward the base of the root. Loss of PGP4 affects LR number, indicating that MDR/PGP-dependent auxin transport is important for LR formation (Santelia et al. 2005; Terasaka et al. 2005).

Taken together, both acropetal and basipetal auxin transport systems with a balance of influx and efflux are crucial for LR initiation and subsequent LR primordium development.

Auxin signaling for LR initiation and LR primordium development

In addition to the auxin transport system, normal auxin signaling mediated by Aux/IAA and ARF families of transcriptional regulators and $SCF^{TIR1/AFBs}$ complexes-dependent protein degradation is required for LR initiation (Dharmasiri et al. 2005b; Fukaki et al. 2002, 2005; Okushima et al. 2005; Vanneste et al. 2005). Auxin is perceived by the F-box auxin receptors, TIR1 and AFBs (AFB1, AFB2, and AFB3), which results in the degradation of Aux/IAA repressor proteins through $SCF^{TIR1/AFBs}$ complexes and 26S proteasomes (Dharmasiri et al. 2005a; Kepinski and Leyser 2005). This causes derepression of the ARF activity that positively or negatively regulates auxin-responsive transcription. A defect in either auxin perception or in the Aux/IAA degradation system inhibits auxin responses including LR initiation (Dharmasiri et al. 2005b; Fukaki et al. 2002). Gain-of-function mutations in the several Aux/IAAs result in the complete or partial loss of

LRs. For example, the *slr-1* mutation that results in stabilization of IAA14 completely blocks LR initiation (Fukaki et al. 2002). In addition, other gain-of-function mutations, including *shy2/iaa3*, *msg2/iaa19*, *axr5/iaa1*, *iaa28*, and *crane/iaa18*, also dramatically decrease the number of LRs (Tian and Reed 1999; Rogg et al. 2001; Tatematsu et al. 2004; Yang et al. 2004; Uehara et al. 2008). Similarly, a mutation in either ARF7 or ARF19 results in mild or almost negligible phenotypes, whereas the *arf7 arf19* double mutant has severely impaired in LR initiation, indicating that these ARFs have redundant roles in auxin-mediated LR initiation (Okushima et al. 2005; Wilmoth et al. 2005). The *ARF7*, *ARF19* and *SLR/IAA14* genes are all expressed in the stele including the pericycle (Fukaki et al. 2002; Okushima et al. 2005). In addition, in the yeast two hybrid system, both ARF7 and ARF19 proteins interact with SLR/IAA14 and the other Aux/IAAs involved in LR initiation (Fukaki et al. 2005; Okushima and Fukaki, unpublished data). These data strongly suggest that SLR/IAA14 and the other Aux/IAAs (AXR5/IAA1, SHY2/IAA3, CRANE/IAA18, MSG2/IAA19, IAA28) negatively regulate LR initiation through the inactivation of ARF7 and ARF19 (Fig. 2).

Detailed analyses of the *slr-1* mutant with the cell cycle markers demonstrate that the *slr-1* mutation blocks early cell divisions during LR initiation (Fukaki et al. 2002; Vanneste et al. 2005). Furthermore, the use of the LRIS in combination with the *slr-1* mutant further revealed the role of auxin signaling in cell cycle activation during LR initiation (Vanneste et al. 2005). In the LRIS, the *slr-1* mutation completely blocks auxin-induced anticlinal cell divisions at the protoxylem pericycle, whereas auxin induces synchronous cell divisions within the wild-type protoxylem pericycle, indicating that the *slr-1* mutation blocks anticlinal cell divisions during LR initiation. Analysis of the expression profiling of wild-type and *slr-1* mutant roots in the LRIS showed that the *slr-1* mutation affects the expression of genes involved in cell cycle regulation, and in auxin biosynthesis, metabolism, transport, and signaling, indicating that these genes are regulated under the control of SLR/IAA14-dependent auxin signaling in the wild type (Vanneste et al. 2005). Particularly, polar auxin transport genes such as *AUX1*, *LAX3* and several *PINs* are positively regulated by SLR/IAA14-dependent auxin signaling, indicating that auxin activates polar auxin transport during LR initiation.

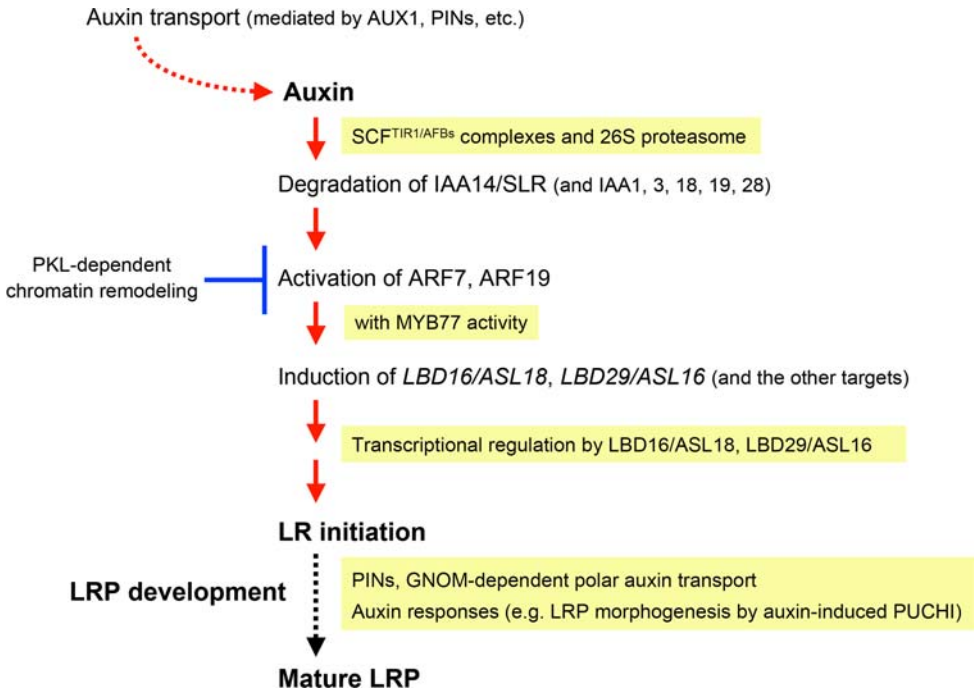

Fig. 2 Auxin signaling pathway model for LR initiation and LR primordium development. Auxin signals that are transported to the protoxylem pericycle cells (mediated by AUX1, PINs, etc.) promote degradation of the Aux/IAAs (IAA14/SLR etc.) involved in LR initiation through $SCF^{TIR1/AFBs}$ complexes and 26S proteasome, resulting in the activation of ARF7/ARF19 function, and allowing ARF7/ARF19 to activate the target genes required for LR initiation (*LBD16/ASL18*, *LBD29/ASL16*, and the other targets). LBD16/ASL18 and LBD29/ASL16 are nuclear proteins that are also involved in transcriptional regulation for LR initiation. This results in anticlinal cell divisions in the protoxylem pericycle for LR initiation. Subsequent LR primordium (LRP) development occurs through PINs, GNOM-dependent polar auxin transport, and morphogenesis mediated by auxin-induced PUCHI signaling. PKL-dependent chromatin remodeling is required for IAA14/SLR-mediated inactivation of ARF7/ARF19 activity during LR initiation

Both ARF7 and ARF19 activate the transcription of many auxin-responsive genes (Okushima et al. 2005), indicating that the genes which are activated downstream of these ARFs are necessary for LR initiation. Okushima et al. (2007) demonstrated that *LBD16/ASL18* and *LBD29/ASL16*, both of which encode nuclear proteins, are the direct targets of ARF7 and ARF19. Both *LBD16/ASL18* and *LBD29/ASL16* are members of the *LBD (LATERAL ORGAN Boundaries-domain)/ASL (AS2-like)* family (Iwakawa et al. 2002; Shuai et al. 2002), and are induced by auxin in roots, if ARF7 and ARF19 are present (Okushima et al. 2005). Overexpression of either *LBD16* or *LBD29* partially rescues the *arf7 arf19* defect in LR formation. Overexpression of *LBD16-SRDX* (LBD16 with a transcriptional repressor domain) strongly suppresses LR formation, also suggesting that endogenous LBD16 positively regulates LR initiation. A loss-of-function of LBD16 mutation slightly decreases the number of LRs, suggesting a redundant role for *LBD/ASL* family members homologous to *LBD16* (Okushima et al. 2007). Further analysis of such LBD/ASL family proteins will be necessary to fully understand the mechanisms of auxin-mediated LR initiation in *Arabidopsis*.

Monocot plants also use a member of the LBD/ASL family for adventitious root formation. In rice, a mutation in the *CRL1/ARL1* (*CROWNROOTLESS1/ADVENTITIOUS ROOTLESS1*) gene, which encodes a LBD/ASL protein homologous to *Arabidopsis* LBD29/ASL16, causes defects in adventitious root formation and lateral root formation (Inukai et al. 2005; Liu et al. 2005). Similarly, the *rtcs* (*rootless concerning crown and seminal roots*) mutation in the maize *CRL1/ARL1* homologue dramatically reduces adventitious root formation (Taramino et al. 2007). These studies in *Arabidopsis* and monocot plants indicate that the basic mechanisms of root formation are highly conserved between dicot and monocot plants.

Novel regulators of auxin-mediated LR initiation and primordium development

Chromatin remodeling is one of the mechanisms that controls gene expression. As described above, stabilized mutant IAA14 (mIAA14) protein in the *slr-1* mutant inactivates ARF7/19 functions, thereby completely blocking ARF7/19-dependent LR initiation (Fukaki et al. 2002, 2005). The mechanism of mIAA14-mediated inactivation of ARF7/19 was studied using suppressor mutants of *slr-1*, *ssl2* (*suppressor of slr 2*) mutants (Fukaki et al. 2006). *ssl2 slr-1* double mutants produce LRs and are sensitive to exogenous auxin in LR formation but *ssl2 slr-1 arf7 arf19* quadruple mutants have no LRs, indicating that SSL2 is necessary for mIAA14-mediated inactivation of ARF7/19 in LR initiation. *SSL2* encodes PICKLE (PKL), a homologue of the animal chromatin-remodeling factor CHD3/Mi-2. *SSL2* is thus an interesting link between auxin-dependent gene expression in plants, and chromatin remodeling in general (Fukaki et al. 2006). In animals, Mi-2 represses transcription as a subunit of the NuRD/Mi-2 complex containing histone deacetylases (HDACs) (Ahringer 2000). Inhibition of HDAC activity by trichostatin A also results in LR initiation in the *slr-1* mutant, but not in the *slr-1 arf7 arf19* triple mutant, suggesting that normal HDAC activity is required for mIAA14-mediated inactivation of ARF7/19 functions in LR initiation. These results suggest that PKL/SSL2-mediated chromatin remodeling negatively regulates auxin-mediated LR initiation in *Arabidopsis*, through the repression of ARF7/19 activity with Aux/IAA repressors including SLR/IAA14 (Fig. 2). A recent finding that the co-repressor TOPLESS (TPL) functions in IAA12/BODENLOS (BDL)-mediated inactivation of ARF5/MONOPTEROS (MP) activity in auxin-regulated embryogenesis suggests that TPL or TPL-related (TPR) proteins may be involved in mIAA14-mediated inactivation of ARF7/19 with PKL/SSL2-mediated chromatin remodeling (Szemenyei et al. 2008).

In *Arabidopsis*, MYB77, one of the R2R3 MYB transcription factors, is involved in auxin response (Shin et al. 2007). A knockout mutation of *MYB77* greatly reduces auxin-inducible gene expression whereas overexpression of MYB77 activates auxin-inducible genes in the absence of exogenous auxin. The *myb77* mutation reduces LR density at low concentrations of IAA. In addition, the *myb77* mutation strongly enhanced the *arf7* phenotype in LR formation, indicating there is a synergistic genetic interaction between MYB77 and ARF7 in LR formation. Interestingly, the activation domain of MYB77 interacts with the C terminus (domains III and IV) of ARF7, and co-expression of MYB77 and an ARF-C terminus increase the auxin-responsive reporter gene expression in protoplasts. These results suggest that the interaction between MYB77 and ARFs including ARF7 also plays a key role in auxin-mediated growth and developmental processes including LR formation (Fig. 2). This work suggests that MYB77 or related MYBs interact with ARFs, thereby regulating a variety of auxin-mediated growth and developmental processes.

A novel regulator of LR primordium development, *PUCHI*, which encodes a putative APETALA2/Ethylene-Responsive Element Binding Protein (AP2/EREBP) transcription factor, has been identified and characterized (Hirota et al. 2007). The recessive *puchi* mutant is defective in the coordinated pattern of cell divisions during LR primordium development; the proximal region of LRs swells with more divided cells. *PUCHI* is expressed in all cells in early stages of LR initiation, but later is restricted to the proximal region of the LR primordium (Hirota et al.

2007). *PUCHI* expression is rapidly induced by auxin, and is downstream of SLR/IAA14 and ARF7/19-dependent auxin signaling (Okushima et al. 2005; Vanneste et al. 2005; Hirota et al. 2007), indicating that auxin regulates the morphogenesis of LR primordia through PUCHI-dependent control of cell division (Fig. 2). It will be important to determine which kinds of genes are regulated by PUCHI during LR primordium development.

Several proteins (NAC1, SINAT5, XBAT32, and CEGENDUO) are also involved in auxin-mediated LR formation in *Arabidopsis* (Xie et al. 2000; Xie et al. 2002, Nodzon et al. 2004; Guo et al. 2005; Dong et al. 2006). In addition, genetic studies in plant species other than *Arabidopsis* have also identified a novel protein in LR formation. The tomato recessive mutant, *diageotropica* (*dgt*), has pleiotropic auxin-related phenotypes including a lack of LR formation (Ivanchenko et al. 2006; Oh et al. 2006). The *DGT* gene encodes a member of cyclophilins, strongly suggesting that the peptidyl-prolyl isomerase activity of DGT may take part in the regulation of auxin transport or in the auxin response necessary for LR formation.

How the LR initiation site is determined?

Although LRs are initiated from founder cells in specific pericycle cell files, it has been a mystery how the position of LR initiation is determined. De Smet et al. (2007) demonstrated that LR sites are determined in a region at the transition between the meristem and the elongation zone, referred to as the basal meristem. When grown on media in an oblique position, wild type *Arabidopsis* roots show gravity-induced waving and develop LRs along the main axis in a regular left-right alternating pattern whereas a mutation in AUX1, an auxin influx carrier, loses these responses, indicating that this pattern correlates with AUX1-dependent gravity-induced waving (De Smet et al. 2007). The correlation between an elevated auxin response in the basal meristem and the subsequent initiation of an LR using the auxin-responsive *DR5::GUS* marker and an LR initiation marker (CycB-GUS), strongly suggests that the oscillation of local auxin response in the basal meristem primes pericycle cells to become LR initiation sites at regular intervals. This priming of pericycle cells occurs before SLR/IAA14-mediated auxin signal triggers cell division at LR initiation, because SLR/IAA14 does not act in the basal meristem. This implies that some unidentified auxin response(s) within the basal meristem is necessary for priming pericycle cells for LR initiation (Fig. 1). Based on this finding, Lucas et al. (2008) also demonstrated that determination of LR initiation sites and root gravitropism are co-regulated in *Arabidopsis*. They examined the effects of several types of gravistimulation on LR initiation density, and found that the determination of LR initiation sites could be modulated by environmental cues such as gravistimulation, probably by affecting endogenous AUX1-dependent basipetal auxin transport in the root. These experiments suggest that local accumulation or a local auxin gradient determines which cells become LR founder cells.

Dubrovsky et al. (2008) recently provided the evidence that local accumulation of auxin in root pericycle cells is necessary and sufficient for the re-specification of these cells into LR founder cells. Time-lapse observations of LR initiation show that the auxin-responsive *DR5* promoter is the earliest marker for founder cells and that its activation correlates perfectly with subsequent LR primordium formation. Interestingly, the Cre-Lox-based mosaic expression of an enzyme for auxin synthesis in the *alf4* (*aberrant lateral root formation4*) mutant that is defective in LR initiation (Celenza et al. 1995; DiDonato et al. 2004) resulted in local auxin accumulation in a single pericycle cell or a group of cells (JG Dubrovsky, personal communication), which converted them into an LR founder cells. These results indicate that auxin is the local signal for acquisition of LR founder cell identity. The next step in understanding this developmental cascade will be to determine what signal causes the accumulation of auxin in specific pericycle cells.

Auxin thus acts as a key hormone for LR formation. Components of auxin transport, signaling including Aux/IAAs, ARFs, and their downstream targets (auxin-inducible LBD/ASLs, and PUCHI, etc.) play important roles in LR initiation and LR primordium development. However, auxin is active during several LR developmental steps such as the initial priming of the basal meristem, the asymmetric pericycle cell division (toward Stage I), the boundary formation at the base of the LR, and the emergence of LRs (Celenza et al. 1995; Bhalerao et al. 2002). Each step requires proper auxin accumulation based on its transport and signaling in the corresponding cell(s)/tissue(s). Furthermore, it is possible that the other hormones modulate these steps (see below).

Cytokinin negatively regulates LR formation

Cytokinin (CK) generally plays an antagonistic role to auxin in plant growth and development including the apical dominance of shoots, and tissue regeneration. Recent studies have indicated that CK is an endogenous negative regulator of LR formation. For example, multiple mutations in *Arabidopsis* CK signaling components such as the Type B ARRs (Arabidopsis Response Regulators), and AHK (Arabidopsis Histidine Kinase) CK receptors, increase the number of LRs (Mason et al. 2005; Riefler

et al. 2006). Moreover, transgenic plants with reduced levels of endogenous CKs due to the overexpression of the CK-degrading enzyme cytokinin oxidase (CKX), exhibit enhanced LR formation (Werner et al. 2001, 2003; Lohar et al. 2004). In contrast, multiple mutations in the Type A ARRs, negative regulators of CK signaling, decrease the number of LRs (To et al. 2004). These observations suggest that endogenous CKs are involved in the inhibition of LR formation. In fact, exogenous CK inhibits LR initiation in *Arabidopsis* (Li et al. 2006; Laplaze et al. 2007; Kuderová et al. 2008) and rice (Rani Debi et al. 2005). Li et al. (2006) demonstrated that CK treatment inhibits LR initiation by blocking pericycle founder cell cycling during the G2 to M transition phase. CK-repression of LR initiation is dependent on CK signaling components, including the CK receptor CRE1/AHK4 (Li et al. 2006), and AHPs (*Arabidopsis* histidine phosphotransfer proteins) (Hutchison et al. 2006). Interestingly, exogenously applied auxin cannot rescue the CK-mediated inhibition of LR initiation *per se* but it can rescue cell divisions (Li et al. 2006; Laplaze et al. 2007). This indicates that CK accumulation in pericycle cells does not prevent the auxin-mediated activation of cell divisions but blocks the developmental program of LR initiation.

Although both exogenous and endogenous CKs inhibit LR initiation, knowing which tissues are responsible for the CK response would help to better understand the interaction of hormone regulation of LR initiation not least because of the local auxin accumulation mechanisms. In other words, CK suppression could also be due to local activity, or it could be by transduction of the CK signal from some other organ. Studies using transgenic *Arabidopsis* plants demonstrated that protoxylem pericycle is the site of CK action (Laplaze et al. 2007). Expression of the *Agrobacterium tumefaciens* CK biosynthesis enzyme isopentenyltransferase (IPT) in protoxylem pericycle cells inhibits the initiation of LRs, whereas expression of the *Arabidopsis* CK degrading enzyme cytokinin oxidase 1 (CKX1) in protoxylem pericycle cells derepresses the initiation of LRs (Laplaze et al. 2007). These experiments provide strong evidence that endogenous CK in the protoxylem pericycle inhibits LR initiation.

Laplaze et al. (2007) also showed that exogenous CK inhibits auxin-induced expression of *PIN* genes and perturbs the establishment of an auxin gradient during LR initiation, strongly suggesting that endogenous CK in the protoxylem pericycle disrupts the PIN-dependent auxin accumulation, thereby inhibiting the asymmetric cell division for LR initiation (Fig. 3).

In addition to its direct roles in LR initiation, exogenous CK affects the spatial expression of several *PIN* genes in LR primordia and prevents the formation of an auxin gradient that is required for normal LR primordium patterning (Laplaze et al. 2007) (Fig. 3). Similarly, conditional CK overproduction by overexpression of *IPT* interferes with early LR primordium patterning, and auxin maximum response during LR primordium development (Kuderová et al. 2008). On the other hand, exogenous auxin rapidly inhibits CK synthesis in *Arabidopsis* seedlings, indicating that CK and auxin can interact also on the metabolic level (Nordström et al. 2004). Although it is unknown whether the auxin-regulated inhibition of CK synthesis affects LR formation, these observations indicate that there is some sort of regulatory interaction between CK and auxin during LR primordium development (Fig. 3).

Similar to *Arabidopsis*, the legume *Medicago truncatula* also uses CK signaling to inhibit LR formation. RNA interference of the CK receptor homolog Cytokinin Response1 (MtCRE1) led to CK-insensitive roots, which had an increased number of LRs (Gonzalez-Rizzo et al. 2006), indicating a common roles for CK in higher plant LR formation.

These discoveries have led to a new model of CK-auxin interaction during LR formation (Laplaze et al. 2007). Further analysis will be necessary to determine how the CK-mediated pathway blocks auxin-mediated LR formation.

Abscisic acid (ABA) negatively regulates the emergence of LR primordia

The involvement of abscisic acid (ABA) in LR formation has been studied mainly using ABA signaling mutants in *Arabidopsis*. These studies showed that ABA is a negative regulator of LR emergence (reviewed in De Smet et al. 2006b). Exogenous ABA inhibits the emergence of LR primordia from the parent root prior to activation of the LR meristem (Fig. 3). ABA-induced LR inhibition could not be rescued by exogenous auxin, indicating that an ABA-sensitive, auxin-independent checkpoint is involved at the post-emergence stage (De Smet et al. 2003). There is also genetic evidence for ABA-auxin regulatory interaction in LR formation. The *ABI3* (*ABA INSENSITIVE3*) gene, encoding a B3 type transcription factor necessary for ABA signaling, is auxin-inducible in LR primordia (Brady et al. 2003). Mutations in *ABI3* attenuate the responsiveness of LR formation to exogenous auxin or auxin transport inhibitor. In contrast, mutations in *ERA1* (*ENHANCED RESPONSE TO ABA1*), which encodes a farnesyl transferase, increase numbers of LRs. Therefore, while exogenous ABA negatively regulates LR emergence as mentioned above, ABA signaling mediated by ERA1 and ABI3 is necessary for auxin-mediated LR formation, probably LR initiation, indicating cross talk between ABA signaling and auxin action (Fig. 3).

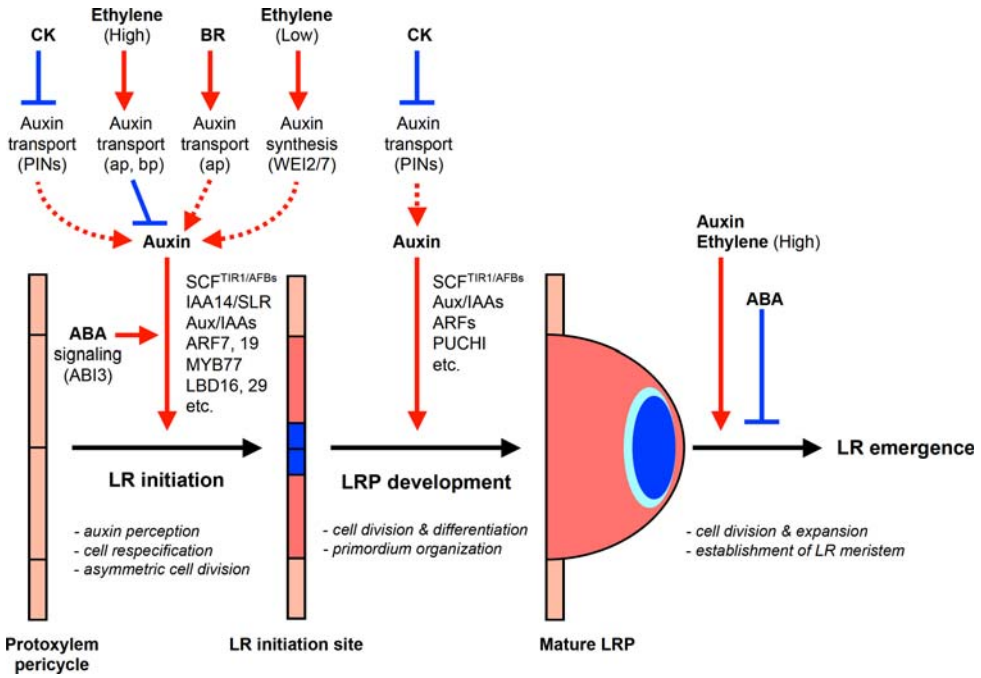

Fig. 3 Hormone interactions during LR formation. LR initiation is positively regulated by auxin but negatively regulated by CK and high concentrations of ethylene (high concentrations of exogenous ACC). The polar auxin transport with a balance of influx and efflux in both acropetal and basipetal directions is necessary for LR initiation and setting up auxin gradient to organize LR primordium (LRP) (blue color in LR initiation site and primordium). CK inhibits auxin maxima by altering the expression of PINs, thereby inhibiting auxin gradient for LR initiation. High concentrations of ethylene (high concentrations of exogenous ACC) enhanced both acropetal (ap) and basipetal (bp) auxin transport, inhibiting LR initiation. BR promotes LR initiation by increasing acropetal (ap) auxin transport. Low concentrations of ethylene (low concentrations of exogenous ACC) promote LR initiation by increasing Trp-dependent auxin synthesis mediated by WEI2 and WEI7. Normal ABA signaling mediated by ABI3 is necessary for proper auxin responsiveness for LR initiation. Auxin also promotes LR primordium development but CK inhibits LR primordium development and affects auxin maxima by altering the expression of PINs. ABA inhibits LR emergence whereas auxin and ethylene (via high concentrations of exogenous ACC) promotes LR emergence. Red arrows and blue bars indicate positive and negative regulation during LR formation, respectively

ABA is also involved in nutrient-mediated LR formation. High concentrations of NO_3^- inhibit LR development immediately after the emergence of LR primordia from the parent root in *Arabidopsis* (Signora et al. 2001). The inhibitory effect of NO_3^- is significantly reduced in several ABA insensitive mutants (*abi4* and *abi5*) and ABA synthesis mutants (*aba1*, *aba2* and *aba3*) but not in other ABA insensitive mutants (*abi1*, *abi2* and *abi3*). This indicates that the inhibitory effect of NO_3^- on LR development involves a specific ABA signal transduction pathway mediated by ABI4 and ABI5 in *Arabidopsis* roots.

The auxin-related mutant *ibr5* (*indole-3-butyric acid response5*) provide evidence for a strong relationship between auxin and ABA signaling; the *ibr5* mutation reduces both IBA-induced LR formation and ABA-induced germination inhibition (Monroe-Augustus et al. 2003; Strader et al. 2008). Interestingly, *IBR5* gene, which encodes a putative dual-specificity protein phosphatase, promotes auxin responses through a novel mechanism distinct from TIR1-mediated Aux/IAA repressor degradation (Strader et al. 2008). On the other hand, a gain-of-function mutant in Aux/IAAs, *axr2-1/iaa7* is resistant to exogenous ABA whereas the *slr-1/iaa14* mutant is hypersensitive to ABA in primary root growth inhibition assays (Wilson et al. 1990; Fukaki et al. 2002), suggesting that Aux/IAA-dependent auxin signaling also affects ABA activity in the roots. Therefore, there may be several types of interactions between ABA and auxin in auxin-mediated root growth and development.

Brassinosteroid (BR) promotes LR formation

Cross talk between brassinosteroid (BR) and auxin regulatory pathways has been demonstrated for several developmental processes and in transcriptional regulation (Nakamura et al. 2003; Li et al. 2005; Nakamura et al. 2006; Nakamoto et al. 2006). Bao et al. (2004) demonstrated that BR and auxin promote LR formation synergistically. The *Arabidopsis bri1* mutant, which is insensitive to BR due to a defect in the BR receptor, has fewer LRs than the wild type. In addition, the *bri1* mutation reduces auxin-responsive *DR5::GUS* expression in root tips whereas exogenous BR promotes *DR5::GUS*

expression in wild-type root tips. Interestingly, BRs promote acropetal auxin transport in the root, leading to the hypothesis that BRs promote LR initiation by increasing acropetal auxin transport (Fig. 3). It will be necessary to determine if and how BR promotes acropetal auxin transport, particularly whether BR affects the cellular localization of components (AUX1/LAXs, PINs, and PGP/MDRs) necessary for auxin transport. The involvement of BR in LR formation was also observed in pea (Ferguson et al. 2005), indicating that the auxin-BR regulatory interaction may be found widely distributed in dicot plants.

Gibberellin (GA) involvement in the regulation of LR formation

The role of gibberellins (GAs) in LR formation has not been studied in detail but there is some evidence that GAs are involved in LR formation. For example, GA-deficient pea mutant lines have fewer nitrogen-fixiing nodules and LRs (Ferguson et al. 2005), suggesting that GAs promote both nodulation and LR formation in pea. *Arabidopsis* GA-deficient mutants have reduced primary root growth (Fu and Harberd 2003) but the LR phenotype has not been reported in detail as far as we know.

Interactions between ethylene and auxin in LR initiation and LR primordium development

Ethylene has a stimulatory effect on adventitious root formation in many plant species (Clark et al. 1999), but its effects on LR formation had not until recently been analyzed in detail. Negi et al. (2008) reported that ethylene negatively regulates *Arabidopsis* LR formation by altering auxin transport. They showed that increased ethylene synthesis either by exogenous application of the ethylene precursor ACC, or by the *eto1* mutation that causes overproduction of ethylene, and enhanced ethylene signaling (the *ctr* mutation) both decrease LR formation. Conversely, blocking ethylene responses by the *etr1* (*ethylene triple response1*) or *ein2* (*ethylene insensitive2*) mutation increases LR formation. Interestingly, the overproduction of ethylene enhances IAA transport in both acropetal and basipetal directions but this enhanced IAA transport is not observed in the ethylene insensitive *etr1* and *ein2* mutants, indicating that ethylene positively regulates IAA transport in both acropetal and basipetal directions. This ethylene-enhanced IAA transport depends on AUX1, an IAA influx carrier, because the *aux1-7* mutant is insensitive to ethylene as an enhancer of acropetal and basipetal IAA transport, and thus for the inhibition of LR formation (Negi et al. 2008). As mentioned above, BR also enhances acropetal auxin transport in the root and promotes LR formation (Bao et al. 2004) but it is unknown whether BR enhances basipetal auxin transport in the root. The opposite effects of BR and ethylene on LR formation might be due to the difference in enhanced auxin transport: ethylene-enhanced auxin transport results in a reduction of auxin accumulation for LR initiation in the protoxylem pericycle.

Furthermore, Ivanchenko et al. (2008) analyzed the role of ethylene-auxin interaction in LR initiation and LR primordium development. As mentioned above, application of high concentrations of ACC strongly inhibits initiation of new LR primordia, but it promotes the emergence of existing LR primordia. In contrast, increased ethylene synthesis provided by low exogenous concentrations of ACC promote LR initiation. A genetic analysis using auxin signaling or ethylene-induced auxin synthesis mutants such as the *wei2* and *wei7* (*weak ethylene insensitive*) which are defective in ethylene-stimulated tryptophan-dependent auxin biosynthesis (Stepanova et al. 2005, 2007) indicates that the effect of ethylene on LR initiation is mediated by both auxin synthesis and signaling (Ivanchenko et al. 2008).

These results indicate that the interactions between ethylene and auxin in LR initiation and primordium development are complex but can be genetically dissected with the use of mutants.

Future perspectives and concluding remarks

Auxin is certainly a central regulator of LR formation but the other hormones also positively or negatively affect LR formation, at various stages. Such positive and negative influences indicate the complexity of interactions between auxin and other hormones, or among multiple hormones in LR formation. For example, the auxin-ethylene interaction that affects AUX1-dependent auxin transport in the root, may also involve BR because the auxin-BR interaction modulates acropetal auxin transport. Moreover, one hormone often affects the synthesis, metabolism, transport, or signaling of other hormones, such as in auxin-CK interactions in CK synthesis: auxin negatively regulates CK biosynthesis (Nordström et al. 2004), auxin-CK interactions in transcriptional regulation: auxin antagonizes CK signaling through direct transcriptional activation of *ARR7/15* genes, repressors of CK signaling (Müller and Sheen, 2008), auxin-ethylene interactions in their synthesis: ethylene induces auxin synthesis as mentioned above (Stepanova et al. 2005, 2007), auxin-GA interactions in root growth: auxin is necessary for GA-mediated root growth (Fu and Harberd 2003), and auxin-BR interactions in auxin-responsive transcription: BR positively regulates auxin-inducible genes (Nakamura et al. 2003). It is highly

probable that LR formation is regulated by a network of interacting plant hormones rather than by any one or two stimuli. This network is further complicated by its sensitivity to environmental factors. These interactions are also likely to play additional, as yet unidentified but important roles during LR formation.

Additional studies will be needed to provide information about how hormone interactions and environmental signals cooperatively regulate LR initiation, LR primordium development, and LR emergence, all of which result in the establishment of a root architecture which is suitable for the soil, nutrient and moisture conditions in which the plant is growing. The use of hormone-related mutants which have distinguishable LR formation phenotype, and new experimental approaches to monitor developmental changes during LR formation, such as genetically modifying specific gene activities within target cell(s)/tissue(s), will be powerful assets for dissecting many interactions among plant hormones in LR formation. Studies on hormone interactions in LR formation will also contribute to our understanding of the common and distinct mechanisms that regulate lateral organ formation in plant roots and shoots.

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