PLETHORA transcription factors orchestrate de novo organ patterning during Arabidopsis lateral root outgrowth

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Plant development is characterized by repeated initiation of meristems, regions of dividing cells that give rise to new organs. During lateral root (LR) formation, new LR meristems are specified to support the outgrowth of LRs along a new axis. The determination of the sequential events required to form this new growth axis has been hampered by redundant activities of key transcription factors. Here, we characterize the effects of three PLETHORA (PLT) transcription factors, PLT3, PLT5, and PLT7, during LR outgrowth. In plt3plt5plt7 triple mutants, the morphology of lateral root primordia (LRP), the auxin response gradient, and the expression of meristem/tissue identity markers are impaired from the “symmetry-breaking” periclinal cell divisions during the transition between stage I and stage II, wherein cells first acquire different identities in the proximodistal and radial axes. Particularly, PLT1, PLT2, and PLT4 genes that are typically expressed later than PLT3, PLT5, and PLT7 during LR outgrowth are not induced in the mutant primordia, rendering “PLT-null” LRP. Reintroduction of any PLT clade member in the mutant primordia completely restores layer identities at stage II and rescues mutant defects in meristem and tissue establishment. Therefore, all PLT genes can activate the formative cell divisions that lead to de novo meristem establishment and tissue patterning associated with a new growth axis.

axis formation | meristem | cell specification | plant architecture | branching

Lateral root (LR) formation in plants represents a remarkable example of developmental plasticity in prepatternning, initiation, and emergence (1–4). Recent studies in Arabidopsis have highlighted temporally oscillating gene activities that lead to LR initiation sites, termed LR prebranch sites, in which a group of xylem-pole-pericycle (XPP) cells are thought to gain competence to form LRs (1, 5–7). The acquisition of competence and the subsequent specification of LR founder cells are associated with distinct auxin signaling events that culminate in auxin accumulation (6–15).

Lateral root primordia (LRP) initiate from LR founder cells that will undergo stereotypical asymmetric cell divisions, forming shorter central cells and longer flanking cells (16–19) (Fig. 1A). Subsequent rounds of anticlinal, periclinal, and tangential cell divisions form a dome-shaped primordium that emerges through the overlying primary root cell layers, possessing a fully functional meristem that is highly reminiscent of the primary root meristem (20, 21) (Fig. 1B–E). In the primary root meristem, the quiescent center (QC; cells with low mitotic activities) and its surrounding stem cells (initials) make up root tissues, forming a stem cell niche that maintains an undifferentiated stem cell pool at the position of an auxin maximum (20, 21) (Fig. 1E). During LR formation, signal exchanges between primordia and their surrounding tissues are important to guide LR emergence (4, 12, 14, 22–25).

The critical factors and mechanisms involved in LR outgrowth, including the establishment of new radial and proximodistal axes, have hitherto remained unknown (4). Although several genes involved in cell cycle reactivation and LRP boundary delineation during LR initiation have been identified (7, 26, 27), cell cycle reactivation in XPP cells is not sufficient to instruct a new LR meristem and distinct tissue identities (12, 28). Here, we reveal that PLETHORA3 (PLT3), PLT5 and PLT7, three proteins within the AINTEGUMENTA-like subclade of AP2 domain transcription factors, represent such key factors in instructing new LR growth axes. During LR outgrowth, PLT3, PLT5, and PLT7 are expressed earlier than the other members of the PLT clade (29). We show that these three “early” PLTs are required for the formative divisions that split inner and outer cell layers in stage II primordia and the establishment of differential gene expression in these layers. In addition, they are required for the activation of “late” PLT1, PLT2, and PLT4 genes that contribute to stem cell maintenance and establishment of de novo meristems.

Results

PLT Genes Are Required for Formative Cell Divisions in LRP. To specify the roles of PLT family transcription factors in LR formation, we compared the morphology of LRP in WT and plt3plt5plt7 triple mutants. LR development is staged according to the number of radial cell layers in primordia (16) (Fig. 1A–D). In WT, incipient stage I LRP are formed after the first asymmetric founder cell divisions (Fig. 1A). The central-most cells in WT stage I LRP reorient their division planes and undergo formative periclinal cell divisions to generate a new cell layer, forming a stage II primordium (17, 18) (Fig. 1B). In plt3plt5plt7, incipient stage I primordia were morphologically indistinguishable from WT (Fig. 1F). However, at the transition from stage I to II, the central-most cells in plt3plt5plt7 LRP frequently lacked periclinal cell divisions and became enlarged, leading to partially undivided layers at stage II (Fig. 1G). Primordia were scored as “delayed” if at least one central cell did not undergo complete periclinal division (Fig. 1G and H). In WT, ~2% stage II LRP

Significance

Root architecture is an important trait that is shaped by the formation of primary roots, lateral roots, and adventitious roots. Here, we show that three PLETHORA (PLT) transcription factors are the key molecular triggers for the de novo organ patterning during Arabidopsis lateral root formation. PLT3, PLT5, and PLT7 redundantly regulate the correct initiation of formative cell divisions in incipient lateral root primordia and the proper establishment of gene expression programs that lead to the formation of a new growth axis.

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showed delayed periclinal divisions, while LRP/LR at later stages showed a normal periclinal division pattern (Fig. 1 I). In contrast, in plt3plt5plt7 mutant primordia, ~60% stage II and ~50% stage III-emerged primordia lacked periclinal divisions (Fig. 1 J). In addition to the conspicuous defects in periclinal division, several other aspects of LR formation were affected in the triple mutant. Cell division planes in plt3plt5plt7 LRP were generally abnormal at later stages, and cell shapes became irregular, leading to a variable mutant primordium morphology (Fig. 1 G–I). In these roots, only a few of the primordia emerged (29% (~3% of the total) (Fig. 1 K and SI Appendix, Fig. S1 A and B). However, plt3plt5plt7 roots displayed more lateral organ initiation events (LRP + LRs) than WT. Consequently, the number and density (number per 1 cm) of stage I, stage II, and emerging LRP were significantly higher in the triple mutant (Fig. 1 K and SI Appendix, Fig. S1 A and B).

WT LRP/LRs form acropetally, with the youngest closest to the root tip. Here, we analyzed all stage I and II primordia located rootward from the youngest stage III primordium. The triple mutant showed a significant increase of new stage I and II primordia (Fig. 1 L), indicating that mutant primordia delayed their entry into the next developmental stage, consistent with the observed impaired periclinal cell division defects in central cells of mutant primordia.

In conclusion, visible morphological defects of plt3plt5plt7 LRP initiate at the transition phase between stage I and stage II, when many cells fail to set up formative cell divisions.

**PLT Genes Are Required to Maintain Auxin Response Maximum During LR Formation.** The establishment of a new auxin response maximum inside LRP is important for their outgrowth, and prior work has shown that the formation of this auxin maximum correlates with relocalization of auxin transport proteins in the PINFORMED (PIN) family (30, 31). In the primary root meristem, the maintenance of PIN gene expression has been shown to require PLT proteins (32-34). We thus asked whether and from what stage onward auxin response patterns in LRP could be mediated by PLT genes.

An introgressed auxin response reporter, DR5::GFP, was expressed in a similar pattern in WT and plt3plt5plt7 stage I primordia (Fig. 2 A and B). At stage II, however, the level of DR5::GFP expression became higher in plt3plt5plt7 primordia than in WT (Fig. 2 A and B). At later stages, DR5 expression in the mutant primordia further increased and rather than becoming restricted to a distal maximum, was dispersed throughout the primordia (Fig. 2 A and B). This indicates that auxin response in the mutant primordia resembles the response in WT at early stage I but that it increases and becomes diffuse at later stages.
PLT3, PLT5, and PLT7 Genes Are Critical for Meristematic Gene Activation During LR Formation. The PLT1 and PLT2 transcription factors are main regulators of primary root meristem maintenance and the position of the meristematic boundary (32, 37, 38); plt1plt2 double mutants can produce emerged LRs, but the continuous growth of these LRs is not maintained over time (37), indicating that PLT1 and PLT2 are required to maintain but not to initiate de novo LR meristems. To visualize PLT gene transcription dynamics, we fused their promoters to CFP (29, 32).

PLT1, PLT2, and PLT4 are expressed at later developmental stages in WT LRP than PLT3, PLT5, and PLT7 (29) (Fig. 2 G, I, and K compared with SI Appendix, Fig. S2 A, C, and E). We first detected PLT1, PLT2, and PLT4 promoter fusion activities in stage II–III LRP (Fig. 2 G, I, and K), pPLT1::erCFP resided in the central cells of the innermost layer and not in the outer layers of stage II and/or stage III primordia (Fig. 2G and SI Appendix, Fig. S2K). PLT2 promoter activity was distributed differently, preferentially in the outer layers of the primordium but absent in the innermost layer (Fig. 2I and SI Appendix, Fig. S2K). The initial domain of PLT4 expression encompassed all layers of stage II and/or III primordia (Fig. 2K). Remarkably, PLT1, PLT2, and PLT4 promoter activities were completely undetectable in plt3plt5plt7 LRP (Fig. 2 H, J, and L), indicating that early PLT3, PLT5, and PLT7 are upstream and essential for late PLT1, PLT2, and PLT4 expression in LRP. Thus, the initiation of key players in stem cell and meristem maintenance, two of which mark the asymmetric identity of inner and outer layers in stage II LRP, is defective in plt3plt5plt7 LRP. This renders the triple mutant effectively a PLT null mutant in the context of LRP.

To determine whether the early expressed PLT3, PLT5, and PLT7 proteins form an autoregulatory loop, we determined whether loss of all PLT expression also affected PLT3, PLT5, and PLT7 expression. During LR outgrowth, PLT3, PLT5, and PLT7 promoters are activated in stage I LRP in WT (29) (SI Appendix, Fig. S2 A, C, and E). At later stages, both pPLT3::erCFP and pPLT7::erCFP expression converged to the new stem cell niche area with a graded pattern in the vasculature (SI Appendix, Fig. S2 A and E). pPLT5::erCFP expression faded away in the central cells of the primordium from stage II onward but was retained in the peripheral cells (SI Appendix, Fig. S2C). In plt3plt5plt7, PLT3, PLT5, and PLT7 promoter expression levels and patterns were normal up to stage I but deviated from WT from stage II onward; nevertheless, all three promoters remained active until later stages (SI Appendix, Fig. S2 B, D, and F). Collectively, our data indicate that PLT3, PLT5, and PLT7 are strictly required for induction of late PLT1, PLT2, and PLT4 gene expression during LR formation but not for the initial onset of their own expression.

PLT Genes Are Essential for Correct Expression of Key Tissue-Specific Regulators During LR Formation. The morphology of plt3plt5plt7 LRP indicated an absence of radial and proximodistal cell type patterning typically associated with the formation of a new growth axis. Intriguingly, several genes encoding transcription factors involved in radial and distal patterning in the primary root meristem, such as SHORT-ROOT (SHR) (39) and FEZ (40), are direct PLT targets (34). To determine whether triple-mutant primordia, which are effectively plt nulls, initiate pattern formation, we selected these and other tissue-specific markers with known developmental roles in primary roots and analyzed their expression dynamics during LR formation.

SHR and SCARECROW (SCR) are required for QC and ground tissue (cortex and endodermis) specification in the primary root meristem (Fig. 1E) and during LR formation (39, 41, 42). In WT, a functional SHR protein fusion started to accumulate in nuclei of stage I LRP cells (Fig. 3A). At stage II, SHR:GFP became asymmetrically expressed in two layers, with nuclear localization in the outer layer and nucleocytoplasmic
localization in the inner layer (Fig. 34 and SI Appendix, Fig. S2K). Similar to WT, SHR:GFP nuclear signal was detected in plt3plt5plt7 stage I LRP (Fig. 3B). However, in the mutant primordia from stage II onward, SHR:GFP signal was greatly reduced in the central cells of the outer and inner layers (Fig. 2B and SI Appendix, Fig. S1E). In line with this, SHR promoter activity and transcript level, as shown by a fluorescently tagged promoter reporter and semi-quantitative RT-PCR, also decreased in plt3plt5plt7 LRP (SI Appendix, Fig. S2 G–I). In WT, SCR promoter activity was initially detected in the outer layer of stage II primordia and hence, is another marker of radial asymmetry between different cell layers in stage II LRP (Fig. 3C and SI Appendix, Fig. S2K). Strikingly,SCR promoter activity was not detectable in plt3plt5plt7 LRP, including the cells that have a reduced expression level of its upstream regulator SHR:GFP (Fig. 3D). Together, our data indicate that, in LRP, the patterned SHR expression and the activation of SCR require PLT3, PLT5, and PLT7.

WUSCHEL-related homeobox5 (WOX5) is required for QC specification in the primary root meristem (20, 43) (Fig. 1E). In WT, a WOX5 promoter fused to a GFP reporter could be detected in the central cells of both layers in a few stage II LRP. This expression pattern was gradually confined to the new QC area (Fig. 3E). In stark contrast, we did not detect pWOX5::GFP expression in plt3plt5plt7 LRP at any stage (Fig. 3F). PLT-dependent WOX5 expression in the LR context is consistent with the detection of PLT2 binding sites in the WOX5 promoter (34).

Two NAC domain transcription factors, FEZ and SOMBRERO (SMB), are required for correct root cap (columella and LR cap) development and mark the distal root cap (40) (Fig. 1E). In WT LRP, pFEZ::GFP:MBD was detected around stage V1 in cells located at the most distal region of the emerging primordium dome (Fig. 3G). SMB::GFP (40) was first detected around stage VII in the central cells at the outer periphery of emerging primordium/LR apex (Fig. 3I). In plt3plt5plt7 LRP, weaker pFEZ::GFP:MBD expression was detected at late stages in a tip region similar to the area in WT LRP (Fig. 3H), showing that, during LR outgrowth, unlike WOX5, FEZ expression does not critically depend on PLT proteins but is enhanced by their presence. This is consistent with the occupation of the FEZ promoter by PLT proteins (34). In contrast, SMB::GFP, which marks differentiated root cap cells, was precociously expressed in the central cell files from the transition phase between stage I and II onward (Fig. 3J and SI Appendix, Fig. S2J).

The MYB-related transcription factor WEREWOLF (WER) is required for patterning and specification of root epidermal cell identities (44) (Fig. 1E). In WT LRP, the expression of WER promoter fusion marker, pWER::erCFP, was detected at the apex of emerging primordia (Fig. 2K). In plt3plt5plt7 LRP, pWER::erCFP expression was not detectable at any stages (Fig. 3L).

In summary, three early-expressed tissue identity genes SHR, SCR, and WOX5 that specify the identity of ground tissue and QC were not properly activated in plt3plt5plt7 LRP. The expression of later induced genes that mark root cap and epidermal cells, FEZ and WER, was either reduced or absent, and the expression of the late root cap differentiation gene SMB was derepressed from the stage I–II transition phase onward. Collectively, our data indicate that PLT3, PLT5, and PLT7 genes are required to orchestrate pattern formation of the LRP at and after their transit from stage I to stage II.

LRP-Targeted Induction of Any PLT Family Member Completely Rescues plt3plt5plt7 LR Outgrowth. As late expressed PLT1, PLT2, and PLT4 genes were not expressed in plt3plt5plt7 LRP, we asked if specific reintroduction of these transcription factors into the mutant primordia could rescue the outgrowth defects. In a prior study, we showed that reintroduction of PLT3, PLT5, and PLT7 proteins fusions under their native promoters rescues the plt3plt5plt7 outgrowth defect (29). Here, we used a 1.5-kb truncated PLT7 promoter fragment (PLT71.5) (29) to target PLT gene expression solely to LRP/LR from incipient stage I onward in young seedlings to see if this localized induction is sufficient to rescue LR outgrowth (SI Appendix, Fig. S3 A–E). This promoter is tightly regulated and activated around the onset of nuclear migration in LR founder cells before the stage where phenotypic defects in plt3plt5plt7 primordia are first observed. When PLT71.5 drives PLT genes during LR outgrowth, the expression pattern of this promoter is maintained in both WT and plt3plt5plt7 (SI Appendix, Fig. S3 F–Q compared with SI Appendix, Fig. S34); fully encompasses the initial expression domains of PLT1, PLT2, and PLT4 (Fig. 2 G, I, and K compared with SI Appendix, Fig. S3 F, H, and L); and does not affect the morphology of WT LRP (SI Appendix, Fig. S3 F–Q). These properties make the PLT71.5 promoter an optimal tool to assess rapid and local complementation within LRP.

Strikingly, reactivation of any PLT family member at this early stage led to a full complementation of the morphological defects in plt3plt5plt7 LRP (Fig. 4A–C and SI Appendix, Fig. S3 F–Q), including pericinal cell division defects at early stages (Fig. 4D and SI Appendix, Fig. S6 A and B). In addition, the expression of auxin-responsive (Fig. 4D), meristematic (Fig. 4B and SI Appendix, Figs. S4 G–L and S5 G–L), and tissue-specific (Fig. 4C and SI Appendix, Figs. S4 A–F and S5 A–F) markers in LRP-targeted PLT-complemented (PLT2 and PLT5 as examples) plt3plt5plt7 LRP and LRs was entirely restored, resulting in
continuously growing LRs and WT-level emerged LR density (Fig. 4E and **SI Appendix, Figs. S6–H and S7**). Hence, we conclude that the role of PLT3, PLT5, and PLT7 to control de novo LR meristem and tissue establishment can be exerted by all of the PLT members.

Notably, the restored expression patterns of meristematic (late PLT1, PLT2, and PLT4) and tissue-specific reporters in PLT-complemented plt3plt5plt7 primordia coincided with their native promoter activity patterns (rather than following PLT7.2 promoter), indicating that they are indirectly downstream of early PLT3, PLT5, and PLT7 during LR formation. Taken together, our analysis identifies PLT genes as critical molecular regulators that activate “primordium-intrinsic” formative cell divisions at the basis of a morphogenetic program, where de novo meristem establishment and tissue patterning are executed in a new developmental axis after LR initiation.

Finally, we asked whether any of the patterning regulators regulated by PLT3, PLT5, and PLT7 proteins, other than late PLT1, PLT2, and PLT4 proteins, was a major effector of the PLT activity in the LRP context. To this end, we reintroduced PIN1, WOX5, SCR, and SHR expression in plt3plt5plt7 LRP by using PTL7.5 promoter (**SI Appendix, Fig. S8**). LRP-targeted induction of the individual genes in the mutant primordia failed to complement the LR outgrowth defect (**SI Appendix, Figs. S8 and S9**), although reactivation of WOX5 in the mutant primordia partially restored periclinal cell division defects (**SI Appendix, Figs. S8 C and D and S9 A and D**).

**Discussion**

**PLT Proteins Are Required to Promote “Symmetry Breaking” at the Stage I to II Transition.** Our analysis uncovers roles for PLT proteins in regulation of asymmetric periclinal cell divisions that generate new radial and proximodistal axes in incipient LRP. Genes that mark the segregation of proximal–distal domains first become detectable at stage II/III with expression patterns that differ between the inner and outer LRP layers, including PLT1, PLT2, SHR, and SCR (Fig. 4F). Notably, in plt3plt5plt7 stage II/III LRP, some central cells are able to divide periclinally to generate “layered” daughter cells (Figs. 1J, 2H, and 3B and D). However, even in these cells, the expression of PLT1, PLT2, SHR, and SCR was never restored. This indicates that early activation of PLT genes in incipient stage I primordia is essential to generate the asymmetric expression of these later-activated genes and at stage II independent of the occurrence of periclinal cell divisions (Fig. 4F). Some of these asymmetrically induced genes may be direct targets, such as SHR, for which the direct regulation by PLT proteins has been shown, and WOX5, which has a promoter that is bound by PLT4 proteins (34). Alternatively, these induced genes may be the targets of PLT targets, such as the SHR-regulated SCR gene. Identification of these critical regulatory steps in the radial and proximodistal asymmetry establishment at the stage I to II transition under control of the PLT genes during LR outgrowth will aid detailed follow-up investigations of molecular mechanisms behind the establishment of new growth axes.

Understanding the sequential molecular events by which a new growth axis is formed is a substantive mission in the field of developmental biology. In plants, this is complicated by redundancy and feedback regulation. Here, we propose that the formation of a new LR meristem proceeds via two steps. The expression of late PLT1, PLT2, and PLT4 genes, particularly PLT1 and PLT2 that have the largest effect on stem cell niche

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**Fig. 4.** Full restoration LR outgrowth in PLT2-complemented plt3plt5plt7 LRP. (A–C) Confocal images of different markers in plt3plt5plt7 crossed with pPLT1,2::cPLT2:mRFP plt3plt5plt7 during LR outgrowth: (A) DRS::GFP, (B) pPLT2::erCFP, and (C) pSCR::H2B::YFP. Arrows indicate rescued marker expression during LR outgrowth in PLT2-complemented plt3plt5plt7. Triangles indicate cell layers in LRP at stage II/III. (D) Periclinal cell division (PeD) counts in LRP central cells at different stages and (E) emerged LR density (number per 1 cm) in WT, plt3plt5plt7, pPLT1,2::cPLT2:vYFP plt3plt5plt7 L-1, and pPLT1,2::cPLT2:vYFP plt3plt5plt7 L-2 roots at 7 d postgermination (n = 20). **P < 0.01 (Student’s t test).** (F) Schematic representation of PLT-regulated auxin response, symmetry breaking markers, and tissue specificity during LR outgrowth. CEI, cortex/endodermis initial; COL, columella; Cor, cortex; Endo, endodermis; Epi, epidermis; LRC, lateral root cap; SCN, stem cell niche. (Scale bar: 100 μm.)
maintenance (32, 37), is strictly dependent on early expressed PLT3, PLT5, and PLT7 but dependent on the upstream auxin response factors ARF7 and ARF19 that are required to activate the expression of early PLT genes (10, 29, 45). These data indicate that additional relevant ARF7/ARF19 targets contribute to the establishment of an early auxin maximum in LR founder cells during LR initiation.

Tissue- and Meristem-Specific Marker Induction Reveals Plasticity of Pattern Formation in LRP

It has been shown that LR outgrowth proceeds through a precise but not completely deterministic pattern of cell divisions (18). Here, we show that this variable pattern of cell division is accompanied by variable expression patterns of several tested meristem and tissue identity genes. For instance, pWOX5::GFP signal may be detected as early as in stage II but is more steadily present in the primordia that are older than stage III. Similarly, initial pPLT1::erCFP, pPLT2::erCFP and pPLT4::erCFP expression is not always detectable in stage II/III LRP. Despite the plasticity of meristem- and tissue-specific marker expression during LR outgrowth, important stem cell niche regulators, including PLT5, SHR, SCR, and WOX5, are mostly all expressed at stage III/IV (Fig. 4F). At these stages, the future stem cell niche can be distinguished as the cells with overlapping PLT, SHR, SCR, and SHR expression domains (Fig. 4F). We speculate that this coincidence may underlie the phenomenon that LRP are able to develop autonomously from stage III onward (46, 47).

Materials and Methods

The description of all plant materials, constructs and plant growth conditions, RT-PCR, the quantification of LRP morphology, phenotypic analysis, and microscopy used for this study is listed in SI Appendix, SI Materials and Methods.

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