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

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Edited by Mark Estelle, University of California, San Diego, La Jolla, CA, and approved September 14, 2017 (received for review August 24, 2017)

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 prepatterning, 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. 1*A*). Subsequent rounds of anticlinal, periclinal, and tangential cell divisions form a dome-shaped primordium that emerges through the overlaying primary root cell layers, possessing a fully functional meristem that is highly reminiscent of the primary root meristem (20, 21) (Fig. 1 *B*–*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. 1*E*). 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 *plt3plt55plt7* triple mutants. LR development is staged according to the number of radial cell layers in primordia (16) (Fig. 1 *A*–*D*). In WT, incipient stage I LRP are formed after the first asymmetric founder cell divisions (Fig. 1*A*). 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. 1*B*). In *plt3plt5plt7*, incipient stage I primordia were morphologically indistinguishable from WT (Fig. 1*F*). 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. 1*G*). Primordia were scored as "delayed" if at least one central cell did not undergo complete periclinal division (Fig. 1 *G* 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.

Author contributions: B.S. designed research; Y.D. performed research; Y.D. and B.S. analyzed data; and Y.D. and B.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1714410114/-/DCSupplemental.

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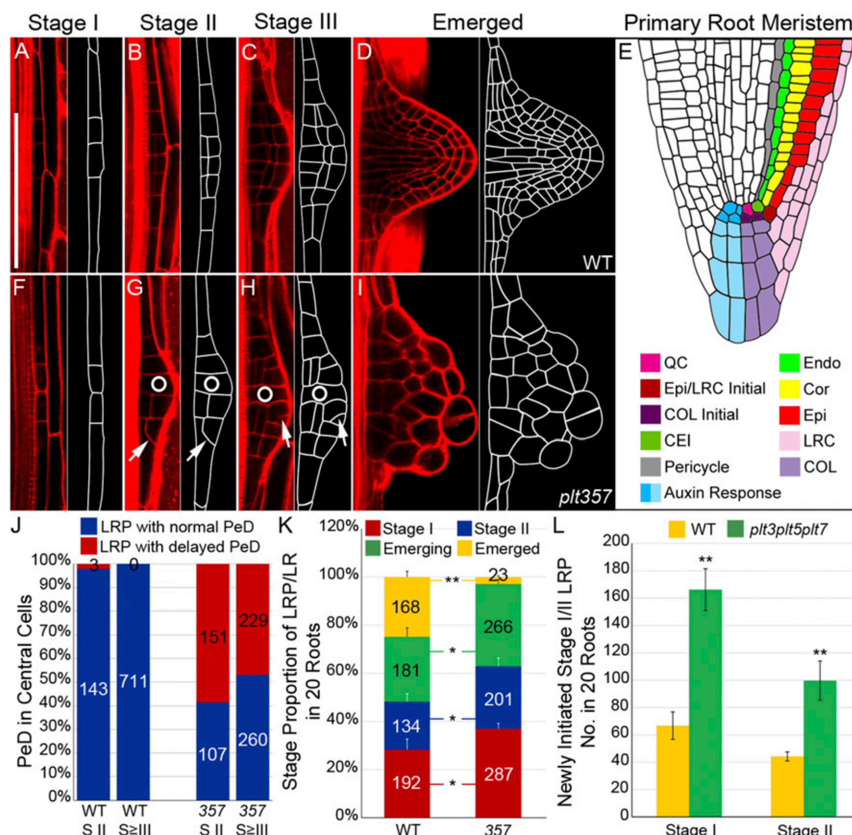

Fig. 1. Critical requirement for three PLT genes in formative periclinal cell divisions. (*A*–*D*) Confocal images and corresponding cell outlines of WT LRP at indicated stages stained with propidium iodide: (*A*) stage I, (*B*) stage II, (*C*) stage III, and (*D*) emerged. (*E*) Schematic representation of auxin response (on the left) and tissue specificity (on the right) in the primary root meristem of WT. (*F*–*I*) Confocal images and corresponding cell outlines of *plt3plt5plt7* LRP at indicated stages stained with propidium iodide: (*F*) stage I, (*G*) stage II, (*H*) stage III, and (*I*) emerged. Circles indicate LRP central cells without PeD. Arrows indicate abnormal cell division planes. (*J*–*L*) Data quantification details are described in *Materials and Methods*. (*J*) Periclinal cell division (PeD) counts in LRP central cell files at different stages in *pPLT3::GUS* marked WT and *plt3plt5plt7* roots at 7 d postgermination (d.p.g.) from left to right: WT LRP at stage II (S II), WT LRP older than stage II (≥III), *plt3plt5plt7* LRP at stage II (S II), and *plt3plt5plt7* LRP older than stage II (≥III). (*K*) Stage proportion of LRP/LR in *pPLT3::GUS* marked WT and *plt3plt5plt7* roots at 7 d.p.g. ($n = 20$). *$P < 0.05$ (Student's *t* test); **$P < 0.01$ (Student's *t* test). (*L*) Number of newly initiated LRP in *pPLT3::GUS* marked WT and *plt3plt5plt7* roots at 7 d.p.g. ($n = 20$). **$P < 0.01$ (Student's *t* test). CEI, cortex/endodermis initial; COL, columella; Cor, cortex; Endo, endodermis; Epi, epidermis; LRC, lateral root cap. (Scale bars: 100 μm.)

showed delayed periclinal divisions, while LRP/LR at later stages showed a normal periclinal division pattern (Fig. 1*J*). 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 in the rootward region compared with the WT (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.

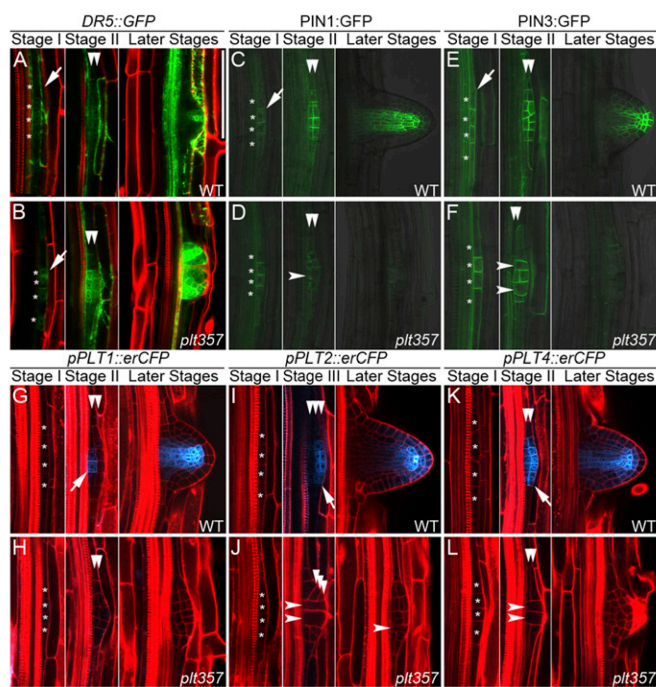

Fig. 2. Auxin response gradually delocalizes, and PLT1, PLT2, and PLT4 promoters are not activated in *plt3plt5plt7* LRP. (*A* and *B*) Confocal images of DR5::GFP in LRP at indicated stages: (*A*) WT and (*B*) *plt3plt5plt7*. (*C* and *D*) Confocal images of PIN1:GFP in LRP at indicated stages: (*C*) WT and (*D*) *plt3plt5plt7*. (*E* and *F*) Confocal images of PIN3:GFP in LRP at indicated stages: (*E*) WT and (*F*) *plt3plt5plt7*. (*G* and *H*) Confocal images of pPLT1::erCFP in LRP at indicated stages: (*G*) WT and (*H*) *plt3plt5plt7*. (*I* and *J*) Confocal images of pPLT2::erCFP in LRP at indicated stages: (*I*) WT and (*J*) *plt3plt5plt7*. (*K* and *L*) Confocal images of pPLT4::erCFP in LRP at indicated stages: (*K*) WT and (*L*) *plt3plt5plt7*. Asterisks indicate stage I LRP. Arrows indicate earliest morphological stages of detectable marker expression during LR outgrowth. Triangles indicate cell layers in LRP at stage II/III. Arrowheads indicate LRP central cells without periclinal cell division. (Scale bar: 100 μm.)

We next asked whether PLT3, PLT5, and PLT7 control PIN expression patterns. PIN1:GFP was polarly localized in the central cells in stage I LRP of both WT and *plt3plt5plt7* (30, 35, 36) (Fig. 2 *C* and *D*). PIN3:GFP was also expressed in stage I LRP of both genotypes (Fig. 2 *E* and *F*). Although PIN3:GFP expression was more evenly distributed throughout the LRP than PIN1:GFP, its level was slightly higher on the membranes surrounding the central short cells (Fig. 2 *E* and *F*). These orientations suggest that PIN1:GFP and PIN3:GFP direct auxin flow from the flanking cells to the central ones in both WT and *plt3plt5plt7*, which accords with the observation of higher *DR5::GFP* level in the central cells (30, 35) (Fig. 2 *A* and *B*). At and after stage II, the expression pattern of PIN1:GFP and PIN3:GFP in WT LRP became gradually concentrated in the more distal area, consistent with the position of the maximum *DR5* expression in those stages (30, 35) (Fig. 2 *A*, *C*, and *E*). In contrast, both PIN1:GFP and PIN3:GFP levels decreased in the mutant primordia at late stages until they became undetectable (Fig. 2 *D* and *F*). Moreover, from stage II onward, polar localization on the plasma membrane of PIN1:GFP, but not PIN3:GFP, was impaired (Fig. 2 *D* and *F* and *SI Appendix*, Fig. S1 *C* and *D*).

Taken together, PLT3, PLT5, and PLT7 do not markedly affect auxin response patterns and PIN protein localization up to stage I but are required to restrict the auxin response to a distal maximum at later stages. This is consistent with previously documented important roles of the PLT genes in regulating the auxin distribution pattern (33).

***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. 2*G* and *SI Appendix*, Fig. S2*K*). *PLT2* promoter activity was distributed differently, preferentially in the outer layers of the primordium but absent in the innermost layer (Fig. 2*I* and *SI Appendix*, Fig. S2*K*). The initial domain of *PLT4* expression encompassed all layers of stage II and/or III primordia (Fig. 2*K*). 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* expressions 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. S2*C*). 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. 1*E*) 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. 3*A*). At stage II, SHR:GFP became asymmetrically expressed in two layers, with nuclear localization in the outer layer and nucleocytoplasmic

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localization in the inner layer (Fig. 3*A* and *SI Appendix*, Fig. S2*K*). Similar to WT, SHR:GFP nuclear signal was detected in *plt3plt5plt7* stage I LRP (Fig. 3*B*). 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. 2*B* and *SI Appendix*, Fig. S1*E*). 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. 3*C* and *SI Appendix*, Fig. S2*K*). 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. 3*D*). 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. 1*E*). 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. 3*E*). In stark contrast, we did not detect *pWOX5::GFP* expression in *plt3plt5plt7* LRP at any stage (Fig. 3*F*). PLT-dependent *WOX5* expression in the LR context is consistent with the detection of PLT2 binding sites in the *WOX5* promoter (34).

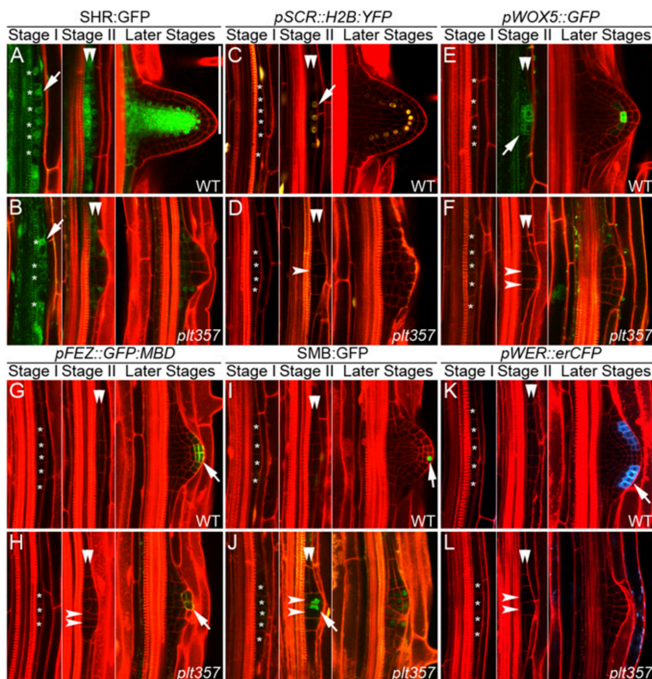

Fig. 3. The expression of key cell fate regulators is disrupted in *plt3plt5plt7* LRP. (*A* and *B*) Confocal images of SHR:GFP in LRP at indicated stages: (*A*) WT and (*B*) *plt3plt5plt7*. (*C* and *D*) Confocal images of *pSCR::H2B:YFP* in LRP at indicated stages: (*C*) WT and (*D*) *plt3plt5plt7*. (*E* and *F*) Confocal images of *pWOX5::GFP* in LRP at indicated stages: (*E*) WT and (*F*) *plt3plt5plt7*. (*G* and *H*) Confocal images of *pFEZ::GFP:MBD* in LRP at indicated stages: (*G*) WT and (*H*) *plt3plt5plt7*. (*I* and *J*) Confocal images of SMB:GFP in LRP at indicated stages: (*I*) WT and (*J*) *plt3plt5plt7*. (*K* and *L*) Confocal images of *pWER::erCFP* in LRP at indicated stages: (*K*) WT and (*L*) *plt3plt5plt7*. Asterisks indicate stage I LRP. Arrows indicate earliest morphological stages of detectable marker expression during LR outgrowth. Triangles indicate cell layers in LRP at stage II/III. Arrowheads indicate LRP central cells without periclinal cell division. (Scale bar: 100 μm.)

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. 1*E*). In WT LRP, *pFEZ::GFP:MBD* was detected around stage VI in cells located at the most distal region of the emerging primordium dome (Fig. 3*G*). SMB:GFP (40) was first detected around stage VII in the central cells at the outermost layer of emerged primordium/LR apex (Fig. 3*I*). 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. 3*H*), 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. 3*J* and *SI Appendix*, Fig. S2*J*).

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

In summary, three early-expressed tissue identity genes *SHR*, *SCR*, and *WOX5* that specify the identity of ground tissue and QC cells 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 protein fusions under their native promoters rescues the *plt3plt5plt7* outgrowth defect (29). Here, we used a 1.5-kb truncated *PLT7* promoter fragment ($PLT7_{1.5}$) (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 promotor 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 $PLT7_{1.5}$ 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. S3*A*); 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 $PLT7_{1.5}$ 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. 4 *A–C* and *SI Appendix*, Fig. S3 *F–Q*), including periclinal cell division defects at early stages (Fig. 4*D* and *SI Appendix*, Fig. S6 *A* and *B*). In addition, the expression of auxin-responsive (Fig. 4*A*), meristematic (Fig. 4*B* and *SI Appendix*, Figs. S4 *G–L* and S5 *G–L*), and tissue-specific (Fig. 4*C* 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. 4*E* and *SI Appendix*, Figs. S6 *C–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 $PLT7_{1.5}$ 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 $PTL7_{1.5}$ 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. 4*F*). Notably, in *plt3plt5plt7* stage II/III LRP, some central cells are able to divide periclinally to generate "layered" daughter cells (Figs. 1*J*, 2*H*, and 3 *B* 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 at and after stage II independent of the occurrence of periclinal cell divisions (Fig. 4*F*). 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 PLT 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

Fig. 4. Full restoration LR outgrowth in PLT2-complemented *plt3plt5plt7* LRP. (*A–C*) Confocal images of different markers in *plt3plt5plt7* crossed with $pPLT7_{1.5}$::cPLT2:mRFP *plt3plt5plt7* during LR outgrowth: (*A*) *DR5::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*, $pPLT7_{1.5}$::cPLT2:vYFP *plt3plt5plt7* L-1, and $pPLT7_{1.5}$::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.)

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maintenance (32, 37), is strictly dependent on early expressed *PLT3*, *PLT5*, and *PLT7* in an LR-specific context. It remains to be established why and how this regulatory chain operates at the molecular level.

We have shown that not only PLT3, PLT5, and PLT7 under their native promoters (29) but also, PLT1, PLT2, and PLT4 proteins, when they are induced in LR founder cells around nuclear migration stage and incipient stage I LRP, can complement the *plt3plt5plt7* LR outgrowth defect. Thus, the orchestration of meristem establishment and tissue patterning in LRP, including the induction of later PLT1, PLT2, and PLT4 function, can be performed by all PLT proteins. We speculate that this broad complementation ability stems from the large overlap between the gene sets regulated by different PLT proteins and the strong overlap in their binding motifs (34). We note that the initial auxin response in LRP is not dependent on the activity of 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 PLTs, SHR, SCR, and WOX5, are mostly all expressed at stage III/IV (Fig. 4*F*). At these stages, the future stem cell niche can be distinguished as the cells with overlapping PLT, SHR, SCR, and SHR expression domains (Fig. 4*F*). 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*.

ACKNOWLEDGMENTS. We thank Ikram Blilou, Stephen P. Grigg, Albert van den Toorn, and Hugo Hofhuis for generous gifts of unpublished materials. We thank Marta Laskowski for critical suggestions on the manuscript and Wenkun Zhou for a Triton-PI staining suggestion. This work was funded by European Research Council Advanced Grant SysArc (to B.S.).

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