

Phyllotaxis and Rhizotaxis in *Arabidopsis* Are Modified by Three PLETHORA Transcription Factors

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Summary

Background: The juxtaposition of newly formed primordia in the root and shoot differs greatly, but their formation in both contexts depends on local accumulation of the signaling molecule auxin. Whether the spacing of lateral roots along the main root and the arrangement of leaf primordia at the plant apex are controlled by related underlying mechanisms has remained unclear.

Results: Here, we show that, in *Arabidopsis thaliana*, three transcriptional regulators implicated in phyllotaxis, *PLETHORA3* (*PLT3*), *PLT5*, and *PLT7*, are expressed in incipient lateral root primordia where they are required for primordium development and lateral root emergence. Furthermore, all three PLT proteins prevent the formation of primordia close to one another, because, in their absence, successive lateral root primordia are frequently grouped in close longitudinal or radial clusters. The triple *plt* mutant phenotype is rescued by PLT-vYFP fusion proteins, which are expressed in the shoot meristem as well as the root, but not by expression of *PLT7* in the shoot alone. Expression of all three *PLT* genes requires auxin response factors ARF7 and ARF19, and the reintroduction of PLT activity suffices to rescue lateral root formation in *arf7,arf19*.

Conclusions: Intriguingly *PLT3*, *PLT5*, and *PLT7* not only control the positioning of organs at the shoot meristem but also in the root; a striking observation that raises many evolutionary questions.

Introduction

Roots and shoots of flowering plants appear to be very different. Their organs display vastly distinct morphologies and arise in different spatial patterns. Through a process called phyllotaxis, leaves and associated lateral branches are initiated in spirals or other precise arrangements from undifferentiated cells around the circumference of apical meristems [1]. In contrast, root branches arise from pericycle cells embedded within the differentiating root. The spacing of these lateral root primordia (LRP) defines the rhizotactic pattern. LRP usually do not form adjacent or opposite to one another, and their spacing along the *Arabidopsis* root correlates with its curvature [2–5].

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The formation of LRP proceeds through a series of steps that are all correlated with activities of the plant growth regulator auxin. In *Arabidopsis*, the level of auxin response just shootward of the root meristem fluctuates [3, 6]. Pulses of auxin response are thought to stimulate lateral root initiation by activating the expression of the lateral-root-promoting transcription factor GATA23 in patches of the pericycle [7]. Subsequently, auxin accumulates in a subset of cells within the pericycle located in the differentiation zone and adjacent to the protoxylem [4, 8]. Auxin accumulation suffices to drive lateral root initiation as shown by experiments in which cell-specific activation of auxin biosynthesis in the pericycle induces LRP formation [9]. The first asymmetric cell divisions in the pericycle founder cells that give rise to LRP are associated with an elevated auxin response. These divisions require the auxin-responsive protein module SOLITARY-ROOT/IAA14 and ARF7 and ARF19, which regulate lateral organ boundary genes to promote LRP formation [10–12]. Despite the identification of several factors involved in LRP formation, the molecular mechanisms that position LRP are not well understood.

Here, we investigate whether mechanisms that control the spacing of leaf and lateral root primordia are conserved. As in rhizotaxis, organ initiation during phyllotaxis is associated with patterns of auxin accumulation [13, 14], and paleobotanical evidence indicates that tip-branching leafless shoots predate roots and leaf-bearing shoots [15, 16]. Therefore, a common origin for root- and shoot-branching systems, or for the mechanisms that pattern them, might be expected. On the other hand, the contexts in which these organs arise and the genetic networks implicated so far in their formation are quite distinct. We recently demonstrated that phyllotaxis is controlled by three redundantly acting *PLETHORA* (*PLT*) AP2-class transcription factor genes, *PLT3*, *PLT5*, and *PLT7* [17, 18]. In this study, we demonstrate that in *Arabidopsis* the same three PLT proteins control rhizotaxis, revealing a shared genetic mechanism between these two processes.

Results

PLT3, PLT5, and PLT7 Influence Rhizotaxis

We examined the expression of *PLT3*, *PLT5*, and *PLT7* during LRP development. Complementing *PLT3*, *PLT5*, and *PLT7*-vYFP protein fusions as well as transcriptional reporters revealed earliest expression in a subset of pericycle cells prior to the first founder cell division (Figures 1A–1C, Figure S1 available online). The *PLT5* protein fusion was also expressed in vascular cells nearby LRP initiation sites (Figure 1B). Protein and transcriptional fusions remained expressed through the early stages of LRP formation, consistent with a potential role in rhizotaxis (Figures 1D–1I and S1). Expression of *PLT3* and *PLT5* protein fusions, but not *PLT7*, was observed in the tip of the main root (Figures 1J–1L). Other genes in the *PLT* family (*PLT1*, *PLT2*, and *PLT4*) were expressed in the root tip, but appeared only at later stages within LRP (Figure S1).

Roots of the triple mutant *plt3,plt5,plt7* exhibited multiple changes in lateral root development, including altered rhizotaxis. Lateral root emergence was severely impaired in *plt3,plt5,plt7* triple mutant and *plt3,plt7* double mutant roots

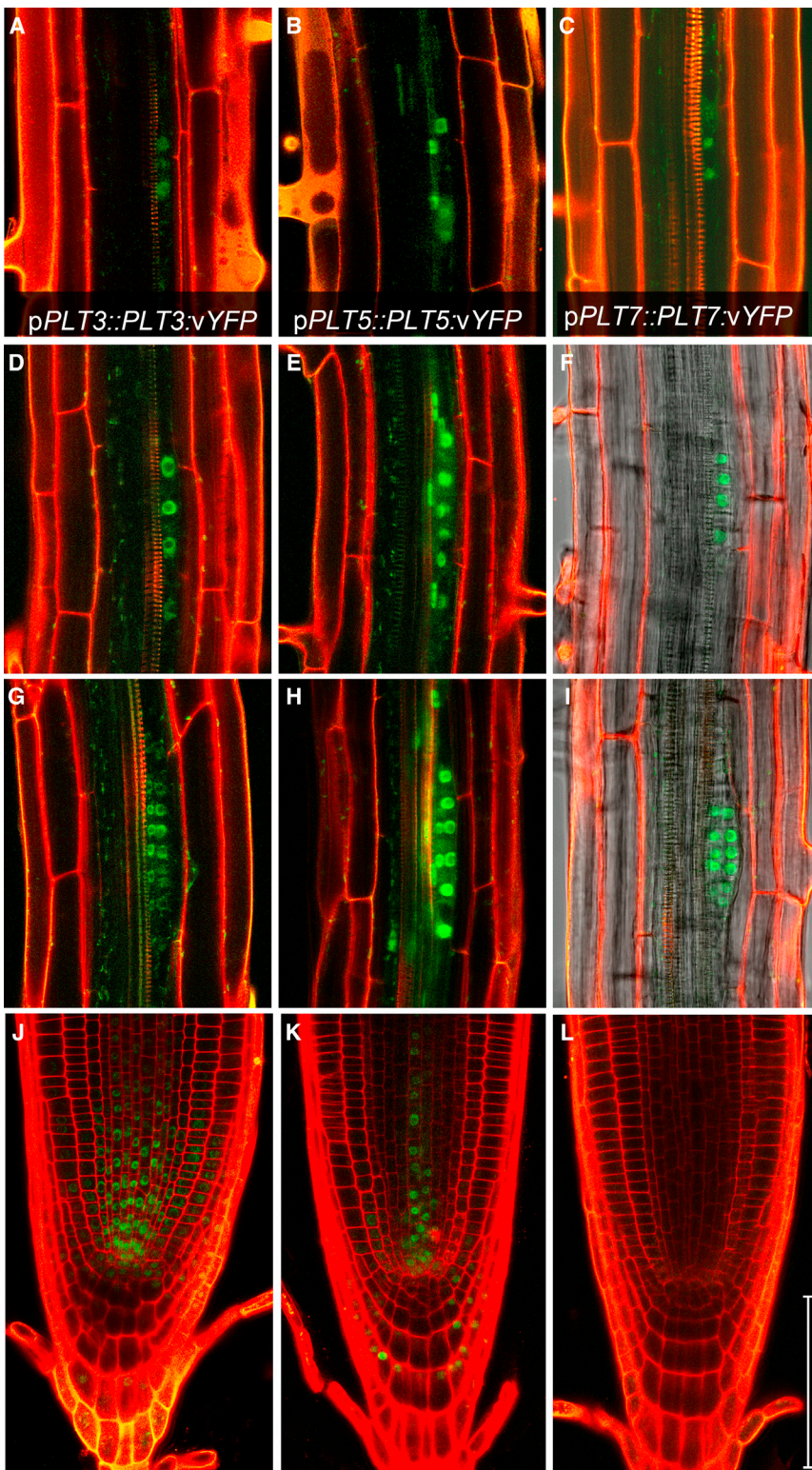


Figure 1. PLT3, PLT5, and PLT7 Are Expressed Early in Lateral root Formation

The accumulation of complementing PLT protein fusions (green signal) in round nuclei of pericycle cells before the founder cell divisions give rise to stage 1 LRP (A–C), within stage 1 LRP (D–F), within stage 2 LRP (G–I), and in the root tip (J–L). Elongated nuclei in (B), (E), and (H) indicate vascular expression. Shown are *PLT3::PLT3-vYFP* in *plt3,plt5,plt7* (A, D, G, and J), *PLT5::PLT5-vYFP* in *plt3,plt5,plt7* (B, E, H, and K), and *PLT7::PLT7-vYFP* in *plt3,plt7* (C, F, I, and L). The red signal marking cell boundaries represents propidium iodide. The scale bar represents 100 μm .

In roots of WT seedlings, LRP typically do not form within 300 μm of one another [2]. On the basis of this observation, longitudinal clusters were defined as those in which two LRP had formed within 300 μm along a single protoxylem pole (Figure S3). Radial clusters are similar, except that LRP or emerged lateral roots had formed along opposite protoxylem poles. In *plt3,plt5,plt7* triple mutants, successive LRP were frequently grouped in longitudinal or radial clusters (Figures 3A–3C). The increase in closely spaced LRP in *plt3,plt5,plt7* was not solely the result of increased organ density, given that, in several rescue experiments (see below), the density of total lateral organs fully reverted to WT levels, whereas the number of clusters remained substantial (Figures 4A and 4C, compare graphs and tables). When clusters formed, they included roots of all developmental stages (e.g., some clusters included two stage 1 LRP and some included two emerging lateral roots). However, approximately 75% of the primordia within a cluster were either at the same developmental stage or differed by just one stage, suggesting that clustered primordia arose nearly simultaneously (Figure 3D). Altogether, these data reveal that the bias against forming clusters of primordia depends on the joint action of PLT3, PLT5, and PLT7.

Rhizotaxis and Phyllotaxis Are Controlled by Root- and Shoot-Specific *PLT* Expression Domains

To investigate whether PLT proteins locally influence LRP clustering, we

(Figures 2A and S2A). LRP in these roots initially resembled those of wild-type (WT) seedlings, but morphological defects became increasingly severe as the LRP developed (Figures 2B–2P). Although only a few lateral roots emerged in the triple mutant, the density of total lateral roots (emerged lateral roots + LRP per cm) was higher than in WT seedlings. Total lateral root density was also increased in *plt3,plt7* and *plt3,plt5* (Figure S2A).

reintroduced PLT-vYFP protein fusions to double and triple *plt* mutants. All three protein fusions rescued the clustering phenotype (Figures 4A and 4B). Specifically, *PLT3::PLT3-vYFP* rescued the average number of clusters in the *plt3,plt5,plt7* triple mutant back to the level found in both *plt5,plt7* and WT roots (Figure 4A). In the same experiment, *PLT5::PLT5-vYFP* rescued clustering of *plt3,plt5,plt7* to a level

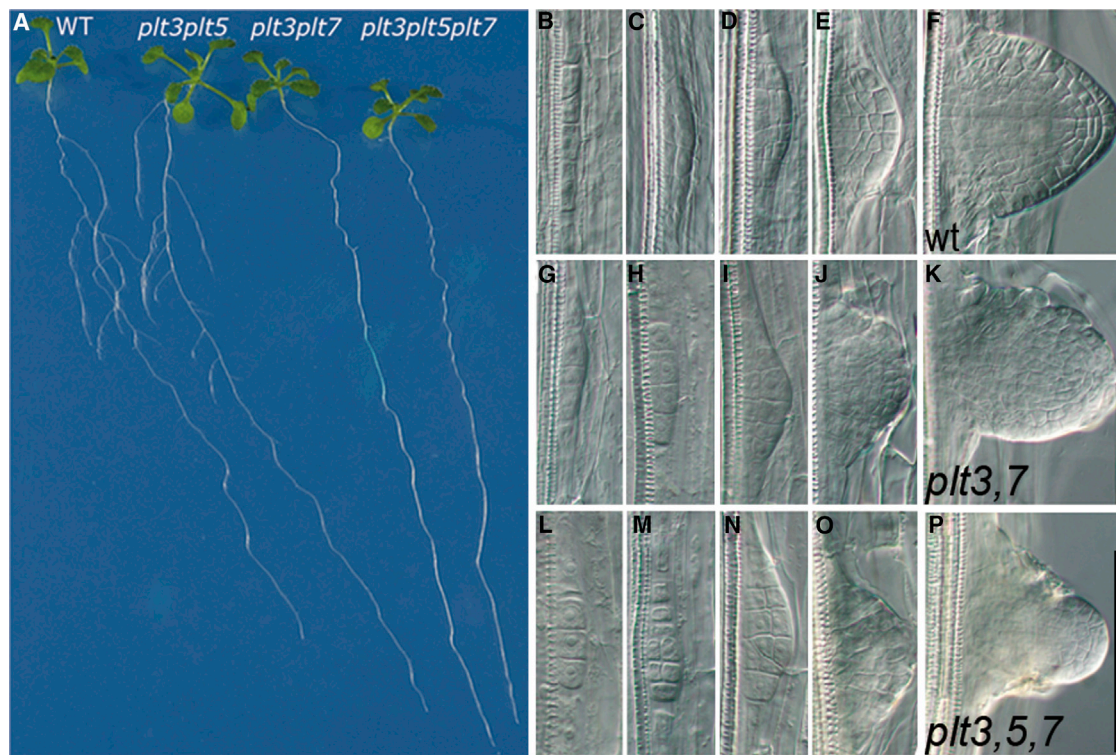


Figure 2. PLT3, PLT5, and PLT7 Influence Lateral Root Development and Outgrowth

(A) An overview of wild-type (WT) and *plt* mutant seedlings. Note that LRP largely fail to emerge from within the *plt3,plt7* and *plt3,plt5,plt7* roots. Morphology of WT (B–F), *plt3,plt7* (G–K), and *plt3,plt5,plt7* (L–P) LRP at progressive stages of development. LRP development was staged in broad categories because of variable developmental defects within the *plt3,plt7* and *plt3,plt5,plt7* backgrounds. Category 1, stage 1 LRP composed of short pericycle cells prior to the first periclinal division; category 2, stage 2 LRP with two cell layers; category 3, LRP has expanded radially but is still confined within the cortex, roughly three to five cell layers thick; category 4 (emerging), LRP that is pushing against the epidermis prior to emergence; and category 5 (emerged), the tip of the LRP extended past the epidermis. (B), (G), and (L) depict category 1; (C), (H), and (M) depict category 2; (D), (I), and (N) depict category 3; (E), (J), and (O) depict category 4; and (F), (K), and (P) depict category 5. Emerged lateral roots as shown in (K) and (P) in *plt3,plt7* and *plt3,plt5,plt7* occasionally occur. The scale bar represents 100 μ m (B–P).

similar to that of *plt3,plt7* (Figure 4A); *PLT7::PLT7-vYFP* restored the average number of clusters in *plt3,plt7* to levels found in *plt3* and WT roots (Figure 4B). In addition, reintroduction of the PLT fusion proteins restored LRP morphology in all lines. Lateral root emergence was more readily rescued than clustering, given that we observed one PLT5 and one PLT7 complementation line in which lateral root emergence was fully or almost fully restored, whereas clustering of LRP remained substantial (Figure S2B).

Because it was striking that the activity of three related transcription factors (PLT3, PLT5, and PLT7) determines primordium spacing in both the shoot [17, 18] and root, we asked if control of rhizotaxis is related to PLT action in the shoot. We observed the expression of *PLT7* only in the shoot apex and in LRP. In the *plt3,plt5,plt7* background, driving the expression of *PLT7* from the shoot meristem-specific *SHOOT MERISTEM LESS* promoter restored shoot primordium spacing [18]. However, despite complementation in the shoot, this re-expression did not restore rhizotaxis (Figure 4D), indicating that inhibition of cluster formation derives from local *PLT7* activity within LRP.

PLT3, PLT5, and PLT7 Function Downstream of ARF7- and ARF19-Mediated Auxin Response

To position the *PLT* genes in the sequence of steps leading to lateral root initiation, we focused on their relationship to the auxin-responsive transcription factors ARF7 and ARF19.

arf7,arf19 double mutant roots are defective in the first asymmetric cell divisions that characterize LRP formation and almost completely lack lateral roots [19, 20]. *SLR* encodes an AUX/IAA protein that represses ARF7- and ARF19-mediated lateral root initiation [20–22]. Microarray data indicated a modification of *PLT3* transcript levels in the auxin perception mutant solitary root (*slr*) [12, 21]. Furthermore, the initial expression of PLT3, PLT5, and PLT7 in founder cells at the developmental stage affected by ARF7 and ARF19 indicated that *PLT* genes might operate downstream of ARF action.

In the mature region of WT roots, the expression of *PLT3*, *PLT5*, and *PLT7* promoter fusions to vYFP or CFP was observed in LRP (Figures 5A, 5C, and 5E), the first marked LRP being located nearby the position of metaxylem differentiation. In contrast, LRP were not typically present in this region of *arf7,arf19* roots, and the enhanced expression of all three promoter fusions in the pericycle was correspondingly absent. Only one LRP was found in 60 roots of *arf7,arf19*, indicating that “escape” LRP did express the *PLT5* promoter fusion. In contrast, primary root tip expression of *PLT3* and *PLT5* promoter fusions was similar in both WT and *arf7,arf19* roots, and *PLT7* promoter fusion was not observed in either case. We concluded that the elevated transcription of *PLT3*, *PLT5*, and *PLT7* in pericycle-derived cells requires ARF7 and ARF19 except in rare escape LRP in the *arf7,arf19* background.

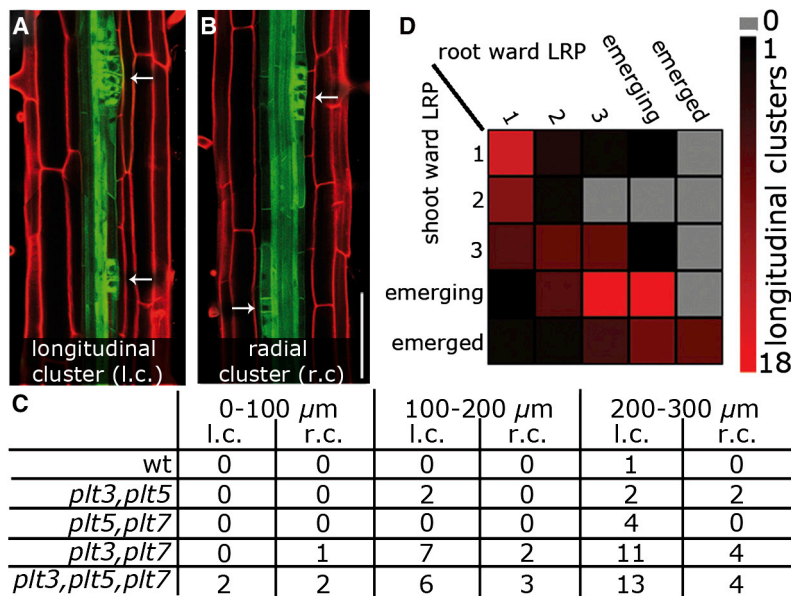


Figure 3. Cluster Formation in the Wild-Type Roots Is Prevented by PLT3, PLT5, and PLT7

(A and B) Examples of longitudinally (A) and radially (B) clustered LRP in a *plt3,plt7* root. The scale bar represents 100 μm .

(C) Frequency of clustered LRP in 20 roots of WT seedlings and *plt* mutants. LRP were also more frequently observed 300–400 μm apart in *plt3,plt7* and *plt3,plt5,plt7* in comparison to WT seedlings.

(D) A diagram plotting the frequency with which LRP of categories as defined in Figure 2 were clustered. Columns and rows indicate the category of the rootward and shootward located LRP, respectively. Note that pairings on and near the diagonal (e.g., clusters that contain LRP of similar stages) are most common.

To determine whether ARF7, ARF19 act through *PLT* genes, we asked whether reintroducing *PLT* expression in the *arf7,arf19* mutant background might rescue aspects of lateral root initiation. Seedlings expressing dexamethasone (DEX)-inducible *35S::PLT-GR* [17, 23] in *arf7,arf19* were transferred to agar plates containing DEX. The region of the pericycle rootward of the first root hair at the time of transfer responded most strongly. *PLT3-GR*, *PLT5-GR*, and *PLT7-GR* induction all resulted in continuous patches of divided pericycle within the *arf7,arf19* root segments upon 24 hr of DEX induction (Figures 5G–5L). Additional divisions of pericycle cells occurred in the region that had differentiated prior to transfer, which resulted in a few lateral roots (Figure S4). Altogether, our data indicate that *PLT3*, *PLT5*, and *PLT7* are downstream components of ARF7- and ARF19-mediated lateral root initiation.

Discussion

Our data reveal that the positioning of root primordia is controlled by the joint action of three partially redundant *PLT* transcription factors that become expressed before the first asymmetric cell division of LRP founder cells. Their activity restricts the region of LRP formation to a single focus, and the result of this restriction is that clusters of adjacent or opposite lateral roots are not formed. *PLT3*, *PLT5*, and *PLT7* expression within pericycle cells depends on the activity of the ARF7, ARF19 auxin response factor pair, which is known to be required for lateral root initiation [19–21, 24–26]. This induction may be indirect, given that *PLT* genes are not included in the list of direct targets of ARF7, ARF19 [19, 20]. Moreover, ectopic expression of *PLT3*, *PLT5*, or *PLT7* suffices to overcome the block to LRP formation in *arf7,arf19* roots, indicating that the expression of these three *PLT* genes is an effective downstream component of ARF7, ARF19-mediated auxin response.

The joint action of *PLT3*, *PLT5*, and *PLT7* inhibits LRP clustering and promotes their emergence, which puts forth the question whether and how these functions are related. Our complementation data indicate that these two processes are separable. Along with the dosage-dependence shown for other combinations of *PLT* genes [27], this may suggest that distinct targets of these transcription factors, which separate

clustering and emergence functions, are activated at different thresholds. This line of thought may also explain why the roles of these *PLT* genes in promoting early-stage primordium initiation seem exaggerated and roles in spacing primordia are masked after their induced expression from the 35S promoter in *arf7,arf19*.

The fact that the local activity of the same three *PLT* genes defines organ spacing in both the shoot and the root hints at unexpected similarities in the mechanisms that generate plant architecture. On the other hand, the topology of *PLT* expression, the arrangement of primordia in roots and shoots, and the topology of the zones competent for organ formation are different in shoots and roots, suggesting the involvement of important context-specific factors for primordium spacing. A comprehensive analysis of downstream targets of *PLT3*, *PLT5*, and *PLT7* in the root and shoot will be necessary for the comparison of gene regulatory networks that regulate primordium spacing in both contexts.

Our observation that *Arabidopsis* phyllotaxis and rhizotaxis require an identical subset of *PLT* genes also raises evolutionary questions. Did the stems of early vascular plants acquire an ancestral *PLT* module influencing branching prior to the invention of roots and laterally branching shoots? Have the dicots—which diversified the *PLT* clade beyond *PLT5* homologs [17]—extended this module? Or was the involvement of the same redundant *PLT* genes in organ spacing recruited independently for patterning of roots and shoots—an equally remarkable event? To resolve this issue, it must be determined whether shared roles of *PLT* clade members in shoot and root patterning are conserved in seed plants, lycophytes, and ferns.

Experimental Procedures

Plant Materials and Constructs

Arabidopsis thaliana

A Columbia-0 (Col-0) background was used for all experiments with mutants and transgenics as indicated. *plt3-1*, *plt5-2*, *plt7* and *plt* mutant combinations were described in [17]. *PLT1,2,3,4::eCFP* are described in [27]. Promoter regions of *PLT5* and *PLT7* were amplified from Col-0 genomic DNA with the following primer combinations and fused to eCFP or vYFP coding sequences: pPLT5-f, GGGGACAACCTTTGTATAGAAAAGTTGTTcagcgttgca gcgttgatattgc; pPLT5-r, GGGGACTGCTTTTTGTACAAACTTGTcatcttggg aataggtttttttttt; pPLT7-f, GGGGACAACCTTTGTATAGAAAAGTTGTTcgtga gtccttggcttccctcatctctt; pPLT7-r, GGGGACTGCTTTTTGTACAAACTTGTtga agcagcagcagcttctatctca; 1.5kbPLT7-f, GGGGACAACCTTTGTATAGAAAAG TTGAGtaggtgagtcacctcgagtgac; 1.5 kbPLT7-r, GGGGACTGCTTTTTTGT ACAAACTTGGcaaaaagattgaactttttt.

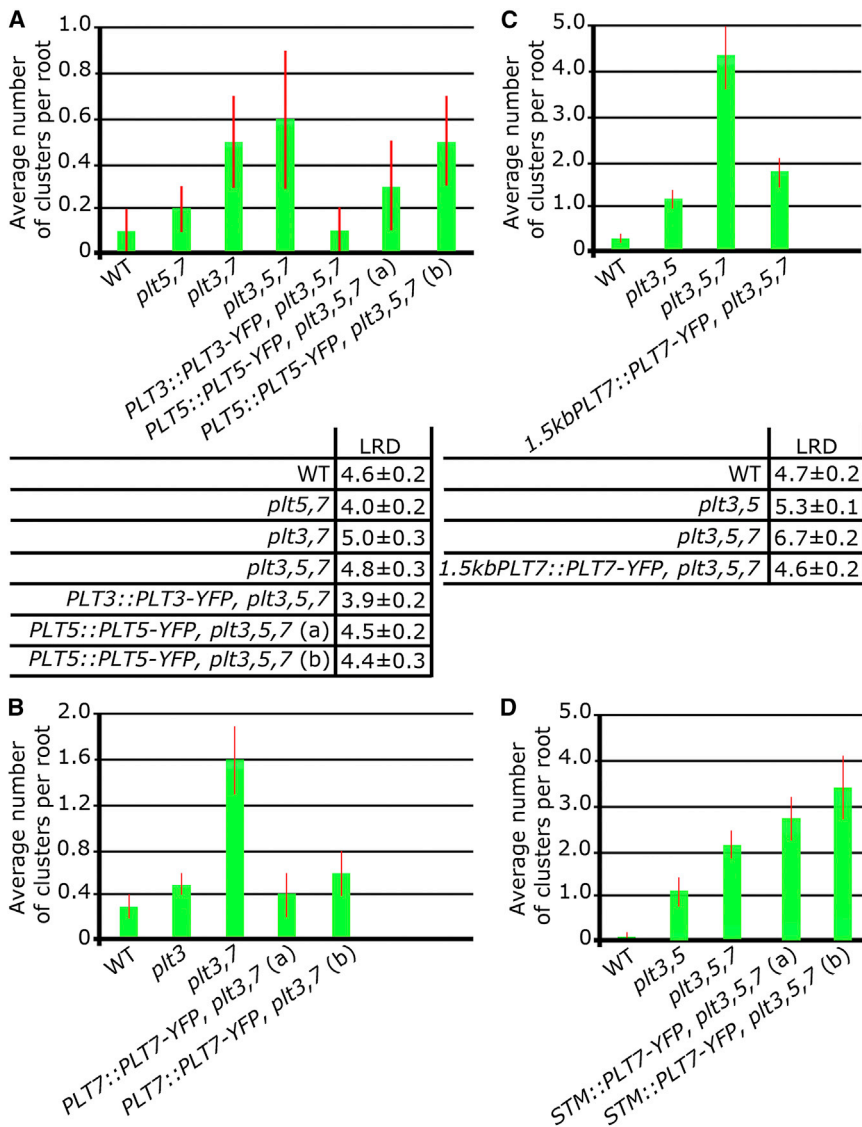


Figure 4. Formation of Clustered LRP in *plt* Mutants Is Rescued by *PLT* Expression under Their Endogenous Promoters but Not by Expression in the Shoot Apical Meristem Alone

Green bars indicate the average number of clusters per root. Associated lateral root densities (LRD) are emerged lateral roots + LRP per cm. (A) Expression of *PLT3::PLT3-YFP* and *PLT5::PLT5-YFP* in *plt3,plt5,plt7* rescue LRP spacing; 8-day-old roots. (B) *PLT7::PLT7-vYFP* in *plt3,plt7* prevents cluster formation; 8-day-old roots. (C) Expression of *PLT7* under the control of a truncated *PLT7* promoter sequence in *plt3,plt5,plt7* restores total organ density to WT levels, whereas clustering of LRP is only partially rescued. Truncation of the *PLT* promoter sequence did not change the pattern of *PLT7-vYFP* expression in the root or shoot; 11-day-old roots. (D) *STM::PLT7-YFP* in *plt3,plt5,plt7* does not prevent cluster formation, indicating that the expression of *PLT7* in location(s) other than the shoot is responsible for the rescue seen in (B); 11-day-old roots. Between-experiment variability is partially correlated with plant age. Red error bars represent SEM; $n \geq 18$ roots per sample.

least 1 hr, dipped in ethanol, and placed on microscope slides with cold chloral hydrate. Roots were viewed and photographed on a Nomarski microscope.

Phenotype Analysis and Microscopy

Light microscopy and confocal microscopy were performed as described previously [27, 28]. Roots were cleared with chloral hydrate, and the relative position(s) of LRP were examined by Nomarski microscopy. When LRP occurred close enough together that any part of the nearest neighbors could be seen within one field of view under the microscope (a distance well in excess of 300 μm), they were imaged and the distance between successive primordia was measured with ImageJ (version 1.6 or 2.0) or camera

software. The relationships between the developmental stages of subsequent primordia were plotted as heatmaps with MultiExperiment Viewer.

Supplemental Information

Supplemental Information contains Supplemental Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2013.04.048>.

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Translational fusions *PLT3::PLT3-vYFP*, *PLT5::PLT5-vYFP*, and *PLT7::PLT7-vYFP* were described in [17]; *STM::PLT7-vYFP* fusion was described in [18]. All constructs were generated in pGreenII [27] and transformed into the indicated genotypes.

PLT-GR Lines and Dexamethasone Inductions

35S::PLT5-GR was described in [17]. To generate *35S::PLT3-GR* and *PLT7-GR* fusions, we amplified *PLT* genomic fragments using the following primers: *PLT3*: SalPLT3-F, GCTGCAGGTCGACATGATGGCTCCGATGACGAAGTGG; BamPLT3-R, ACGGATCCGACTGATTAGGCCAGAGGAAGAACTC. *PLT7*: SalPLT7-F, GCTGCAGGTCGACATGGCGGATTCAACAACCTTACTAC; BamPLT7-R, ACGGATCCGACTGGTTAGGCCACAAGAAAACTCAGC.

The amplified PCR fragments were cloned as Sall-BamHI into the plant binary vector pGII227-GR for the generation of in-frame fusions with the glucocorticoid receptor fragment under the control of the 35S promoter and transformed into Col-0 WT plants. WT seedlings expressing *35S::PLT3-GR*, *35S::PLT5-GR*, and *35S::PLT7-GR* were crossed with *arf7,arf19* and maintained as heterozygous stocks, owing to what may have been a poor germination of homozygous GR lines. Seeds were placed on 1/2 MS medium with hygromycin for 4 days and transferred to 1/2 MS medium with or without 10 μM DEX for the indicated time. At the time of transfer, the location of the root tip and the first root hair were marked with the use of a dissecting microscope. At the end of the treatment period, roots were placed in cold fixative (50% methanol and 10% acetic acid in water) for at

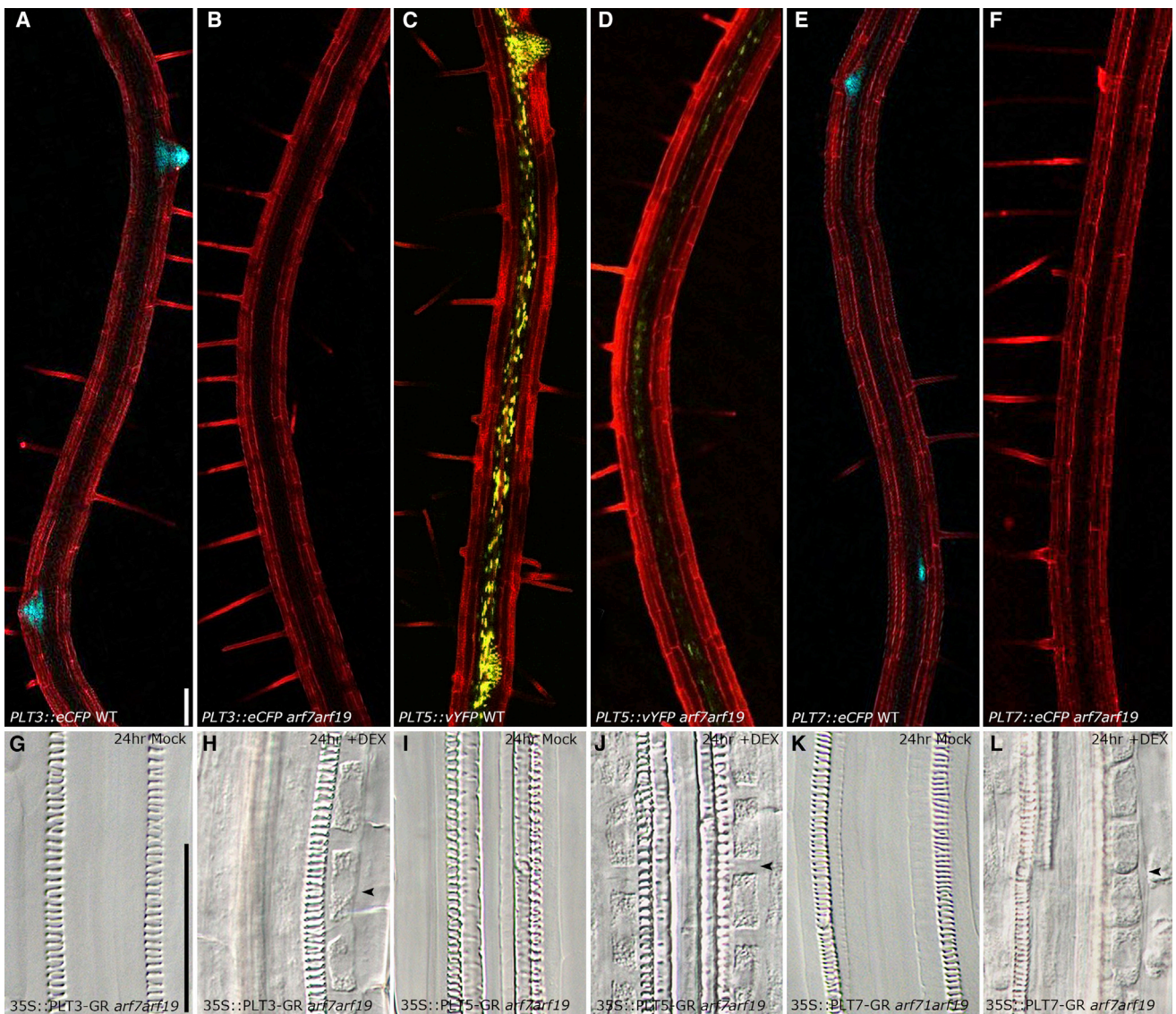


Figure 5. *PLT3*, *PLT5*, and *PLT7* Expression in Pericycle-Derived Cells Requires *ARF7* and *ARF9*, and the Ectopic Expression of *PLT-GR* Overcomes the Block in LRP Formation in *arf7,arf9*

Top, *PLT* promoter fusions are expressed in (A), (C), and (E) WT LRP but are absent from pericycle in mature regions of (B), (D), and (F) *arf7,arf9*. Shown are *PLT3::eCFP* (A and B), *PLT5::vYFP* (C and D), and *PLT7::eCFP* (E and F). Note the *PLT5::vYFP* expression in *arf7,arf9* vascular cells. Bottom, Nomarski images of cleared *arf7,arf9* roots expressing indicated *PLT-GR* proteins after 24 hr of growth on 1/2 MS media containing mock (G, I, and K) or 10 μ M DEX (H, J, and L). DEX, but not mock, treatment induces cell divisions in the pericycle. Nomarski images are from regions that were newly grown on the DEX plates. Shown are *35S::PLT3-GR* (G and H), *35S::PLT5-GR* (I and J), and *35S::PLT7-GR* (K and L). The red signal depicts propidium iodide. Scale bars represent 100 μ m (A–F) and 50 μ m (G–L). Arrowheads indicate additional pericycle cell division.

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