Auxin acts as a local morphogenetic trigger to specify lateral root founder cells

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Plants exhibit an exceptional adaptability to different environmental conditions. To a large extent, this adaptability depends on their ability to initiate and form new organs throughout their entire postembryonic life. Plant shoot and root systems unceasingly branch and form axillary shoots or lateral roots, respectively. The first event in the formation of a new organ is specification of founder cells. Several plant hormones, prominent among them auxin, have been implicated in the acquisition of founder cell identity by differentiated cells, but the mechanisms underlying this process are largely elusive. Here, we show that auxin and its local accumulation in root pericycle cells is a necessary and sufficient signal to specify these cells into lateral root founder cells. Analysis of the alf4–1 mutant suggests that specification of founder cells and the subsequent activation of cell division leading to primordium formation represent two genetically separable events. Time-lapse experiments show that the activation of an auxin response is the earliest detectable event in founder cell specification. Accordingly, local activation of auxin response correlates absolutely with the acquisition of founder cell identity and precedes the actual formation of a lateral root primordium through patterned cell division. Local production and subsequent accumulation of auxin in single pericycle cells induced by Cre-Lox-based activation of auxin synthesis converts them into founder cells. Thus, auxin is the local instructive signal that is sufficient for acquisition of founder cell identity and can be considered a morphogenetic trigger in postembryonic plant organogenesis.

Results and Discussion

Local Auxin Response Correlates with Founder Cell Specification. To gain insights into the mechanism of cell reprogramming and founder cell specification we analyzed available molecular markers and screened enhancer trap libraries for reporter expression associated with early stages of lateral root primordium (LRP) initiation. The earliest activity identified was that of the synthetic promoter DR5, which is an established marker for auxin-responsive cell cycle because of sustained expression of cell cycle genes such as CycA2;1 and until now no founder cell-specific marker or mutants affected specifically in founder cell specification have been found (14).

Here, we demonstrate that the plant hormone auxin is the local instructive signal for specification of founder cells that give rise to lateral roots. Our analysis of the alf4–1 mutant suggests that acquisition of founder cell identity and activation of patterned cell division can be genetically separated. Time-lapse experiments show that the auxin-responsive promoter DR5 is the earliest marker for founder cells and its activation absolutely correlates with subsequent primordium formation. Furthermore, a Cre-Lox-based mosaic expression of an enzyme for auxin synthesis in β-glucuronidase (GUS)-labeled sectors demonstrates that local auxin accumulation in a single pericycle cell converts it into a founder cell. Thus, auxin is sufficient to trigger acquisition of founder cell identity in postembryonic organogenesis in plants.

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responsive and indirectly for auxin accumulation (16, 17). DR5 is active at all stages of LRP development in both Arabidopsis (ref. 6 and Fig. 1A) and tomato (Fig. 1B). The earliest detectable DR5 (DR5rev::GFP; ref. 18) expression was either in single or two longitudinally abutted xylem-adjacent pericycle cells (Fig. 1C and D). These DR5-expressing cells exhibited all attributes of LRP founder cells: they were found only in the xylem-adjacent pericycle where all LRP initiate and were distal to the youngest LRP founder cells: they were found only in the xylem-adjacent and D longitudinal abutted xylem-adjacent pericycle cells (Fig. 1C and D). These DR5-expressing cells exhibited all attributes of LRP founder cells: they were found only in the xylem-adjacent pericycle where all LRP initiate and were distal to the youngest LRP, which is consistent with the acropetal pattern of LRP initiation (12, 14, 19). To test whether these DR5-expressing pericycle cells were in fact founder cells, we followed the fate of GFP-positive cells by performing a time-lapse experiment in live roots. In intervals of ~15 h, we scanned roots of DR5rev::GFP seedlings and closely followed fate of all DR5-positive cells and the origin of all initiating LRP. In 13 tested roots, all pericycle cells that showed DR5rev::GFP expression developed into LRP (Fig. 1C and D); conversely, not a single LRP was initiated from a GFP-negative cell. These data show that DR5 activation completely correlates with the acquisition of founder cell identity and that the local activation of auxin response precedes the initiation of LRP formation.

Acquisition of Founder Cell Identity Is Genetically Separable from Activation of Cell Division. To further analyze whether specification of founder cells precedes cell division in primordium morphogenesis, we analyzed DR5 activity in the alf4–1 Arabidopsis mutant, which is blocked in pericycle cell division that leads to LRP formation (20, 21). In alf4–1 roots, we consistently observed the presence of DR5-active pericycle cells (Fig. 1E) that were distributed along the root in a pattern comparable to LRP distribution in WT roots; however, these cells did not develop into LRP (data not shown). In 10-day-old homozygous alf4–1 plants, the number of pericycle DR5 activation events (21.0 ± 4.0, n = 3, mean ± SD) was similar to the number of lateral roots and LRP in 10-day-old WT plants (19.9 ± 3.7, n = 11, mean ± SD, Student’s t test P = 0.412). This observation suggests that acquisition of founder cell identity marked by DR5 activation precedes activation of patterned cell division for LRP development.

Auxin Production in a Single Pericycle Cell Triggers Its Conversion into a Founder Cell. As established, DR5 is expressed in response to activated auxin signaling in a given cell and thus, indirectly, DR5 expression positively correlates with cellular auxin levels (6, 16, 17). Indeed, increased DR5 activity in embryos and roots has been previously correlated with local auxin accumulation as visualized by anti-indole-3-acetic acid (IAA) antibody (6, 18). In line with this, we observed uniform activation of DR5rev::GFP expression in all xylem-adjacent pericycle cells after treatment with different natural and synthetic auxins such as IAA, 1-naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D), demonstrating comparable auxin response in these cells [Fig. 1F and supporting information (SI) Fig. S1]. This observation suggests that increased DR5rev::GFP activity in single pericycle cells at the positions of lateral root initiation (as shown in Fig. 1A–C and E) does not reflect higher sensitivity of auxin signaling but rather increased cellular auxin levels in these cells. In addition, these DR5-positive cells after auxin treatment become proliferatively active, and eventually, formed lateral root primordia (6). Based on these results, we propose a scenario where local accumulation of auxin in single pericycle cells is the signal that induces specification of LRP founder cells.

To test this model, we created transgenic plants that allowed us to stimulate auxin production in random single cells and identify these same cells and their progeny by GUS activity. In brief, these plants carry a heat shock-inducible Cre recombinase gene that, when induced, creates clonal sectors that simultaneously express indoleacetic acid tryptophan monoxygenase

Fig. 1. Auxin response-marked specification of lateral root founder cells precedes cell cycle activation in the pericycle. (A and B) DR5 activity throughout the lateral root formation (starting at 8 h; roman numbers are developmental stages in accordance with ref. 30. (A) Arabidopsis roots of the DR5rev::GFP line (n = 58). (B) Tomato roots of the DR5::GUS line (n = 46). Red arrowheads denote end walls of pericycle founder cells; black arrowheads denote periclinal cell walls. (A) CLSM images. (B) Nomarski optics. 0, merged Nomarski and CLSM; 0 and II, live unstained roots; I and II, live roots stained with neutral red; IV–VII, fixed roots. (C and D) Time-lapse analysis of live roots showing that pericycle founder cells are always accompanied by increased DR5 auxin response. Longitudinal unicellular (C) and biccicular (D) types of lateral root initiation. (Left) Pericycle cells at the beginning of the experiment. (Right) Images taken at the same focal plane 15 h later, showing primordia formed. Red arrowheads indicate end walls of founder cells. Yellow arrowheads indicate new cell walls formed. At the beginning of the experiments plants were 6 days old (C) and 7 days old (D). (E) DR5 activation in presumptive founder cell in 10-day homozygous alf4–1 mutant plant (n = 10); p, pericycle; px, protoxylem. (F) In Arabidopsis, DR5 can be activated in all pericycle cells by auxin treatment (10 μM NAA, 6 h); live roots were stained with neutral red. (Scale bars: A, B, and F, 20 μm; C–E, 25 μm.)
(iaaM) and the GUS reporter. iaaM catalyzes a critical step in the conversion of tryptophan (Trp) into auxin (22). Where Trp levels are limiting such as in roots, exogenous Trp application to iaaM-expressing lines has been shown to increase auxin production (23). Thus in these Arabidopsis Cre/Lox lines after heat shock treatment, seedlings will randomly form sectors that both express iaaM and can be visualized with the GUS reporter (Fig. 2A). These same sectors will have increased auxin production in the presence of Trp. Such a system would, in theory, allow inducible activation of auxin production in marked random sectors.

First, we tested in detail different aspects of this system. The generated expression sectors were, as expected, originally of single cell size. No sectors of GUS-marked iaaM expression were observed in untreated seedlings (Fig. 2B). The iaaM expression under more general RPS5 promoter (Fig. S2) or when large sectors were induced (data not shown) indeed lead to increased auxin production as manifested by typical auxin overproduction phenotypes, including long hypocotyls. These phenotypes were shown to correlate with higher levels of free auxin in iaaM-activated lines (22, 23). To optimize the conditions for in vivo auxin biosynthesis in the random sectors, the effects of different heat shock and Trp treatments on LRP initiation were tested. LRP initiation was not significantly affected by 30- or 45-min heat shock durations, nor by 10- or 50-μM Trp treatments alone (Fig. 2C). Simultaneous iaaM activation by 45-min heat shock and 50-μM Trp treatment led to a significant increase of LRP initiation over the heat shock alone (Student’s t test 4, $P < 0.0004$) or Trp alone (Student’s t test 3, $P < 0.0003$). The frequency of LRP initiation was scored 62 h after the heat shock in 10–15 seedlings per treatment (mean ± SE).

To examine the consequences of locally stimulated auxin production on lateral root formation, we scored the frequency of LRP initiation 48 h after the heat shock in the absence or presence of Trp. In addition, GUS staining revealed which of the LRPs originated from iaaM-expressing cells. Indeed, among the LRPs that arose after Trp treatment, GUS-positive LRPs were found as expected for primordia arising from one activated founder cell (Fig. 3A–C). In some cases, half-stained primordia were found corresponding to only one of two adjacent founder cells being activated through iaaM expression (Fig. 3D and E), confirming the clonal character of the sectors and showing that auxin production in a single cell leads to the recruitment also of the neighboring cells into founder cells. In total, Trp-treated roots formed 48% more LRP than untreated roots (Figs. 2C and 3F). This increase in primordia initiation proportionally correlated with an increase of GUS-labeled LRPs (Fig. 3G). Thus, the additional LRPs that were initiated after Trp treatment originated mostly from the auxin-producing pericycle sectors, showing that auxin production in pericycle cells triggers LRP initiation.

Next, we addressed the positions, where these additional LRPs were initiated. It has been shown that LRP initiation follows a regular left and right alternating pattern (24). In Trp-treated seedlings with random activation of iaaM expression, we observed increased frequency of deviations from this natural positioning pattern. These included initiation of two LRPs in
acquisition of lateral root founder cell identity. This process is not directly coupled to subsequent division of the founder cells, as the specification event can be genetically separated from the patterned division during primordium morphogenesis. The local accumulation of auxin in individual xylem pericycle cells could result from either directed transport or local synthesis and serves as a local instructive signal for cell fate reprogramming and the onset of organogenesis. This mechanism of local auxin maxima can thus “select” given pericycle cells and convert them into founder cells, thereby determining a spatial pattern of lateral root formation.

The fact that lateral root founder cell specification and patterned cell division in the pericycle can be separated both temporally and genetically indicates that the primary event during LRP initiation is not an auxin-induced activation of the cell cycle as has been proposed previously (14). Instead, we propose a model whereby auxin serves as a morphogenetic trigger. Although the criteria defining a morphogen in animal systems (25, 26) do not completely fit to plant systems (3, 18), we define a morphogenetic trigger as the factor or signal that induces, through unequal distribution of its activity, acquisition of a new developmental fate in a cell or a group of cells. The current study shows that auxin IAA is such morphogenetic trigger for lateral root initiation.

Previous studies showed that local auxin application on the shoot apical meristem of tomato was sufficient to induce leaf formation from the adjacent peripheral zone of the meristem (5). Taken together with the detection of auxin response maxima correlating with shoot- and root-derived organ initiation in different plant species (5, 6, 7), the results presented here suggest a general, evolutionary conserved auxin-based mechanism for acquisition of founder cell identity in plant organogenesis.

Materials and Methods

Transgenic Lines and Growth Conditions. Drsrev::GFP and Drs::GUS Arabidopsis lines have been described (6, 18). The male-sterile alf4–1 mutant was

Conclusion

In summary, our results show that an increase in auxin levels and signaling in individual pericycle cells always accompanies lateral root organogenesis, and that such increases are sufficient for the

close proximity at the same xylem pole (Fig. 4) or directly oppose each other (data not shown). Activation of iaaM in Trp-treated seedlings increased frequency to 0.63 positioning defects per seedling (LRP, \( n = 145 \)) as compared with 0.30 positioning defects per seedling (LRP, \( n = 94 \)) in untreated seedlings. Thus, as expected for random activation of iaaM expression, the regular patterning of LRP initiation was disrupted and some primordia initiated irregularly. Notably, similar defects, albeit with lower frequency, can be induced by a general increase of auxin levels either by auxin or Trp treatment, which result in increased LRP initiation (0.39 defects in Trp-treated seedlings, LRP, \( n = 125 \)). Altogether, these results suggest that an increased auxin level in individual pericycle cells is a sufficient signal for the pericycle cell fate change into founder cell identity.
crossed with the DR5rev::GFP line, and individual homozygous alf4–1 plants expressing DR5rev::GFP were selected from the F2 generation. Plants were genotyped (Fig. 5C) by PCR. DNA was isolated from rosette leaves with a PUREGENE kit (Genta Systems); the 137-bp product for the alf4–1 allele and the 149-bp product for the WT allele were amplified by using 5’-GTAATTTGTTTTCTGGTGTG-3’ forward and 5’-CACAGTTGTTAAATCCTCG-3’ reverse primers that span a 12-bp deletion in the alf4–1 mutant allele. The PCR products were resolved in 10% polyacrylamide gel (PAAG). Plants were grown on vertical Petri dishes on solid medium under conditions described (27). The DR5::GUS construct (17) was introduced into Agrobacterium tumefaciens strain EHA 105, and stable transformation of tomato (Solanum lycopersicum) was performed as described (28). The lines with the strongest GUS expression were used. Lateral root development and founder cell specification were analyzed within the primary root of Arabidopsis plants and within first-order lateral roots of tomato plants. GUS staining was performed as described (19). Auxin treatments were performed with 10 μM NAA or 20 μM IAA for 6 h and 2.5 μM 2,4-D for 6.5 h.

Cre-Lox-Based Stimulation of Auxin Biosynthesis. Transgenic plants harboring a CRE recombine under control of a heat shock promoter and an empty pCB1 vector [a 35S promoter separated from GAL4::VP16 coding sequence by a spacer flanked with lox P sites (29)] were crossed with pEF iaaM plants that contain both the iaaM and GUS genes under control of an UAS promoter (pSDK7010) (23) to obtain lines with a heat shock-inducible, CRE recombinase-mediated mosaic of GUS-labeled cells expressing iaaM. Seedlings homozygous for the above constructs were germinated and grown for 4 days on MS medium without or with 5 μM N-1-naphthylphthalamic acid NPA to prevent LRP initiation before iaaM activation. The seedlings were then heat shock-treated for 45 min (unless otherwise noted) and subsequently incubated in liquid MS medium or MS medium supplemented with 50 μM tryptophan (Trp) for 48 or 60 h. Roots were stained for GUS and cleared as described (30). LRP initiation was scored with a Zeiss Axioshot microscope using Nomarski optics. To examine positioning defects in lateral root pattern formation, frequencies of nonstandard initiation events were scored like two LRPCs in close proximity at the same xylem pole or directly opposite each other.

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Seedlings 48 and 60 h after heat shock treatment were analyzed. The number of analyzed plants is given in the figure legends.

Microscopy and Time-Lapse Experiments. Live or fixed (4–6 h in 4% formaldehyde either in PBS or in phosphate buffer, pH 6.5, supplemented with 1 μM 2′-propidium iodide) roots expressing DR5rev::GFP were analyzed with a Zeiss LSM 510 Meta confocal laser scanning microscope (CLSM) equipped with an argon laser. Zeiss ×40 (NA 0.75, Plan Neofluor) dry and ×63 (NA 1.2, C-Apochromat 100X water immersion) objectives were used. Some live roots were stained with Neutral red at pH 5.6 (27). For time-lapse experiments, 6- or 7-day-old plants were mounted over a thin layer of 0.4% agar plant growth medium in a custom-made chamber with a coverslip at the bottom. The roots of the plants were covered with solidified 0.4% agar medium, and liquid medium was added to the bottom of the chamber. To minimize evaporation, the chamber’s borders were sealed with Vaseline and covered with a piece of glass. Founder cells were detected in the young root differentiation zone by GFP fluorescence. A total of 32–40 serial optical sections were taken both above and below the middle image. In the same focal planes, Nomarski CLSM images were acquired. The chamber was left overnight on the microscope stage, and the last 6 h later images were taken within the same focal planes and the same confocal settings. To confirm that a GFP-expressing cell became a part of an LRP, merged Nomarski and CLSM images were analyzed, and root hairs or cell walls were used as landmarks to verify that the focal plane was not changed over the experimental period (Fig. S4). For illustration purposes, the signal of the green channel was increased (Fig. 1 C and D) by using Adobe Photoshop. Images with original signal intensity are shown in Fig. S5. The number of plants analyzed is indicated in the figure legends.

References

Fig. S1. Auxin activates DR5 response in all xylem-adjacent pericycle cells. Treatment with natural IAA (A) and synthetic 2,4-D (B) induces DR5 response in all pericycle cells adjacent to the xylem pole. Shown is neutral red staining of live roots; red shows protoxylem. (Scale bars: 25 μm.)
Fig. S2. Activation of iaAM expression by RPSS promoter leads to increased hypocotyl elongation. (A) Control seedling without iaAM activation. (B) Activation of iaAM expression leads to increased hypocotyls elongation. GUS staining accompanies iaAM expression. (Magnification: ×5.)
Fig. S3. Genotyping of F2 segregating plants. PCR products from *alf* 4–1 homozygous (lanes 2, 3, 5, and 6), *ALF4*/*alf* 4–1 heterozygous (lane 1), and *ALF4* Col-0 (lane 4) plants are shown.
Fig. S4. Time-lapse analysis of live roots shows that pericycle founder cells can be defined by their increased DR5 activity ($n = 13$). Longitudinal unicellular (A) and bicellular (B) longitudinal types of lateral root initiation are shown. (Left) Confocal images showing GFP expression in founder cells or primordia. (Right) The same images merged with a phase-contrast image (A) or DIC (B) image taken at the same focal plane. Numbers indicate time in hours and minutes from the beginning to the end of the observations. Red arrowheads indicate end walls of founder cells. Yellow arrowheads indicate new cell walls formed as a result of cell division. Asterisks show reference points (cell walls or root hairs) used to verify that images are taken at the same focal plane at the beginning and the end of observations. At the beginning of the experiments plants were 6 days old (A) and 7 days old (B). (Scale bars: 25 μm.)