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 respecify 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.

cell identity | branching | development | pericycle | plant hormones

Plants, unlike animals, exhibit the remarkable ability to continue organogenesis throughout their entire life cycle. During embryogenesis only shoot and root apical meristems are formed; however, lateral organs such as axillary shoots, lateral roots, leaves, and flowers initiate during the subsequent growth and development of the adult plant (1). The first event in the formation of a new plant organ involves specification of founder cells, which upon activation start to divide and form a primordium (2). Although specification of founder cells is a key event in postembryonic organ formation, there is very little knowledge about the mechanisms regulating this process. It has been known for decades that plant organogenesis is under the control of long-range signaling by plant hormones, prominent among them auxin (3). Auxin promotes organ formation (1, 4, 5), and locally increased levels of auxin response have been reported to mark positions of organ initiation and distal tips of developing organ primordia (6, 7). In shoot apical meristem, specification of founder cells for leaf formation involves down-regulation of a highly conserved class of homeobox genes related to *KNOTTED1* (*KNOX*) (8). It has been shown that the *ASYMMETRIC LEAVES1* (*AS1*) gene, which encodes a Myb protein, restricts *KNOTTED* expression to cells that are destined to form leaf primordia and is a key factor in leaf founder cell recruitment (9). Thus, this interaction between *AS1* and *KNOX* functions to distinguish between stem cells and founder cells within the shoot apical meristem. Similar functions have been revealed for *AS1* gene orthologs *NARROW SHEAT1* in maize (10) or *PHANTASTICA* in *Anthirinium* (11). In roots, knowledge concerning founder cell specification is less consistent. Lateral root founder cells are recruited from the pericycle cells adjacent to the xylem pole. In contrast to leaf initiation, which in *Arabidopsis* involves recruitment of ≈30 founder cells from the periphery of the shoot apical meristem (10), lateral roots are formed from a minimum of three or six founder cells depending on whether the initiation is of a longitudinal unicellular or bicellular type (12). Xylem pole pericycle cells, from which founder cells are recruited, carry cytological features such as dense cytoplasm, large nuclei, and small vacuoles typical of meristematic cells (13). In addition, it has been shown that these cells are capable of fast entry into the cell cycle because of sustained expression of cell cycle genes such as *CDKA;1* or *CycA2;1* (14, 15). However, only very few cells of the pericycle tissue layer are recruited to become founder cells, 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.

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 re-

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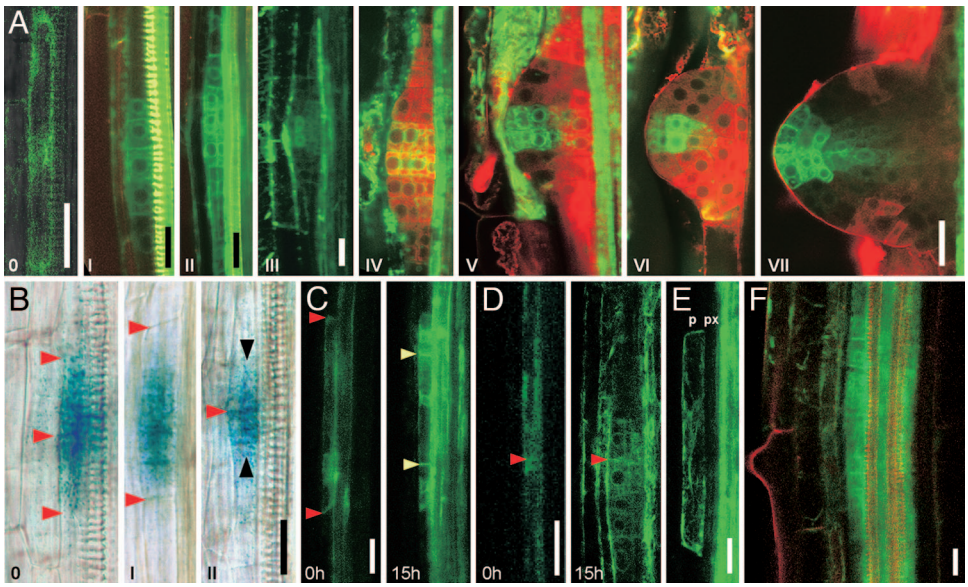

Fig. 1. Auxin response-marked specification of lateral root founder cells precedes cell cycle activation in the pericycle. (*A* and *B*) *DR5* activity throughout the lateral root formation starting from a presumptive founder cell (0); roman numbers are developmental stages in accordance with ref. 30. (*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 III, 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 bicellular (*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.)

sponse and indirectly for auxin accumulation (16, 17). *DR5* is active at all stages of LRP development in both *Arabidopsis* (ref. 6 and Fig. 1*A*) and tomato (Fig. 1*B*). The earliest detectable *DR5* (*DR5rev*::*GFP*; ref. 18) expression was either in single or two longitudinally abutted xylem-adjacent pericycle cells (Fig. 1 *C* and *D*). These *DR5*-expressing cells exhibited all attributes of LRP founder cells: they were found only in the xylem-adjacent pericycle where all LRPs initiate and were distal to the youngest LRPs, 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 LRPs (Fig. 1 *C* 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. 1*E*) that were distributed along the root in a pattern comparable to LRP distribution in WT roots; however, these cells did not develop into LRPs (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 LRPs 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. 1*F* 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. 1 *A–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 monooxygenase*

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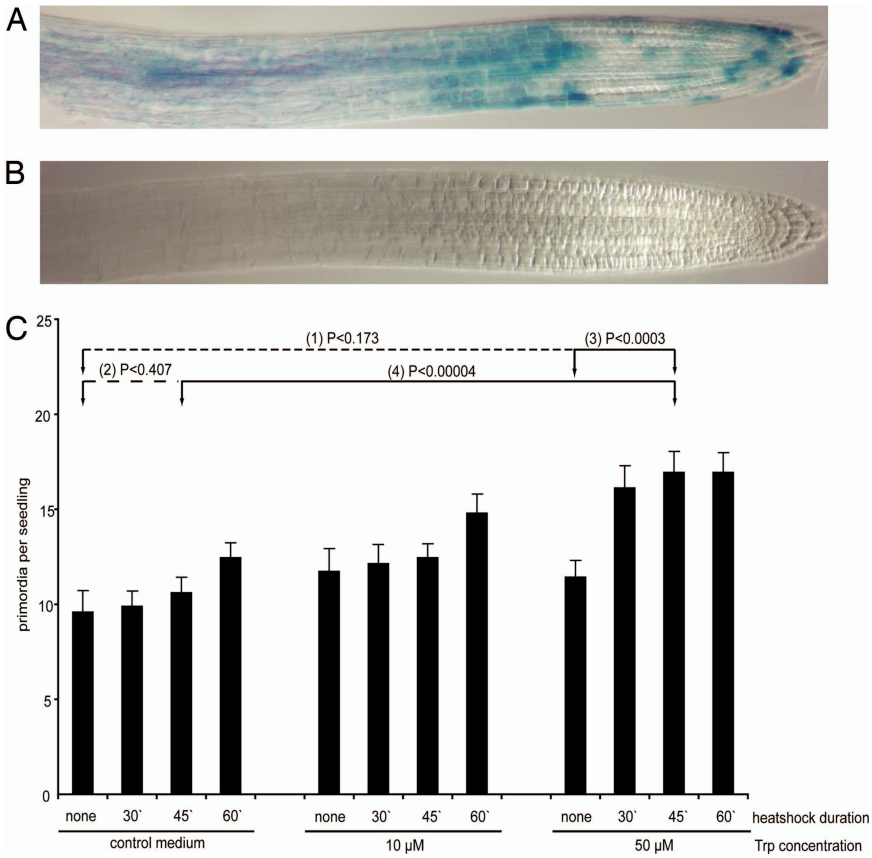

Fig. 2. Heat shock-induced Cre/Lox based random *iaaM* expression. (*A*) Sectors of GUS-marked *iaaM*-expressing cells in root meristem induced by heat shock. (*B*) No sectors of GUS-marked *iaaM* expression were observed without the heat shock treatment. (*C*) LRP initiation was not affected by 30- or 45-min heat shock durations (Student's *t* test 2, $P = 0.407$), nor by 10- or 50-μM Trp treatments (Student's *t* test 1, $P = 0.173$). 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, $P < 0.00004$) 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).

(*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≫iaaM* lines after heat shock treatment, seedlings will randomly form sectors that both express *iaaM* and can be visualized with the GUS reporter (Fig. 2*A*). 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. 2*B*). 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. 2*C*). Simultaneous *iaaM* activation by 45-min heat shock and 50-μM Trp treatment led to a significant increase of LRP initiation over heat shock alone or Trp alone. Based on these analyses, 45-min heat shock and 50-μM Trp treatment were used for all further studies (Fig. 2*C*).

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. 3 *A–C*). In some cases, half-stained primordia were found corresponding to only one of two adjacent founder cells being activated through iaaM expression (Fig. 3 *D* 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. 2*C* and 3*F*). This increase in primordia initiation proportionally correlated with an increase of GUS-labeled LRPs (Fig. 3*G*). 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

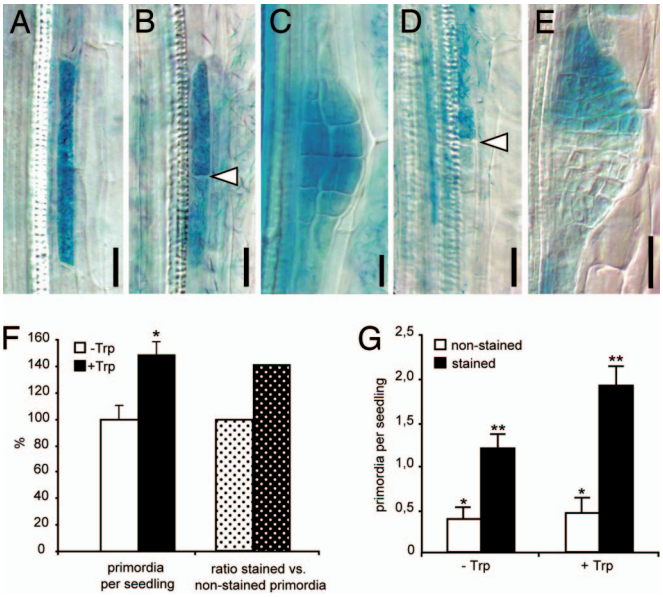

Fig. 3. Local auxin production in pericycle cells triggers primordium formation. (*A–E*) Examples of sectors of GUS-marked *iaaM*-expressing cells induced by heat shock. (*A*) Sector of a single xylem-adjacent pericycle cell is shown. (*B*) After cell division two longitudinally abutted cells are formed; arrowhead indicates the new cell wall formed. (*C*) Such activation leads to formation of a fully stained primordium. (*D* and *E*) Activation of iaaM expression in only one of two longitudinally abutted cells can recruit the neighboring cell as the second founder cell and leads to staining of the progeny of only one founder cell (*D*) and development of a half-stained primordium (*E*). Arrowhead in *D* shows the position of end walls of a heat shock-activated and an independently specified pericycle founder cells. (*F*) Seedlings with random sectors of *iaaM* expression initiate 48% more primordia when auxin synthesis is induced by Trp treatment (black bar) as compared with untreated controls (white bar) (Student's *t* test; *, $P < 0.0008$). Accordingly, the proportion of GUS-positive primordia (indicating auxin-producing sectors) is 40% higher in Trp-treated seedlings (dotted black bar) as compared with untreated controls (dotted white bar). (*G*) Scoring of stained (*iaaM* expressing) and unstained primordia reveals that Trp treatment does not affect the iaaM-independent initiation of primordia, as the number of unstained primordia is not changed compared with the untreated controls (white bars) (Student's *t* test; *, $P = 0.6$,). The significant increase in the number of stained primordia (black bars) upon Trp treatment (Student's *t* test; **, $P < 0.005$) suggests that the local increase in the auxin level as a consequence of iaaM activity in pericycle sectors stimulates primordia initiation. The number of primordia was scored in 35–40 seedlings from two independent experiments (mean ± SE). (Scale bars: *A–C*, 25 μm; *D* and *E*, 40 μm.)

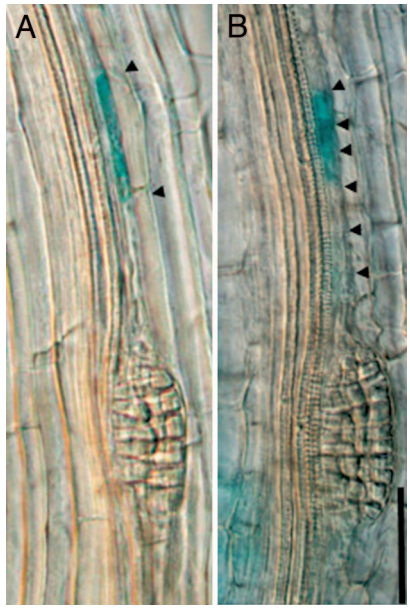

Fig. 4. Random activation of *IaaM* leads to increased frequency of defects in LRP positioning. (*A*) In non-Trp-treated control, *IaaM* activated pericycle cell in close proximity of other LRP at the same xylem pole without LRP iniation. (*B*) In Trp-treated seedlings coincidental activation of *IaaM* in pericycle cell in close proximity of other LRP at the same xylem pole results in primordia initiation. Arrowheads indicate pericycle end walls. A total of 30–35 seedlings were analyzed 48 h after heat shock incubation. (Scale bar: 55 μm.)

close proximity at the same xylem pole (Fig. 4) or directly opposite 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.

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 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. *DR5rev*::*GFP* and *DR5*::*GUS Arabidopsis* lines have been described (6, 18). The male-sterile *alf4–1* mutant was

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crossed with the *DR5rev*::*GFP* line, and individual homozygous *alf4–1* plants expressing *DR5rev*::*GFP* were selected from the F_2 generation. Plants were genotyped (Fig. S3) by PCR. DNA was isolated from rosette leaves with a PUREGENE kit (Gentra Systems); the 137-bp product for the *alf 4–1* allele and the 149-bp product for the WT allele were amplified by using 5′-GTAATTT-GTTTTCTGGGTGG-3′ forward and 5′-CAAAGTCTTGAAATCCTCCG-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 in ref. 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 recombinase 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 (pSDM7010) (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-naphthyl)phthalamic 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 Axiophot microscope using Nomarski optics. To examine positioning defects in lateral root patterning, frequencies of nonstandard initiation events were scored like two LRPs in close proximity at the same xylem pole or directly opposite each other. 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 μg·ml^{-1} 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 Neofluar) dry and ×63 (NA 1.2, C-Apochromat) 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 14–16 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. S3. The number of plants analyzed is indicated in the figure legends.

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