Auxin-dependent regulation of lateral root positioning in the basal meristem of *Arabidopsis*

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In plants, the developmental mechanisms that regulate the positioning of lateral organs along the primary root are currently unknown. We present evidence on how lateral root initiation is controlled in a spatiotemporal manner in the model plant *Arabidopsis thaliana*. First, lateral roots are spaced along the main axis in a regular left-right alternating pattern that correlates with gravity-induced waving and depends on AUX1, an auxin influx carrier essential for gravitropic response. Second, we found evidence that the priming of pericycle cells for lateral root initiation might take place in the basal meristem, correlating with elevated auxin sensitivity in this part of the root. This local auxin responsiveness oscillates with peaks of expression at regular intervals of 15 hours. Each peak in the auxin-reporter maximum correlates with the formation of a consecutive lateral root. Third, auxin signaling in the basal meristem triggers pericycle cells for lateral root initiation prior to the action of INDOLE-3-ACETIC ACID14 (SOLITARY ROOT).

INTRODUCTION

Lateral roots maximize the ability of a root system to acquire nutrients and water. In several plant species, lateral roots along the main root axis seem to be formed according to a regular pattern (Mallory et al., 1970; Charlton, 1983; Barlow and Adam, 1988). New lateral roots are continuously initiated at a predictable distance above the growing root tip (reviewed by Charlton, 1996). Lateral root primordia and the youngest lateral roots can be found nearest to the root tip, whereas more mature lateral roots are encountered higher in the root (Fahn, 1974).

Prior to emergence in the mature zone, lateral root primordia go through an extensive series of cell divisions (Malamy and Benfey, 1997). Due to the acropetal development of lateral roots, early stages can be traced back at more distal positions. Furthermore, a G2-to-M-specific promoter-reporter construct, *CYCB1;1::GUS*, marks the very first divisions in the pericycle during lateral root initiation. In *Arabidopsis thaliana*, the first lateral root that is initiated after embryogenesis is observed in the differentiation zone, at a fixed distance above the root tip (Casimiro et al., 2001). This position corresponds with the region where pericycle cells progress via S phase to G2 (Beeckman et al., 2001). Initiation of *Arabidopsis* lateral roots occurs in a strict acropetal pattern and only in a relatively short zone distal to the youngest lateral root primordium (Dubrovsky et al., 2006).

Here, we provide evidence that the events which determine lateral root positioning take place in a region at the transition between the meristem and the elongation zone, referred to as the basal meristem (Beemster et al., 2003), where several other physiological and growth responses occur, including responses to gravity, touch and moisture (Ishikawa and Evans, 1995). The data presented suggest that auxin signaling in the central cylinder of the basal meristem correlates with regular lateral root spacing. Furthermore, we show that lateral root initiation is regulated by periodic fluctuations in DR5 activity. We hypothesize such fluctuations might be representative for fluctuations in auxin distribution mediating regular longitudinal spacing of lateral roots. Our data provide a new model for root branching that highlights the involvement of the root apex.

MATERIALS AND METHODS
Used materials
We analyzed *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0); mutants *aux1-7* (Pickett et al., 1990) and *aux1-22* (both null alleles), *axr3-1*; promoter fusions CYCB1;1::GUS (Colón-Carmona et al., 1999), DR5::GUS (Ulmasov et al., 1997), IAA2::GUS (Swarup et al., 2001), IAA14::GUS (Fukaki et al., 2002); GAL4-GFP enhancer trap lines J0121, J0951, J1701, M0013 and Q1220 (http://www.plantsci.cam.ac.uk/Haseloff/geneControl/catalogues/Jlines); the promoter trap QC184 (Sabatini et al., 2003); UAS:AUX1,*aux1*-22, J0951>>AUX1,*aux1*-22, UAS:*axr3-1*, J0121>>UAS:*axr3-1* (Swarup et al., 2005), and *pIAA14::mIAA14-GR* lines (Fukaki et al., 2005).

Growth conditions and drug treatments
Seeds were germinated on standard Murashige and Skoog (MS)-derived medium on vertically or at 45° oriented square plates (Greiner Labortechnik, Kremmünster, Austria) under growth conditions described by Vanneste et al. (Vanneste et al., 2005). Supplements were 10 μM *N*-1-naphthylphthalamic acid (NPA; Duchefa, Haarlem, The Netherlands), 10 μM α-naphthaleneacetic acid (NAA; Sigma-Aldrich, St Louis, MO), or 1 μM dexamethasone (Dex; Sigma-Aldrich).

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For all time-course experiments, the highest synchronization level was obtained by incubating the agar plates, after sowing, for 2 days at 4°C in the dark and then under continuous light at 22°C. Under these conditions, germination started at the earliest 20 hours after transfer to the growth chamber and was nearly 100% at 48 hours. After transfer to the growth chamber, the plates were screened for germinated seeds with a dissecting microscope, to indicate the early (at 24 hours) and late (at 34 hours) germinating population. The positions of germinating seeds (i.e. seeds with a radicle protruding the seed coat) were marked on the plate using a felt-tip pen. Only the marked seedlings were used for further analyses. In each time course, samples were taken at intervals of 7.5 hours (see Fig. S1 in the supplementary material for corresponding seedling stages). By considering the appearance of the radicle as time 0 hours, we obtained highly uniform seedling stages as supported by the homogenous seedling size at each time point determined by time-lapse recordings (see Fig. S1 in the supplementary material).

Histochemical and histological analysis
The β-glucuronidase (GUS) assays were performed as described by Beeckman and Engler (Beeckman and Engler, 1994) or according to the protocol of Malamy and Benfey (Malamy and Benfey, 1997). For anatomical sections, GUS-stained samples were treated as described previously (Beeckman and Viane, 2000; De Smet et al., 2004).

Microscopic analyses
For whole-mount microscopic analysis, samples were cleared by mounting in lactic acid (Acros Organics, Geel, Belgium) or according to Malamy and Benfey (Malamy and Benfey, 1997). All samples were analyzed by differential interference contrast microscopy (DMLB; Leica Microsystems). For fluorescence microscopy, whole seedlings were stained with 10 μg/mL propidium iodide (Sigma-Aldrich) and mounted in water under glass coverslips for green fluorescent protein (GFP) signal analysis with a confocal microscope 100M with software package LSM 510 version 3.2 (Zeiss, Jena, Germany). Images were collected with a 488-nm emission filter.

Imaging and root length measurements
Photographs were taken with a CAMEDIA C-3040 zoom digital camera (Olympus, Tokyo, Japan) and processed with Photoshop 7.0 (Adobe Systems, San José, CA). Whole plates were scanned on a color copier CLC-iR C3200 (Canon, Tokyo, Japan). For measuring the interlateral root distances, the positions of lateral roots and emerged primordia were indicated under a dissecting microscope (Stemi SV11 Apo, Zeiss) on the back of the plates using a felt-tip pen prior to scanning. Plate scans were measured with ImageJ (http://rsb.info.nih.gov/ij/).

Toner labeling
Distances from root tips (including root cap) to the start of DR5::GUS expression at 10, 25, 40 and 55 hours after germination (HAG) were first determined using a stereomicroscope (Stemi SV11 Apo, Zeiss) with an eyepiece and measurement unit. To label that part of the root tip where DR5::GUS expression is anticipated, black toner particles from a copier were positioned with an eyelash on the root. Likewise, roots at off-peak time-points (17.5, 32.5 and 47.5 HAG) were labeled at a position that was extrapolated from the measurements at the high-level time-points. In this way, the epidermal cells of this region become permanently marked (Beemster and Baskin, 1998).

RESULTS
Arabidopsis roots exhibit alternating left-right lateral root positioning correlated with AUX1-dependent root waving
In response to gravity, roots display positive gravitropic growth (reviewed by Morita and Tasaka, 2004), resulting in an enhanced waving of the root when seedlings are grown at a 45° angle (Okada and Shimura, 1990). On agar plates, vertically grown *Arabidopsis* roots also display a wavy pattern (Fig. 1A), which is accompanied by lateral root development at outer sides of bends (Fig. 1B). To

Table 1. Correlation between curve tops and lateral root positioning

n	42
Main-root length (mm)	50.6±2.2
Total number of emerged primordia and lateral roots	10.8±1.0
Total number of curves (amplitude of at least 0.4 mm)	20±1
Number of lateral roots positioned precisely on top of curve	5.5±0.4

investigate the correlation between these two processes, *Arabidopsis* seedlings were grown on 1.5% hard agar at an inclination of 45° (Fig. 1C). At 12 days after germination (DAG), seedlings showed on average 20 measurable curves per root representing 16% of the total root length (Fig. 1D; Table 1). Of the total number of lateral roots, approximately 51% were positioned precisely in this 16% region of the root (Fig. 1D, red mark). A χ^2 test (with one degree of freedom) revealed that this peculiar lateral root distribution does not occur by chance in wild type ($P<0.001$) and suggested a correlation between lateral root formation and root waving.

In vertically grown Col-0 seedlings, the root length between two consecutive curve tops (Fig. 1D) was 2740±146 μm ($n=35$). Because under our growth conditions the growth rate of Col-0 is fairly constant and represents 182±11 μm h^{-1} (Beemster et al., 2002), the time to bridge this distance could be calculated to approximately 15 hours.

The wavy growth pattern is the consequence of an alternation between right-turn and left-turn root bending (Rutherford and Masson, 1996). As lateral roots are formed on top of the bends, the wavy growth will result in a left-right alternation of lateral roots and in an equal distribution of laterals over both sides of the root. In vertically grown 10-DAG-old *Arabidopsis* seedlings ($n=11$), lateral roots (including primordia) were indeed distributed equally at both sides (49.6% left and 50.4% right) (Fig. 1B,E), with 66% of the roots in a strict left-right alternating sequence. This result is in agreement with previous analyses in tomato, another species with lateral roots positioned on two longitudinal rows (Newson et al., 1993).

The agravitropic *aux1* mutant (Bennett et al., 1996) lacks the normal wavy growth pattern. Instead of the left-right bending found in wild-type roots, *aux1* roots mainly bent constitutively to the right, with a right-handed root coiling as a consequence (Fig. 1F). In 10-DAG-old *aux1* roots ($n=18$), lateral roots predominantly appeared on the outer (left) side of the coiling root (69.7% left and 30.3% right; Fig. 1E). This uneven positioning of lateral roots resulted in a clear deviation from left-right alternation in 66% of the successive lateral roots investigated, representing a significantly higher percentage than was found in wild-type roots as determined by a Student's *t*-test ($P<0.001$).

Both lateral root initiation and gravitropic response depend on AUX1-facilitated auxin transport (Casimiro et al., 2001; Swarup et al., 2005), so we asked whether lateral root initiation might be controlled by local activity of AUX1. Targeted expression of *AUX1* to the lateral root cap and epidermal tissues of *aux1* roots fully restores the *aux1* agravitropic defect (Swarup et al., 2005). Hence, we analyzed whether the same targeted expression of *AUX1*, using a GAL4 driver line (J0951; Fig. 2A) could also restore the lateral root initiation defect of *aux1* (Marchant et al., 2002). Seedlings expressing *UAS:AUX1* under the control of the GAL4 driver line J0951 in an *aux1-22* mutant background (Swarup et al., 2005) were grown for 10 DAG on 1.5% agar at 45° inclination. The number of lateral roots per cm in the *aux1-22* mutant was significantly reduced compared with that of the Col-0 control (Fig. 2B; Table 2). In contrast, targeted expression of *AUX1* to the lateral
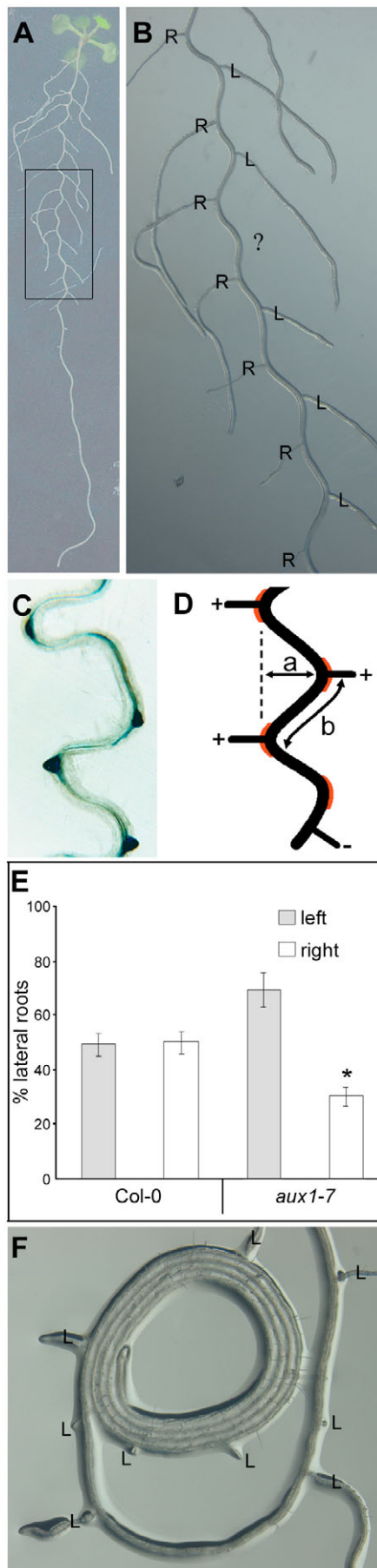
Fig. 1. Correlation between *Arabidopsis* root waving and lateral root positioning. (**A**) Vertically grown Col-0 seedling with wavy growth pattern of the root. (**B**) Detail of boxed area in A showing the left (L) - right (R) alternating lateral roots. ?, wave where lateral root is not apparent. (**C**) Root grown at 45° to enhance waving. Positions of lateral root primordia are marked by the *CYCB1;1::GUS* activity. (**D**) Schematic illustrating the method to determine lateral root positioning/root wave relationship. a, amplitude of the wave; b, root length between the curve tops; the red line marks the top of the wave; +, lateral roots perfectly on top of the wave; –, lateral root at the side of a wave. (**E**) Quantification of left-right alternation of lateral roots in vertically grown seedlings with an equal and dissimilar percentage for wild type (Col-0) and *aux1-7*, respectively. *, statistically significant difference for right side values as determined by Student's *t*-test ($P<0.001$). Error bars, s.e.m. (**F**) Vertically grown agravitropic *aux1-7* seedling root with curled main root. The left (L) position of lateral roots is indicated.

root cap and epidermis of *aux1* restored the lateral root number to that of the wild type (Fig. 2B; Table 2). Furthermore, the left-right alternation in lateral root formation could be rescued in vertically grown J0951>>AUX1,*aux1-22* plants to levels similar to those of the Col-0 control (Fig. 2C; Table 2). Other GFP driver lines restoring AUX1 functioning only in the lateral root cap (M0013) or in stele and columella tissues (J1701) (Swarup et al., 2005) did not complement the lateral root initiation defect in the mutant background, but complementation could be obtained with another lateral root cap and epidermis-specific driver line (Q1220) (see Fig. S2 in the supplementary material). In the absence of an epidermis-specific driver line, we conclude that AUX1 action in lateral root cap and/or epidermal cells influences lateral root initiation and positioning.

The basal meristem exhibits an auxin reporter maximum

The basal meristem has been recently proposed to recycle auxin coming from the root tip via the root cap (Blilou et al., 2005), and the basipetal transport towards this region also involves AUX1 (Swarup et al., 2001; Swarup et al., 2005). Therefore we investigated whether the basal meristem displayed an increased susceptibility for auxin-induced lateral root initiation. Wild-type seedlings (5 DAG) that had already formed a few lateral roots in a left-right alternating fashion were transferred to a high concentration of NAA (10 μM) and incubated for 1 week. Although lateral roots were induced along the entire length of the root, proliferation was especially excessive in the basal meristem (Fig. 3A). The same experiment was repeated with a quiescent center-expressed promoter trap (QC184) (Sabatini et al., 2003). Even though no clear discrete quiescent centers could be visualized in the proliferating cell population in the basal meristem, the marker line had an intensely stained band at the periphery of the structures (Fig. 3B), reflecting some level of apical differentiation and suggesting intensive primordia formation in this part of the root with fused structures as a consequence. We analyzed whether this increased sensitivity was also reflected in the expression of the synthetic auxin-responsive marker *DR5::GUS* (Ulmasov et al., 1997). At 40 HAG, transgenic seedlings were transferred from MS medium to medium containing 10 μM NAA. On MS medium, after 20 minutes of GUS staining, the auxin reporter *DR5::GUS* was detected in two short strands, just above the
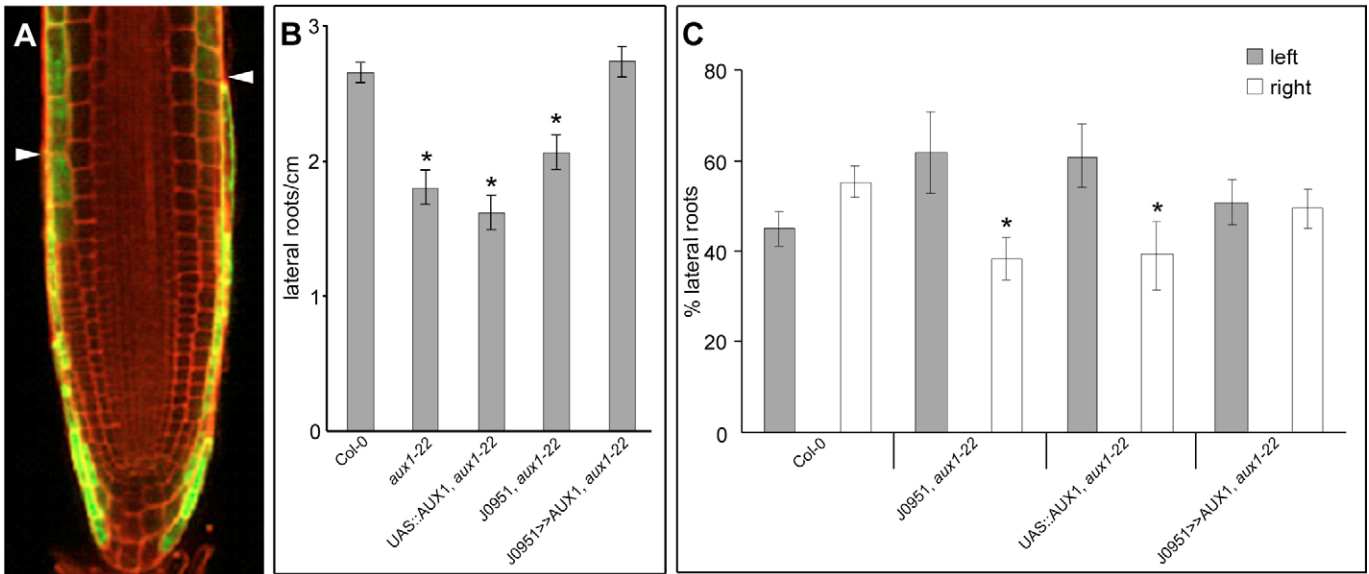
Fig. 2. Role of targeted *AUX1* expression in lateral root formation. (**A**) Root tip of a GAL4-GFP-expressing line (J0951) used for targeted expression of *AUX1* to the lateral root cap and epidermis. Arrowheads mark the end of the root cap. (**B**,**C**) Analysis of lateral root initiation and positioning in wild type (Col-0), *aux1-22*, both parental lines (UAS:AUX1,*aux1-22* and J0951,*aux1-22*), and transactivation line (J0951>>AUX1,*aux1-22*). (B) Lateral root density (seedlings grown at 45°). *, statistically significant differences for values compared with wild type as determined by Student's *t*-test (*P*<0.001). (C) Quantification of left-right alternation of lateral roots in vertically grown seedlings. *, statistically significant difference for right side values compared with wild type as determined by Student's *t*-test (*P*<0.001). Error bars, s.e.m.

meristem (Fig. 3C). After 6 hours of NAA treatment, *DR5::GUS* expression had increased all over the root of the seedlings, but more intensely in the basal meristem (Fig. 3D).

An important regulatory mechanism to obtain the recycling of auxin in the basal meristem is auxin transport (Blilou et al., 2005). Therefore, the *DR5::GUS* reporter line was grown under conditions where auxin transport was blocked with 10 μM NPA. After 72 hours on NPA and subsequent GUS staining, no expression was detected in the basal meristem (Fig. 3E), arguing for auxin transport-dependent expression of *DR5::GUS* in the basal meristem.

A detailed anatomical analysis of *DR5::GUS* stele expression on MS medium revealed that the GUS reporter was restricted to the two protoxylem strands and was absent from the adjacent pericycle cells (Fig. 3F). To confirm this pattern, the expression of a more sensitive auxin-responsive marker, *IAA2::GUS* (Swarup et al., 2001), was analyzed in detail in the basal meristem of 72-HAG-old seedlings grown in the presence or absence of the auxin transport inhibitor NPA. *IAA2* had been shown previously to be expressed in the root meristem and in both protoxylem poles (Swarup et al., 2001). In our analysis of the basal meristem, *IAA2::GUS* was expressed in the central cylinder at and around the phloem and xylem poles. Strong staining, in agreement with the radial pattern of *DR5::GUS* expression could be observed in the protoxylem cells

of *IAA2::GUS* lines (Fig. 3G). When seedlings were grown on NPA, staining was equal, although weaker, in the entire central cylinder (Fig. 3H) in contrast to the pattern observed when grown without NPA.

A recurrent auxin signal in the basal meristem controls regular longitudinal lateral root initiation

The above lines of evidence suggested that the basal meristem might represent a site of auxin accumulation distinct from the distal auxin maximum in the quiescent center and surrounding cells (Sabatini et al., 1999). To elaborate on its potential significance for lateral root initiation, we monitored spatial and temporal expression patterns of the *DR5::GUS* reporter line in the basal meristem. Starting from 10 HAG, seedlings were harvested every 7.5 hours and subsequently stained for GUS activity. Temporal changes were observed in the staining pattern of the GUS-positive strands in the stele of the basal meristem. The recorded temporal variations revealed an oscillating *DR5::GUS* expression pattern in the basal meristem with an interval of approximately 15 hours (Fig. 4A; Table 3). Over the entire time course we obtained two populations of seedlings, one with a high and one with a low percentage of strong *DR5::GUS* staining.

Table 2. Involvement of AUX1 in lateral root initiation and positioning

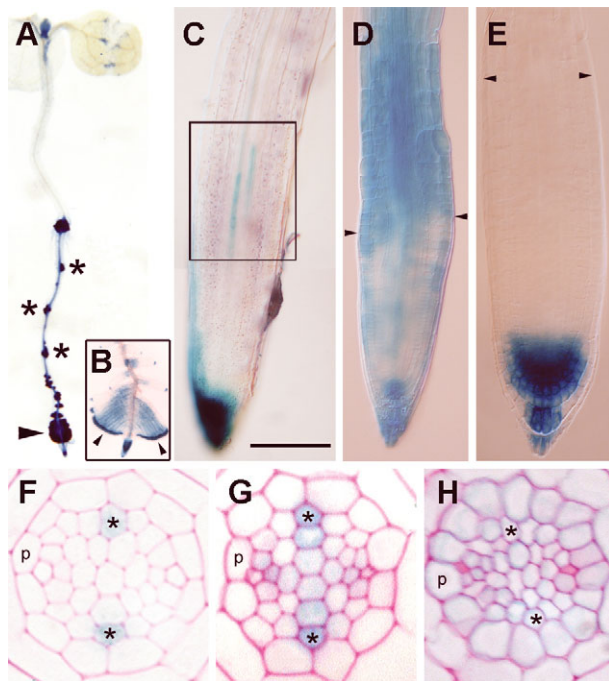|	Lateral root density		Lateral root positioning		
	n	Lateral roots (cm)	*n*	Left (%)	Right (%)
Col-0	32	2.7±0.1	25	44.7	55.3
aux1-22	32	1.8±0.1	n.d.		
J0951,*aux1-22*	32	2.1±0.1	11	61.7	38.3
UAS:AUX1,*aux1-22*	31	1.6±0.1	15	60.9	39.1
J0951>>AUX1,*aux1-22*	32	2.7±0.1	23	50.8	49.2

n.d., not determined.

Fig. 3. Responsiveness of the basal meristem to auxin and specificity of DR5 auxin reporter maximum. (**A**) Transfer from MS medium to 10 μM NAA: induction of lateral roots all over the root (*, visualized using CYCB1;1::GUS) and emergence of a big cluster in the basal meristem (arrowhead). (**B**) Quiescent center marker line (QC184) demonstrating differentiation in fused lateral root primordia (arrowheads). (**C-E**) *DR5::GUS* expression in apical part of root grown on MS medium for 40 HAG (C); transferred from MS medium to 10 μM NAA for 6 hours (D); and grown for 72 HAG on NPA (E). Scale bar: 100 μm. The boxed area in C indicates *DR5::GUS* expression in the basal meristem. Arrowheads mark the end of the root cap and start of the basal meristem. (**F-H**) Transverse sections through the basal meristem of seedling roots expressing *DR5::GUS* (F) and *IAA2::GUS* (G,H) grown on MS for 40 HAG (F,G) and on NPA for 72 HAG (H). *, protoxylem cells; p, pericycle layer.

Having determined the timing of the *DR5* activity in the basal meristem, we designed a toner ink labeling experiment to assess the possible correlation between lateral root initiation and the observed DR5 activity. As the size of the root apical meristem is increasing during early seedling growth (Beemster and Baskin, 1998) we observed a basipetal shift of the *DR5::GUS* expression in the later time points compared with the early ones. To be able to label the correct zone of the root tips where DR5 activity is expected to occur, the distances between the root tip, including the root cap, to the start of the DR5::GUS staining were measured at the time points with high level of expression (Table 3). Taking these distances into account, toner ink particles were positioned on the roots of seedlings of the *CYCB1;1::GUS* reporter line, as described in Materials and methods. After the seedlings had been labeled, they were allowed to grow for another 30 hours, sufficient for development of at least one lateral root initiation site (Dhooge et al., 1999), and subsequently stained histochemically for GUS (Fig. 4B). By assuming that the auxin signal in this part of the root that corresponds to the basal meristem triggers lateral root initiation, the label that remained attached to the epidermis after 30 hours of growth would be expected to colocalize with an early lateral root initiation site (Fig. 4B,C). Indeed, toner particles were detected at the position of a lateral root
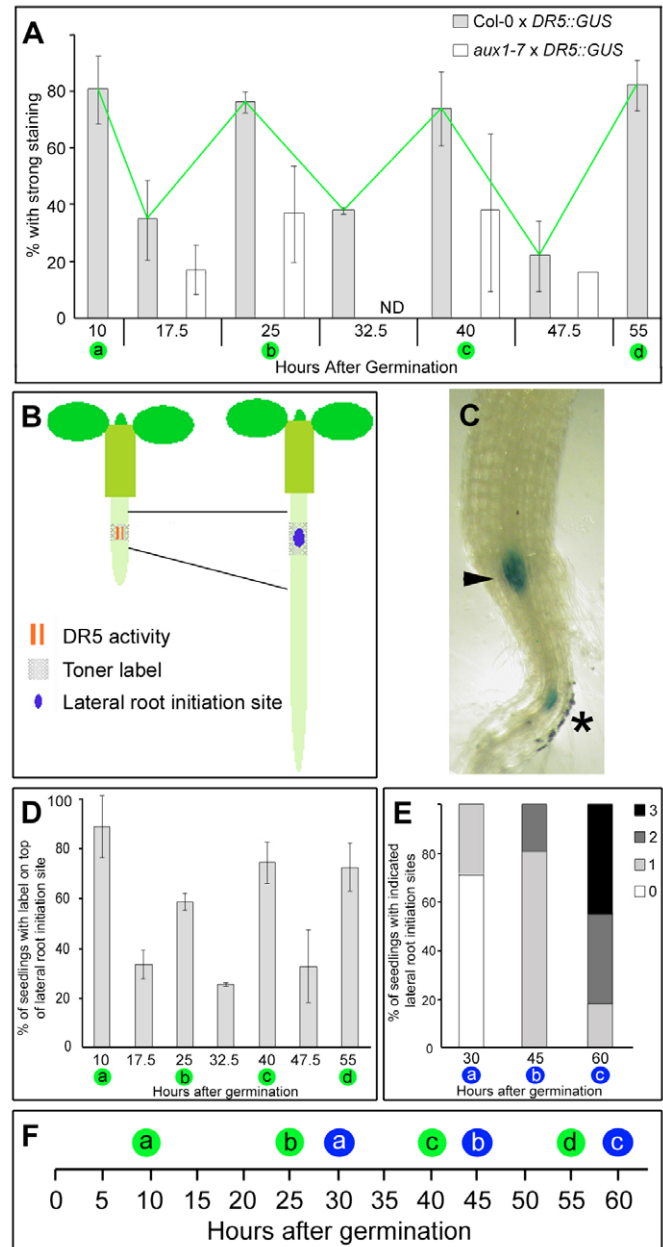

Fig. 4. Regular lateral root spacing and oscillation in DR5::GUS activity in the basal meristem. (**A**) *DR5::GUS*-activity in a 7.5-hour time course in Col-0 (gray) and *aux1-7* (white) expressed as percentage of seedlings with strong GUS staining at each time point. The green line highlights the oscillation in DR5 activity in Col-0. Labels a-d (green circles) mark the time points with high DR5 activity. ND, not determined. (**B**) Schematic representation of toner label experiment. *CYCB1;1::GUS* seedlings were labeled with toner particles (left) and after 30 hours stained for GUS (right). (**C**) Detail of seedling root with toner particles attached on top of the lateral root initiation site (*). Arrowhead, adventitious root. (**D**) Percentage of seedlings with toner label on top of lateral root initiation site. Labels a-d (green circles) mark the time points with higher percentage of seedlings having toner label on top of lateral root initiation site. (**E**) Percentage of seedlings with 0-3 lateral root primordia at 30, 45 and 60 HAG. Labels a-c (blue circles) indicate time points at which the next lateral root appears at the earliest. (**F**) Timeline indicating the intervals between four successive maxima of DR5 activity (green circles) and the periodicity of initiation of the first three lateral roots after germination (blue circles). The 15-hour periodicity in the *DR5::GUS* expression matches the timing of lateral root initiation.

Table 3. Oscillating *DR5* activity in the basal meristem correlates with lateral root initiation

	Seedlings with DR5::GUS-positive strands			Distance from root tip, including the root cap, to the start of DR5::GUS staining		Seedlings with toner particles on top of the LRI site after labeling region with DR5::GUS-positive strands		
	n			*n*		*n*		
HAG	Exp. 1	Exp. 2	%	*n*	Distance (mm)	Exp. 1	Exp. 2	%
10.0	56	10	80±12	52	0.121±0.050	12	15	89±13
17.5	51	24	34±20		n.d.	21	15	33±6
25.0	54	76	76±3	44	0.169±0.053	28	13	59±3
32.5	38	31	38±1		n.d.	31	12	26±0
40.0	34	50	74±13	19	0.198±0.063	18	21	74±8
47.5	91	30	21±12		n.d.	34	24	33±15
55.0	85	22	82±8	21	0.221±0.046	44	36	73±10

HAG, hours after germination; n.d., not determined; Exp. 1/Exp. 2, number of individuals for two independent experiments; LRI, lateral root initiation.

in a high percentage of the cases for 10, 25, 40 and 55 HAG (Fig. 4D; Table 3). At 17.5, 32.5 and 47.5 HAG, in-between two predicted auxin signals and, hence, with low probability to detect the auxin signal, the percentage of the lateral root initiation sites that correlated perfectly with the toner particles was strongly reduced (Fig. 4D; Table 3). In conclusion, significant colocalization between toner particles and lateral root primordia could be obtained by labeling the basal meristem at the time when we recorded the *DR5::GUS* expression.

The correlation between the recurrent *DR5::GUS* expression in the basal meristem and the initiation of lateral roots implies an oscillating auxin response driving the process of lateral root initiation. To evaluate whether a similar oscillation was present in the formation of lateral roots, the timing of the initiation of consecutive lateral roots was determined. A time-series experiment with Col-0 seedlings containing the *CYCB1;1::GUS* marker was performed from 10 HAG until 60 HAG and samples were taken and stained for GUS every 5 hours. Up to 25 HAG, no initiation site could be detected. At 30 HAG, 71% of the seedlings contained only one lateral root initiation event (*n*=43). Seedlings with two lateral root initiation sites appeared at the earliest at 45 HAG in 20% of the analyzed population (*n*=31), and three lateral root initiation sites were observed at the earliest at 60 HAG (*n*=11; Fig. 4E). This order of sequence fits well with a period of 15 hours between the initiations of consecutive lateral roots and is in agreement with the periodicity found with the DR5 activity in the basal meristem. In this respect, the first auxin signal at 10 HAG might correspond to the first primordium at 30 HAG, the second auxin signal at 25 HAG to the second primordium at 45 HAG, and so on (Fig. 4F). Based on these data, a time period of 20 hours between the auxin signal in the basal meristem and the corresponding lateral root initiation event can be deduced.

By assuming that the auxin response and lateral root initiation are correlated, the recorded periodicity would imply a regular longitudinal spacing of lateral roots. In Col-0 seedlings (10 DAG, *n*=37) the distances between two neighboring lateral roots or emerged primordia, irrespective of their radial position were measured. The distance between consecutive primordia was relatively constant along the primary root, namely 2225±35 μm (*n*=856).

Having emphasized the AUX1-dependency of lateral root spacing (see above), we analyzed whether the recorded oscillations in the *DR5::GUS* expression pattern were affected in the *aux1* mutant. Indeed, the number of *aux1* seedlings with *DR5::GUS* expression in the basal meristem was severely reduced at all time points investigated (Fig. 4A).

Priming xylem pole pericycle cells for lateral root initiation depends on intact auxin response but is independent of IAA14

In *Arabidopsis*, pericycle founder cell identity and, hence, lateral root initiation are limited to the pericycle cells opposite the xylem pole (Casimiro et al., 2003). However, the presented data suggested that the auxin response was stronger in the protoxylem elements than in the adjacent pericycle cells.

To show that auxin response in the xylem pole pericycle cells from the basal meristem onward is required for lateral root initiation, this response was selectively disrupted by regional expression of a dominant mutant version of the IAA17 protein (*axr3-1*) (Leyser et al., 1996). An UAS:*axr3-1* construct was expressed under the control of the GAL4 driver line J0121 that is active in the main root xylem pole pericycle from the basal meristem onward (Fig. 5A) (Laplaze et al., 2005). Detailed analysis of the lateral root number revealed a strong reduction in J0121>>UAS:*axr3-1* compared with the controls (Fig. 5B; Table 4). A microscopic analysis (*n*=10) showed that the number of lateral roots was reduced clearly in all developmental stages [as defined by Malamy and Benfey (Malamy and Benfey, 1997)] and that lateral roots rarely developed beyond stage I (Fig. 5C). Interestingly, pericycle cells with nuclei displaced toward each other could be observed in J0121>>UAS:*axr3-1* (Fig. 5D). Longitudinal sections of four segments (with 5 mm intervals) in the

Table 4. Effects of abolished auxin response in xylem pole pericycle cells during lateral root initiation

	Lateral root density		Lateral root stages		
	n	Lateral roots (cm)	*n*	LRI events	Displaced nuclei
Col-0	33	3.60±0.08	n.d.		
J0121	32	3.07±0.06	5	11.0±1.4	0.4±0.2
UAS:*axr3-1*	33	3.73±0.09	n.d.		
J0121>>UAS:*axr3-1*	33	0.04±0.02	5	10.6±1.7	6.80±1.6

n.d., not determined; LRI, lateral root initiation.
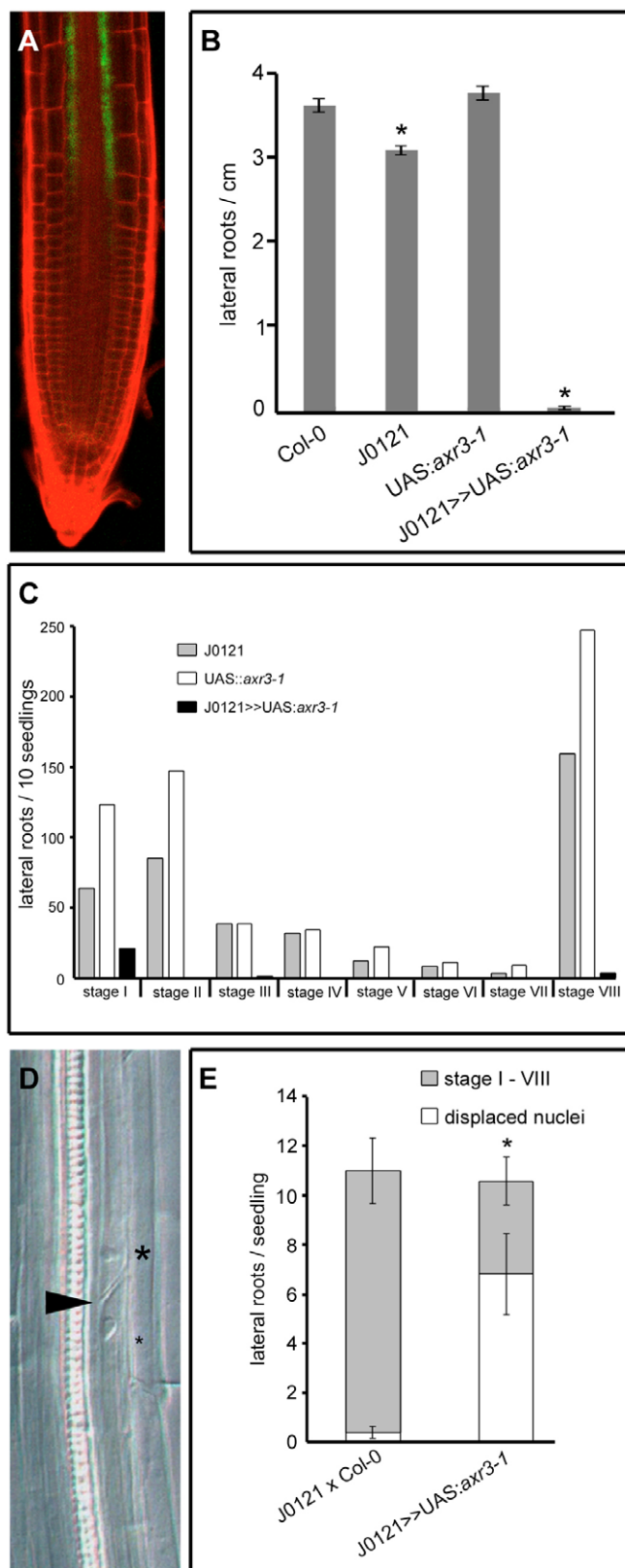
Fig. 5. AXR3 dependency for asymmetric cell division in the xylem pole pericycle. (**A**) J0121 with specific expression in the xylem pole pericycle starting from the basal meristem onward. (**B-E**) Analysis of lateral root formation in wild-type control (Col-0), the parental lines (J0121 and *UAS:axr3-1*), and the transactivation line (J0121>>*UAS:axr3-1*). (B) Lateral root densities. *, statistically significant differences for values compared with wild type as determined by Student's *t*-test (*P*<0.001). (C) Lateral root stages [total number of detected primordia from ten individual roots at each developmental stage according to Malamy and Benfey (Malamy and Benfey, 1997)]. (D) Differential interference contrast image of adjacent pericycle cells with migrated nuclei in J0121>>*UAS:axr3-1*. *, nuclei; arrowhead, cell wall. (E) Average number of lateral roots per seedling at stage I-VIII (gray) and arrested at the stage of displaced nuclei (white). *, statistically significant difference for values for displaced nuclei as determined by Student's *t*-test (*P*<0.02). Error bars, s.e.m.

The strong reduction in the number of lateral roots in J0121>>UAS:*axr3-1* is in agreement with recent data obtained with xylem pole pericycle-specific expression of a stabilized form of IAA14 also resulting in the absence of lateral root initiation (Fukaki et al., 2005). IAA14 (SOLITARY ROOT, SLR) has been shown to play a prominent role in lateral root initiation (Fukaki et al., 2002; Fukaki et al., 2005; Vanneste et al., 2005). Although *IAA14* is expressed in the pericycle and during lateral root initiation (Fukaki et al., 2002) expression analysis of *IAA14::GUS* revealed that *IAA14* is not expressed in the basal meristem (Fig. 6A). Therefore, auxin response in the xylem pole pericycle cells in the basal meristem is most likely still intact in the *slr-1* mutant and initial priming of pericycle cells might still occur. To demonstrate this possibility we made use of plants expressing the stabilized mutant *mIAA14* under the native *IAA14* promoter controlled by an inducible system with the glucocorticoid receptor (*pIAA14::mIAA14-GR*) (Fukaki et al., 2005). This allows the evaluation of a temporal effect of the mutated IAA14 protein on the initiation of lateral roots, as release from medium containing Dex to medium without Dex restores the wild-type IAA14 functioning. We asked more specifically whether pericycle cells that passed through the basal meristem in plants having a stabilized mutant *mIAA14*, were still competent to initiate lateral roots when the wild-type IAA14 was restored. As reported previously, 10-DAG-old seedlings expressing *pIAA14::mIAA14-GR* in the presence of Dex did not form lateral roots (Fukaki et al., 2005). Subsequently, seedlings (10 DAG) grown in the presence of Dex were transferred to media with and without Dex. Seedlings that continued to grow on Dex had no lateral roots (Fig. 6B), but *pIAA14::mIAA14-GR* seedlings that had been transferred onto Dex-free media formed lateral roots (Fig. 6C). Interestingly, also in the distal part of the region previously subjected to Dex (8.3±0.7 mm, *n*=10), roots could be formed whereas this was not the case in the more proximal regions of the roots where the inhibition appeared to be permanent (Fig. 6C).

DISCUSSION
Auxin accumulation in the basal meristem is essential for lateral root initiation

Both lateral root initiation and gravitropic response rely on unimpaired auxin transport and redistribution (Casimiro et al., 2003; Swarup et al., 2005). Here, we demonstrate that the processes for lateral root initiation and gravitropic response are intertwined and
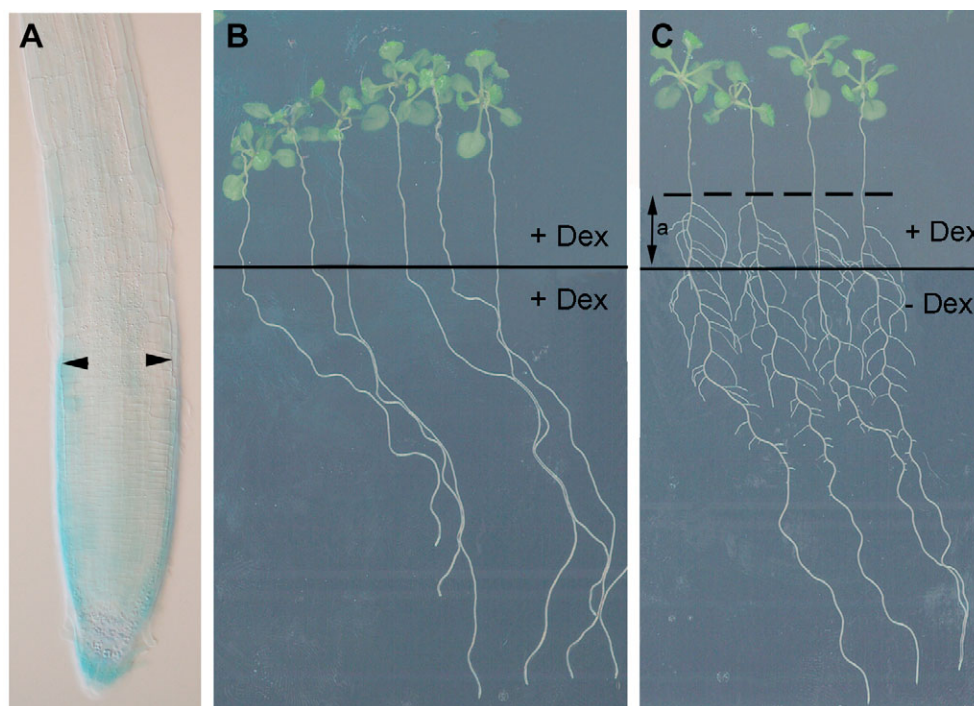
region just above the root meristem demonstrated that both J0121 and J0121>>UAS:*axr3-1* revealed the same frequency of lateral root initiation events per seedling, but the portion of displaced nuclei per seedling in J0121 was significantly lower than that in J0121>>UAS:*axr3-1* (Fig. 5E; Table 4).

Fig. 6. Involvement of IAA14/SLR in lateral root initiation above the basal meristem. (**A**) *IAA14::GUS* expression in the root tip limited to the root cap. Arrowheads point to the edge of the root cap. (**B,C**) Analysis of *pIAA14:mIAA14-GR* seedlings upon transfer from media with Dex to media with (B) and without (C) Dex. Above the black line, lateral roots are formed in the region previously subjected to Dex after transfer to Dex-free medium (C), whereas this is not the case after transfer to Dex medium (B). The region 'a' in C between solid and broken line is the distal part of the root that can form lateral roots after transfer to Dex-free medium.

operate in the same zone of the root tip: gravitropic response-mediated waving of the primary root is correlated with the formation of lateral root primordia. As a consequence, lateral root development displays a left-right alternating pattern, which is disturbed in *aux1* mutants. Several mutations that simultaneously affect lateral root initiation and gravitropic response corroborate this connection (Hobbie and Estelle, 1995; Muday et al., 1995; Simmons et al., 1995; Marchant et al., 2002; Benková et al., 2003; Lin and Wang, 2005).

A temporal delay exists between the fastest auxin responsive gene expression and the initial divisions (Himanen et al., 2002; Vanneste et al., 2005) that could hitherto not be explained. Our new data support the physiological relevance of this time lag. The initial auxin signal transduction takes place in the basal meristem already at 10 HAG, subsequently priming divisions of founder cells will occur at late time stages higher up in the root. Recently, the basal meristem has been shown to recycle auxin channeling back from the root tip through the root cap (Blilou et al., 2005; Leyser, 2005). Redistribution of this recycled pool might generate an auxin response in the basal meristem as visualized by the *DR5::GUS* reporter, which also revealed the existence of an auxin maximum in columella initials (Sabatini et al., 1999). Fundamental changes in cell fate, cell division plane, and cell polarity have been observed when this root tip auxin maximum is disturbed. Although we could not demonstrate directly the existence of a second concentration maximum in the basal meristem, it is tempting to speculate that auxin accumulation itself primes the initiation of a new lateral organ. The occurrence of an auxin maximum in the basal meristem fits with the auxin signaling center defined in the basal part of the elongation zone based on genome-wide expression profiles in the root (Birnbaum et al., 2003; Beeckman, 2004) and is in agreement with the previously proposed inductive signal (hormonal or environmental) that affects founder cell formation and/or division of pericycle cells close to the root tip (Barlow and Adam, 1988; Dubrovsky et al., 2001; Dubrovsky et al., 2006).

At the anatomical level the *DR5::GUS* staining is restricted in the basal meristem to the two protoxylem cell files neighboring the pericycle cells (Fig. 7A). This peculiar radial staining pattern is easily disturbed in the presence of NPA (Fig. 7B), a treatment known to inhibit lateral root initiation (Casimiro et al., 2001). We hypothesize that a radial gradient with a maximum in the protoxylem cells might be required for lateral root initiation to take place. However, this interpretation is still very speculative and only based on the radial expression pattern of GUS markers. Further studies of the radial auxin distribution patterns and mechanisms in the basal meristem are required to support this hypothesis.

A recurrent auxin signal in the basal meristem controls longitudinal lateral root distribution

We demonstrated that an auxin response reporter in the basal meristem shows rhythmic expression with the same periodicity as lateral root initiation. This phasing of approximately 15 hours is in agreement with the temporal window between the initiation of two successive lateral roots as was recently calculated for *Arabidopsis* by Dubrovsky et al. (Dubrovsky et al., 2006). The recurrence of the auxin signal may be caused (at least in part) from periodic gravitropism-induced fluctuations in auxin redistribution within the root apex. The existence of an auxin signal in the basal meristem stresses the importance of the root tip for the regulation of root branching and supports the idea that the auxin pool in the root tip drives the initial stages of lateral root primordia formation (Bhalerao et al., 2002). As lateral roots are almost never found in opposite positions, the appearance of the auxin signal simultaneously at both protoxylem poles (Fig. 7A) necessitates an attenuation determining the left-right positioning of lateral roots. How this attenuation is brought about is not known.

Auxin-dependent signaling in the basal meristem presumably represents the very first checkpoint toward lateral root initiation (Fig. 7C,D). It cannot be neglected that other auxin sources, such as shoot-derived auxin, play a role in later steps of lateral root formation (Reed et al., 1998; Bhalerao et al., 2002), for instance in triggering the asymmetric division and further primordium development.

Fig. 7. Hypothetical model for auxin signaling in the basal meristem and subsequent lateral root initiation. (**A**,**B**) Possible presence of an auxin (response) gradient based on the staining patterns of auxin reporters. In accordance with the auxin gradient in the quiescent center and surrounding initials, the lateral root initiation 'stem cells' are triggered by the neighboring xylem cell showing high auxin concentration and/or response (A). When this gradient disappears (as is the case of auxin transport inhibition), auxin is distributed equally (even into the pericycle) and no lateral root initiation can take place (B). Light to dark blue shades reflect low to high auxin content. Xylem pole pericycle cells that will initiate a lateral root are in red. (**C**) Scheme of two adjacent pericycle cells on the same cell file undergoing early developmental steps prior to lateral root initiation: from priming by auxin in the basal meristem (associated with migration of nuclei as indicated with the dotted arrows) to the auxin response required for asymmetric cell division. (**D**) Hypothetical scheme showing the longitudinal progression of pericycle cells in time and space in the main root with the indication of the major developmental steps toward lateral root initiation. First, auxin (blue arrows) is targeted to the basal meristem (BM) from the root apical meristem (RAM) via AUX1-mediated transport in the lateral root cap (dark gray). Subsequently, reflux (PIN-mediated) is presumably involved in generating the auxin maximum in the protoxylem cells (blue strands) adjacent to the pericycle cells (green). Later, in the differentiation zone, the primed pericycle cells (light blue) are exposed again to auxin response and signaling mechanisms (Aux/IAAs, for instance IAA14/SLR) before *CYCB1;1* becomes expressed and the actual division occurs (light-blue cells with dark-blue center)
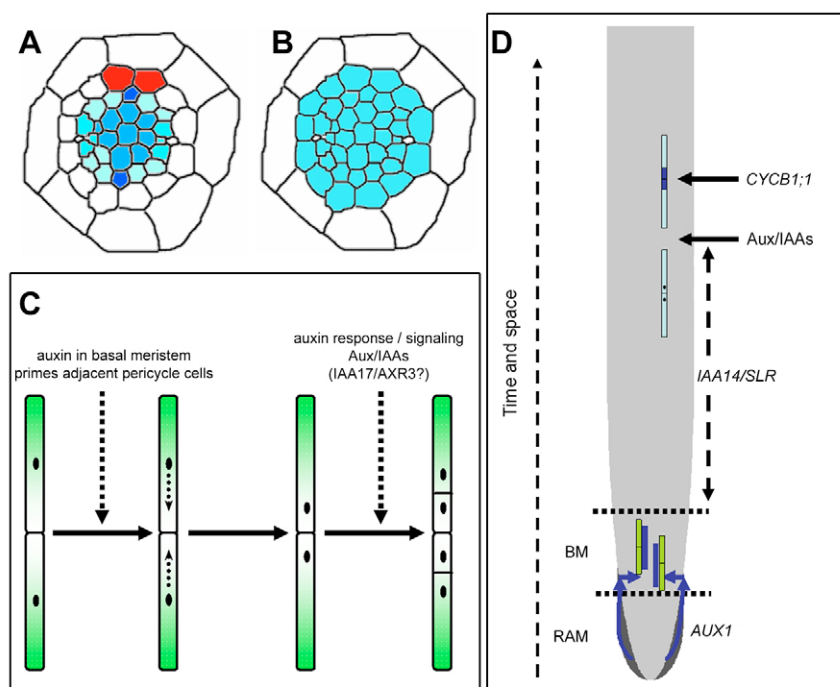
Auxin response of xylem pole pericycle cells in the basal meristem required for determination of founder cell identity is independent of IAA14/SLR

Lateral roots were nearly totally absent when auxin response in the xylem pole pericycle cells was abolished by specific expression of a stabilized form of IAA17 (AXR3). Microscopic inspection of such roots revealed a pre-mitotic stage of *Arabidopsis* lateral root initiation that has, until now, only occasionally been reported (Casero et al., 1993; Barlow et al., 2004). Just prior to the asymmetric cell division, the nuclei of two neighboring pericycle cells migrate to the common anticlinal cell wall (Fig. 7C). In wild-type roots, this process is probably rapidly followed by the division event, explaining the lack of reports on this stage in the literature.

In lateral root initiation, a crucial role has been assigned to IAA14 (Fukaki et al., 2002; Fukaki et al., 2005; Vanneste et al., 2005). Xylem pole pericycle-specific expression of a stabilized form of IAA14, which is closely related to IAA17, can repress lateral root formation (Fukaki et al., 2005). Our data suggest that IAA14 control of lateral root initiation acts downstream of auxin signaling in the basal meristem and is not required for the priming of the founder cells. First, *IAA14* is not expressed in the basal meristem. Second, releasing pericycle cells from the repression of a stabilized form of IAA14 (*mIAA14*) results in the formation of lateral roots even in a region of the root that was earlier subjected to *mIAA14*. We conclude that expressing the stabilized form of IAA14 from its native promoter does not interfere with the very early phases of lateral root initiation and that priming of the pericycle cells could take place in the absence of normal IAA14 functioning.

In Fig. 7D a model is proposed that illustrates the possible spatiotemporal events that occur in the root tip prior to lateral root initiation. Our results suggest that via AUX1 action in lateral root cap and/or epidermal cells, the pericycle cells in the basal meristem

might be primed through an IAA14-independent pathway (Fig. 7D). Next, in more proximal regions of the root, an IAA14-dependent auxin response is required for initiation of cell division of pericylce cells as can be visualized by the expression of *CYCB1;1*. It is likely that IAA14 is not the only Aux/IAA protein involved in this process but further functional characterization of other Aux/IAA proteins such as IAA17 during lateral root initiation is required.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/4/681/DC1

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