PLANT DEVELOPMENT

Cyclic programmed cell death stimulates hormone signaling and root development in Arabidopsis

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The plant root cap, surrounding the very tip of the growing root, perceives and transmits environmental signals to the inner root tissues. In Arabidopsis thaliana, auxin released by the root cap contributes to the regular spacing of lateral organs along the primary root axis. Here, we show that the periodicity of lateral organ induction is driven by recurrent programmed cell death at the most distal edge of the root cap. We suggest that synchronous bursts of cell death in lateral root cap cells release pulses of auxin to surrounding root tissues, establishing the pattern for lateral root formation. The dynamics of root cap turnover may therefore coordinate primary root growth with root branching in order to optimize the uptake of water and nutrients from the soil.

The root cap is the outermost tissue covering the root tip and represents a major root-rhizosphere interaction site (1–3). It is commonly recognized as a protective tissue for the meristematic cells of the root apex and as a sensory organ that perceives environmental signals such as gravity, water, and nutrients to direct root growth (4–6). Although it persists during the life span of roots, it is subjected to a regeneration process in which new cell layers are continuously produced internally while superficial cell layers are regularly sloughed off. In Arabidopsis, the root cap consists of a central columella and peripheral lateral root cap cells (7). Programmed cell death (PCD) of lateral root cap cells occurs when they reach the onset of the elongation zone (8, 9) (fig. S1A). This region is also designated as the oscillation zone because it displays massive oscillations in gene expression (10). These oscillations periodically define the prebranch sites, which may further develop as lateral roots (10). Root cap–specific conversion of the auxin precursor indole-3-butyric acid (IBA) into indole-3-acetic acid (IAA) creates a local auxin source that is essential for the oscillating transcriptional mechanism, which installs the regular spacing of lateral roots (11, 12).

Analysis of the transcriptional auxin signaling output reporter DR5rev::VENUS-N7 (13) by means of stereomicroscopy revealed a striped DR5 pattern in the most distal lateral root cap cells, a pattern that could also be observed for the root cap–expressed early-stage PCD marker pPASPA3::H2A-GFP (GFP, green fluorescent protein) (Fig. 1A and B, and fig. S1) (8). In vivo time-lapse imaging of vertically growing roots showed that the most distal stripe of DR5 expression faded out every ~4 hours (Fig. 1C; fig. S2, A and B; and movie S1). When tracing back the site of origin of lateral root primordia (n = 96 primordia) (Fig. 1C), we found that all primordia were initiated at positions where a distal DR5 stripe had vanished. Furthermore, the disappearance of the DR5 signal from the lateral root cap preceded the DR5::Luciferase maximum in the oscillation zone (fig. S3) and occurred with a similar periodicity (fig. S2B). By rotating the roots by 135°, the orientation of root growth is corrected toward the gravity vector, and a bend is formed. During the reorientation, the period of DR5 oscillations in the oscillation zone is transiently shortened, and lateral root formation is stimulated (10, 14–16). Likewise, the period between successive losses of DR5 stripes was also shortened from ~4 to ~2 hours (fig. S2, C and D, and movie S2). Altogether, these results show that the disappearance of the DR5 signal from the lateral root cap, the DR5 oscillations in the oscillation zone, and the formation of lateral root primordia are temporally and spatially interconnected.

The longitudinal extent of the lateral root cap is developmentally restricted by induction of PCD in the most distal lateral root cap cells (8), raising the possibility that the periodic disappearance of the DR5 signal coincides with PCD in the lateral root cap. Consistently, pPASPA3::H2A-GFP showed a striped pattern in the lateral root cap (Fig. 1B). Moreover, coexpression of the DR5rev::VENUS-N7 reporter with pPASPA3::NLS::ttTomato revealed overlapping expression in the most distal lateral root cap cells (Fig. 2A and fig. S4A). Time-lapse movies revealed a pattern of ~4 hours (fig. S2B) and spatially relating to the inner root tissues. In

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Supplementary materials

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Materials and Methods

S1 to S18

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Movies S1 to S3

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with sites of new lateral root primordia (movie S3). Moreover, a 135° gravistimulation also transiently decreased the periodicity of disappearance of \(PASPA3\) stripes to ~2 hours (fig. S2D and movie S4). Thus, PCD in the lateral root cap is predictive of lateral root formation.

In \textit{Arabidopsis}, the accurate timing of PCD in the lateral root cap requires the transcription factor SOMBRERO (SMB) (8, 17). \(pSMB\text{-}NLS\)-\textit{GFP} stripes overlapped with \(pPASPA3\text{-}NLS\)-\textit{tdTomato} stripes in the most distal lateral root cap and disappeared every ~4 hours (figs. S2B and S4C). The \textit{smb-3} mutant exhibits delayed PCD of the lateral root cap cells (8, 17) and as a result has an increased number of the lateral root cap cells that ectopically extend into the elongation zone (fig. S5, A and B) (8, 17). In this mutant, the typical stripe-like pattern of \(DR5\) expression had disappeared (Fig. 2B), whereas the signal intensity was reduced and extended into the elongation zone (fig. S5, A and C). Moreover, we observed a more variable periodicity of \(DR5\text{-}Luciferase\) oscillations (fig. S5D), fewer prebranch sites, and lateral roots (fig. S5, E to H). Strong activation of SMB-GR (10 \(\mu M\) Dex-amethasone (Dex)) triggers ectopic formation of tracheary element–like cells and growth arrest of all tissues (17). Over a 2-day treatment with \(\leq 1\) \(\mu M\) Dex, root growth was maintained (fig. S6A) while showing a pronounced and

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**Fig. 1.** Periodic disappearance of lateral root cap–\(DR5\) expression correlates with the sites of lateral root initiation. \(A\) and \(B\) Three-dimensional (3D) projection of confocal z-stacks [propidium iodide (PI) stained, left] and macroview stereo microscope images (right) of, respectively, \(A\) the auxin-responsive marker \(DR5\text{-}VENUS\)-N7 expression and \(B\) the PCD marker \(pPASPA3\text{-}H2A\)-\textit{GFP} in seedling root tips. EZ, elongation zone; MZ, meristematic zone. \(C\) Time-lapse imaging of growing \(DR5\text{-}VENUS\)-N7 roots over a growth period of 18 hours. Red arrows indicate the disappearance of a \(DR5\) stripe in the lateral root cap. White arrow indicates a lateral root initiation site at the site where the \(DR5\) stripe disappeared previously. (Insets) The gradual reduction of \(DR5\) signal intensity in the most distal lateral root cap cells is highlighted by the red arrows during the first 3 hours. “g” and associated arrow indicate the gravitational vector. Scale bars, 100 \(\mu m\).
specific PCD in the lateral root cap cells (fig. S6B). Additionally, these roots lacked $DR5$ stripes (Fig. 2C) and $DR5$:Luciferase oscillations (Fig. 2, D and E, and movie S5), and the numbers of prebranch sites and lateral roots were reduced, respectively, by 79.4 and 87.5% at 0.3 μM Dex (Fig. 2F and fig. S6, A and C). When plants were transferred back to control medium, the newly formed root segment re-established normal growth with the production of a new lateral root cap and lateral roots (fig. S6, D to F). In contrast, the part of the root that was formed during Dex treatment remained devoid of lateral roots (fig. S6, D and E). These results indicate that the controlled and recurrent PCD of the lateral root cap cells is the driving factor for gene expression oscillations in the oscillation zone and subsequent lateral root induction.

Oscillations are modulated by a local auxin source in the root cap, derived from the auxin precursor IBA (11, 12). Moreover, genetic ablation of the lateral root cap cells repressed the capacity to produce extra lateral roots in response to exogenous IBA application in Dex-treated $35S$:SMB-GR (fig. S6G). Therefore, we asked whether the auxin response that we observed in the root cap itself could be required for lateral root patterning. We conditionally repressed the auxin response in the lateral root cap cells through activation of a stabilized allele of the auxin response repressor IAA17/AXR3 ($p$SMB:axr3-1-GR) (5, 18). Dex treatment resulted in agravitropic root growth (fig. S7A) and loss of $DR5$ expression in the lateral root cap cells (fig. S7, B and C), but this did not alter the PCD process (fig. S7D) and did not affect the lateral root number (fig. S7E). Constitutive transactivation of $UAS$:axr3-1 in the lateral root cap only slightly reduced lateral root formation, whereas transactivation of $UAS$:axr3-1 in xylem pole pericycle cells blocked lateral root formation (fig. S7F) (19). Therefore, a transcriptional auxin response in the lateral root cap itself is not a decisive factor for lateral root patterning.

**Fig. 3. Predicted auxin distributions and dynamics in the root tip.** (A) 3D projection of confocal z-stacks of a R2D2 root tip; costained with PI, imaged over time. (Middle and right) A close-up of the yellow boxed region at left over an 80-min time frame. White arrows indicate the disappearance of yellow fluorescent protein (YFP) signals in epidermal cells, and blue arrows indicate the appearance of PI-stained nuclei in lateral root cap cells, as a late-stage PCD marker. At positions where R2D2 signals in the epidermis changed at earlier time points (movie S6). (B) Predicted steady-state distribution of auxin within a 3D axisymmetric cellular geometry, for wild type, and in the situations with defects in IBA-to-IAA conversion, AUX1-mediated influx, and polar carrier-mediated efflux, respectively. Auxin concentrations are color-coded according to the rainbow scale to the right. (C and D) Predicted auxin dynamics in the (C) epidermal cells and (D) stele cells underlying the most distal LRC cells after PCD. Results show the auxin concentrations relative to that at time (t) = 0, taken as an average of the four epidermal cells (marked in red) or stele cells (marked in yellow) highlighted in (B). Scale bars, 50 μm.

**Fig. 4. Auxin flux carriers facilitate auxin transport from the lateral root cap into the oscillation zone.** (A) $DR5$:VENUS-N7 expression in 3-day-old Col-0, ibr1ibr3ibr10 and aux1 mutants in control conditions, in macroview stereomicroscope images. White arrows indicate the nuclear $DR5$ signal in lateral root cap cells. (B) Quantification of $DR5$:VENUS-N7 signal in lateral root cap, prebranch site number per root, and lateral root density in Col-0, ibr1ibr3ibr10, and aux1 seedlings ($P < 0.05$ by one-way analysis of variance and Tukey’s test as post hoc analysis, $n > 10$ seedlings). (C and D) $DR5$:VENUS-N7 expression and quantification in 3-day-old Col-0 germinated on 10 μM NPA, 0.3 μM BUM, and 3 μM BZ-IAA expanded $DR5$ expression in WT compared with Col-0 in (A). (E) Quantification of prebranch site number in 5-day-old $DR5$:Luciferase seedlings treated for 2 days with 10 μM NPA, 0.3 μM BUM, and 3 μM BZ-IAA. The prebranch sites formed in the newly grown primary root after transfer were measured. *$P < 0.05$ and **$P < 0.01$ compared with Mock-treatment Col-0 in (A) by Student’s t test ($n > 30$ seedlings). Data are means ± SD. Scale bars, 200 μm.
Alternatively, auxin transport from the root cap to the root proper could be the connecting element for the oscillatory behavior in gene expression in the elongation zone. Consistently, time-lapse analyses of the semiquantitative auxin input reporter R2D2 (20) revealed a marked increase in auxin levels in epidermal cells, before loss of cellular integrity of adjacent lateral root cap cells (Fig. 3A and movie S6). This suggests that auxin released from lateral root cap cells during a late stage of PCD is efficiently taken up by the abutting epidermal cells. To understand how this could result in auxin signaling in stele cells of the oscillation zone, we adopted an in silico auxin-transport model (27) to simulate the auxin dynamics in the root apex (further details are available in the supplementary materials). Simulating the PCD of distal lateral root cap cells, under the assumption that PCD leads to a release of auxin into the surrounding apoplast, generated a transient auxin peak in stele cells in the elongation zone (Fig. 3, B to D; fig. S8; and movie S6). We further tested the hypothesis of lateral root cap cells (Fig. 3A and movie S6). This suggests that auxin released from lateral root cap cells closely associated with the elongation zone (Fig. 3, B to D; fig. S11, A and B). Moreover, PCD in the most distal lateral root cap cells was closely associated with increased auxin in the underlying epidermal cells (Fig. 3A and movie S6). We further tested the contribution of auxin transport within the lateral root cap by means of tissue-specific complementation of the aux1 mutant. In agreement with model predictions (fig. S9, C to E), transactivation of AUX1 in the root cap rescued the defect in lateral root formation and agranuloropic growth of aux1 mutants (fig. S9, F and G). Thus, auxin transported within the (lateral) root cap allows the root cap to communicate with the elongation zone for establishing sites for lateral roots to develop. This process ensures that IBA-derivived auxin can be transported toward the oscillation zone. The auxin-transport topologies in our model also include carrier-mediated efflux and apoplastic diffusion. In the presence of influx carriers and auxin production, simulations lacking carrier-mediated efflux failed to generate an auxin transient in the elongation zone but generated an auxin accumulation in the lateral root cap (Fig. 3, B to D; fig. S8; and movie S7). In the model, diffusion rates were positively correlated with the strength of the auxin peak in the stele. However, apoplastic diffusion could not compensate for a lack in auxin efflux in our simulations (fig. S10). To attempt to identify the components of this auxin transport machinery, we analyzed pin2 and pin2 abc1 abc19 mutants. Although these mutants are severely defective in shootward auxin transport and gravitropism, similar to aux1 (22), they did not show defects in lateral root formation, nor did they have a reduced sensitivity to IBA (fig. S11, A to D), raising the possibility that this reflux model requires the global features of the PIN and ABCB localization for directing auxin into the oscillation zone (25, 24). We could find further evidence by using three chemically unrelated auxin transport inhibitors—1-N-naphthylphthalamic acid (NPA), 2-[4-(diethylamino)-2-hydroxybenzoyl]benzoic acid (BUM), and benzoxyl-IAA (BZ-IAA)—that target mainly ABCB-type transporters (NPA and BUM) (22, 25) or generally interfere with AUX1-, PIN-, and ABCB-based auxin transport (BZ-IAA) (26). Consistent with our simulations, treatments with any of these inhibitors preserved the occurrence of PCD in the lateral root cap (fig. S11E) but resulted in ectopic DR5 activity in the lateral root cap and epidermis (Fig. 4, C and D; fig. S11, F and G; and movie S9), as well as impaired DR5:Luciferase oscillations (movie S9) and lateral root formation (Fig. 4E and fig. S11, H and I), corroborating the auxin reflux model (27). Although we could not completely resolve the molecular mechanism for auxin efflux at present, our data underscore the necessity of auxin transport in the coordination of PCD in the most distal lateral root cap cells with oscillatory gene expression in the oscillation zone for lateral root spacing (fig. S12).

During the exploration of the soil, root tips sense, through the root cap, the nutrient and water status of the soil they are traversing, as well as obstacles they may encounter (6, 28). Transduction of that information may serve to control the periodicity of programmed cell death, thus altering the frequency of lateral root development. In this way, root systems may adjust development according to the quality of the soils they are passing through.

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Supplementary Text

S1 to S12

Table S1

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Movies S1 to S9

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