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Organ regeneration does not require a functional stem cell niche in plants

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Abstract

Plants rely on the maintenance of stem cell niches at their apices for the continuous growth of roots and shoots. However, while the developmental plasticity of plant cells has been demonstrated1, it is not known whether the stem cell niche is required for organogenesis. Here we explore the capacity of a broad range of differentiating cells to regenerate an organ without the activity of a stem cell niche. Using a root-tip regeneration system in *Arabidopsis* to track the molecular and functional recovery of cell fates, we show that re-specification of lost cell identities begins within hours of excision and that the function of specialized cells is restored within one day. Critically, regeneration proceeds in plants with mutations that fail to maintain the stem cell niche. These results show that stem cell-like properties that mediate complete organ regeneration are dispersed in plant meristems and are not restricted to niches, which nonetheless appear necessary for indeterminate growth. This regenerative reprogramming of an entire organ without transition to a stereotypical stem cell environment has intriguing parallels to recent reports of induced transdifferentiation of specific cell types in the adult organs of animals2,3.

The indeterminate growth of plant organs arises from the activity of a localized stem cell niche, a micro-environment that supports stem cells4,5. In the plant root, longitudinal cell files converge on a stem cell niche comprised of a set of initials (stem cells) that are maintained in an undifferentiated state by contact with the quiescent centre (QC), a group of cells with low mitotic activity (Fig. 1a). A newly formed QC is detected early after root-tip excision in pea and maize, and after QC laser ablation in *Arabidopsis*, which is consistent with the role of the niche as a pattern reorganizer in regeneration6–8. However, is the

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Author Contributions G.S. performed all experiments with help from X.W. and J.H.L on dissections and microarrays. H.H. generated *PLT* reporter constructs. K.D.B. and G.S. conceived the project, planned all experiments, performed data analysis, and wrote the manuscript.

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reconstitution of the stem cell niche the basis for the plant's high capacity to regenerate? Alternatively, can a wider population of cells exhibit stem cell-like properties regenerating an organ independently of an actively dividing stem cell niche? Here, we address the requirement for stem cell niche activity as a pattern organizer for organ regeneration.

To develop a comprehensive analysis of regeneration, we adapted root-tip excision techniques utilized in maize and pea6,7 to *Arabidopsis,* enabling the examination of regeneration with high resolution using confocal imaging of cell-identity marker lines and well characterized mutants with meristematic defects. In combination, we used cell type-specific transcriptional profiles generated previously9–11 in order to track cell identities from microarray analysis of regenerating root tissue at specific time points after excision.

We performed standard excisions at 130 μm from the root tip resulting in the complete removal of QC, all surrounding stem cells along with several tiers of daughter cells, and the root cap, including all of the columella and most of the lateral root cap (Fig. 1a; Methods). The standard excisions were made in a zone of proliferative cells that already express cell-specific markers9. No hormones or exogenous treatments were applied. Competence to regenerate extended to at least 200 μm from the QC, with the frequency of regeneration dropping sharply at the proximal end of meristematic zone, indicating an extended region of regeneration competence in the root tip (Fig. 1b).

Cell divisions during regeneration occurred in all major tissues comprising the root tip, as shown by analysis of a cell-cycle marker in five cell types or tissues (Fig. 1c and Supplementary Table 1, n=12 roots). In addition, none of the fate-specific markers that we tracked by time-lapse imaging showed expanded expression patterns that could correlate with tissue-specific proliferation (Fig. 1d,e and Supplementary Fig. 1,2). Cell division was required, since inhibition of the cell cycle prevented regeneration (Supplementary Fig. 3). However, re-patterning during regeneration did not appear to follow a stereotypical sequence of cell divisions, as in embryogenesis or lateral root formation. Taken together, these observations suggest that the meristematic zone as a whole, and not any specific tissue or cell type within it, contributes to root-tip regeneration.

To resolve the early timing of cell identity reappearance, we compared global transcriptional analysis of regenerating stumps with an existing library of cell type-specific transcriptional profiles9–11. We sampled stumps for microarray analysis at 0 h, 5 h, 13 h, 22 h, and 7 days after initial tip excision at 130 μm (Methods). Using cell type-specific transcriptional analyses of the root, we identified sets of markers that were highly enriched in specific cell types and analyzed their activity during regeneration (Supplementary Table 2, Methods). This technique permitted a highly sensitive measure of cell identity since early and late differentiation stage markers could be tracked given about 100 markers for each cell type (Fig. 1f). This global analysis of cell fate showed that molecular recovery of the excised cell identities had begun within five hours after cutting (Fig. 1f). For columella, the percent recovery of enriched markers increased steadily compared to the stump at 0 h, reaching 21% at 5 h, 32% at 13 h and 55% at 22 h ($q<5\%$, Methods), with demonstrated columella differentiation regulators, such as *Auxin Response Factor 10*, induced at these early stages12. About 22% of QC identity recovered at 5 h ($q<5\%$) without any further increase at

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13 h and 22 h (q<5%). Thus, we can track the ordered re-establishment of cell identity, which shows the rapid re-specification of lost cell fates and identifies new candidate regulators for specification of cell identity (Supplementary Table 2). These results do not rule out that some QC-specific genes may play a critical role in early regeneration but they raise the question of whether differentiated cell types can be restored before the cell stem niche becomes functional.

We established the precise timing of the functional recovery of a completely excised cell type by focusing on columella cells, which reside at the tip of the root. In intact roots, differentiated columella cells accumulate starch within amyloplast organelles, a process required for root gravitropism13,14. By one day post cut (dpc), Lugol staining confirmed *de novo* starch accumulation above the cut site (Fig. 2a and Supplementary Fig. 4). More intense staining was observed at 2 dpc (Fig. 2a and Supplementary Fig. 4). To test for recovery of columella function, we subjected regenerating roots to a standard gravitropism assay by reorienting them perpendicularly to the gravity vector and scoring response over time. All wild type roots show a clear gravitropic response within 12 hours. While cut roots did not respond to gravity in the first 12 hours after excision when cut at 130 μm, 13.8% of the cut roots exhibited a clear gravitropic response at 1 dpc, 55.4% at 2 dpc and 89.2% at 3 dpc (n=65, for all time points). However, the QC-specific marker *WOX5* was either ectopically expressed in the endodermal file or, at times, expressed in differentiated columella cells at 1 dpc (Fig. 2a and Supplementary Fig. 4). Thus, as early as one day after complete columella excision, a new set of cells were expressing columella markers and performing columella specific functions while the morphology of the stem cell niche had not yet recovered.

Given the early re-establishment of a differentiated cell type, we tested the requirement for functional stem cells by using mutants in which post-embryonic root growth ceases due to the failure to maintain the stem cell niche. The *PLETHORA* (*PLT*) gene family has been shown to be critical for root formation15 with the double mutant *plt1plt2* showing differentiation of stem cells at 3 days post germination5 (dpg), as verified under our conditions (Fig. 2b, note the lack of stem cell layer between QC and starch-stained columella). The uncut double mutant root has abnormal tip and stem cell niche morphology but normal gravitropism and convergent longitudinal cell files5. Surprisingly, *plt1plt2* roots cut at 4 dpg quickly regenerated by re-establishing the U-shaped convergent pattern of longitudinal cell files at the tip (Fig. 2c,e and Supplementary Fig. 5a). Moreover, starch granules accumulated in the regenerating double mutants (Fig. 2d) and gravitropic response was re-established (Fig. 2c and Supplementary Fig. 6), indicating that functional columella cells were re-specified during regeneration. Similarly, *scarecrow* (*scr*) mutants, which fail to maintain root stem cell function through a pathway independent of *PLT1* and *PLT2*5,16,17, were also able to restore their pre-cut pattern, starch staining, and gravitropism (Fig. 2b–d,f and Supplementary Fig. 5b,6). *PLT1/2* are expressed early in regeneration in wild type roots (Supplementary Fig. 7). However, using microarray comparison of *plt1plt2* mutant and wild type roots, we ruled out that *PLT1/2*-dependent genes were induced by alternative mechanisms in regenerating double mutants (Supplementary Fig. 8). We note that a lower percentage of *plt1plt2* and *scr* mutants regenerated compared to wild type roots (Fig. 2c),

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which we hypothesize is due to the documented effect of both mutants in reducing cell divisions in the meristematic zone[15,17], the pool of cells recruited for regeneration. Together, these results show that stem cell niche activity is not necessary for early root-tip regeneration and they imply the existence of an independent mechanism for cell-specification and patterning in the meristematic region.

Several results suggest that auxin, which has been shown to position the root stem cell niche and to form a potentially instructive concentration gradient[18,19], may be a critical component of the mechanism that coordinates organogenesis[20]. First, roots failed to regenerate beyond the earliest stages when we blocked auxin transport during regeneration using NPA (Supplementary Fig. 9). Second, auxin efflux carriers and an auxin-responsive reporter re-established their excised domains at the root tip within a day of their excision (Fig. 3a–d). Third, many but not all genes induced in the first 24 h after excision have been shown previously to respond to auxin (Supplementary Table 2).

If organ regeneration does not require the activity of a stem cell niche, we hypothesized that other determinate organs should be capable of regeneration after excision. We developed a set of markers to distinguish competent *vs*. non competent tissue using transcriptional data on root developmental zones and a time-course induction of pluripotent callus from mature tissue[21] (Supplementary Methods). Intriguingly, many of these markers showed high expression in young *Arabidopsis* leaves (9 days), compared to older leaves (15 and 22 days)[22] (Fig. 4a), indicating that young but not old leaves may be competent to regenerate, as suggested by historic reports[23]. Consistent with this prediction, we observed leaf regeneration in *Arabidopsis* after excising half of the leaf perpendicular to its midvein, in leaves corresponding to young stages (33.3%, n=27) but never in leaves corresponding to older stages (n=10, Fig. 4b,c). These observations suggest that the competence to re-pattern complex tissues may be a feature of many differentiating plant cells that share a common set of molecular properties.

What distinguishes these regeneration-competent cells from the stereotypical stem cells of the niche? In the *Arabidopsis* root, a body of work has shown that the stem cell niche is critical for indeterminate growth[5,8,17], which was not restored during regeneration in the *plt1plt2* and *scr* mutants. This suggests that continuous growth may be a unique feature of the stem cell niche while organogenesis is not.

The convergence of organ patterning and growth at the stem cell niche of *Arabidopsis* has made it difficult to separate these two fundamental processes. Taken together, our results separate a widely dispersed capacity for pluripotency and patterning during organogenesis from the narrowly located capacity for indeterminate growth within the stem cell niche. The extension of stem cell-like properties that mediate organogenesis into maturing tissues may predispose the plant for a high capacity to regenerate. Recent work has shown that adult mammalian cells may also be induced to directly switch fates without stem cell intermediates[2,3]. Plants and perhaps other highly regenerative organisms appear to be able to reprogram entire organs in this way. These findings provide a new basis to search for mechanisms that coordinate organogenesis independently of a central organizer.

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METHODS SUMMARY

Mutant alleles used were *plt1–4 plt2–2* (Ws) and *scr-4* (Ws). Seedlings at 4 dpg were excised by hand under a dissecting microscope using a 30G sterile dental needle (ExelInt). The frequency of regeneration was defined as the fraction of the plants that showed root regeneration at 6 dpc, measured by gravitropic response and confirmed by tip morphology. For microarray analysis, 130 μm of root tips were removed to instigate regeneration and then, during tip regeneration, 70 μm of regenerating stumps were manually dissected at indicated time points.

Further methods are included in the Supplementary Information.

METHODS

Plant Material and Root Excisions

Mutant alleles used were *plt1–4 plt2–2* (Ws) and *scr-4* (Ws). Seedlings at 4 dpg were excised by hand under a dissecting microscope using a 30G sterile dental needle (ExelInt). The frequency of regeneration was defined as the fraction of the plants that showed root regeneration at 6 dpc, measured by gravitropic response and confirmed by tip morphology. For microarray analysis, 130 μm of root tips were removed to instigate regeneration and then, during tip regeneration, 70 μm of regenerating stumps were manually dissected at indicated time points.

Microarray and Statistical Analysis

Microarray profiles were normalized using the MAS 5.0 method with a target intensity of 250. Cell type-specific markers sets were generated by identifying transcripts whose signal was significantly enriched in a given cell type compared to all other cell types, using Significance Analysis of Microarrays (SAM) with a false discovery rate (q) cut-off < 5%[24] and a two-fold enrichment cut off. To increase stringency for cell specificity and assure no overlap between columella and QC markers, we also required a two-fold enrichment in columella average signal over the average signal in each of the other cell types of the root-tip (for example, columella markers were two-fold enriched over QC and lateral root cap, respectively). The same procedure was followed for QC markers, ensuring a two-fold enrichment over columella and lateral root cap. In addition, the root-tip specific cell types also needed to show a two-fold enrichment in root tip over proximal meristem expression.

For analysis of percent columella and QC identity recovery, ranked gene expression was tested for a significant fit to modelled expression patterns representing an increase in expression at either 5 h, 13 h, or 22 h using the quantitative test in SAM (q < 5%). For example, genes that increase significantly at the 5 h regeneration time point fit the pattern 1 2 2 2, where 1 represents expression of replicates at time 0 and 2 represents replicates at the subsequent time points of regeneration (5, 13, and 22 h). The rank method in SAM was used.

For evaluating *PLT* downstream markers, a two-class unpaired test in SAM (q < 5%) was used to find QC markers significantly down-regulated in the *plt1plt2* mutant tips compared

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to wild type tips (termed the *PLT1/2*-dependent set). Subsequent analysis tested whether any members of the *PLT1/2*-dependent set were significantly up-regulated in wild type (WT) stumps at 0 h *vs*. WT stumps at 24 h (testing for early regulation of *PLT1/2*-dependent set in WT) or WT stumps at 0 h *vs. plt1plt2* stumps at 24 h (testing for potential regulation of the *PLT1/2* dependent set in the *plt1plt2* mutant during regeneration, *i.e.*, alternate regulatory mechanisms) using the two-class unpaired test in SAM ($q < 5\%$).

Lists of competence markers for root, callus, and leaves were generated sequentially and the intersection of each set was taken. To generate root competence markers, a two-class unpaired test in SAM was performed to find genes significantly up-regulated in tissue freshly harvested at 130–200 μm (competent zone) *vs*. tissue freshly harvested at 270–340 μm (non-competent zone) with a $q < 5\%$. This procedure yielded 1,538 genes (root competence markers). To identify competence markers in tissue explants undergoing auxin treatment to generate callus, a quantitative analysis in SAM ($q < 5\%$) was used querying for genes that showed a monotonic increase in the callus induction samples over days 0, 2, 4, 7 and 10 on Callus Inducing Media (CIM) with data from previous work21 using the rank method so that replicates for each time point were labelled: 1, 2, 3, 4, 5 respectively (callus competent markers). The intersection of the root and callus competent sets was 647 genes. To identify potential competence markers in leaf, genes significantly up-regulated in 9-day-old leaves *vs.* to 22-day-old leaves22 were determined using a two-class unpaired test in SAM ($q < 5\%$) (leaf competent markers). The intersection of the root, callus, and leaf competent marker sets was 209 genes.

To find all genes that were significantly regulated in regenerating stumps 5 h after tip-cutting, we used a two-class unpaired test in SAM ($q < 5\%$) comparing replicates in regenerating tips at 0 h *vs*. 5 h. We found the intersection of that list and the list of auxin-induced genes25 to generate the list of genes induced at 5 h after tip cutting that were also induced by auxin. Among the genes that were differentially regulated in root stumps in the first five hours after cutting (n=182, Supplementary Table 2), 22 have been shown to respond to auxin25.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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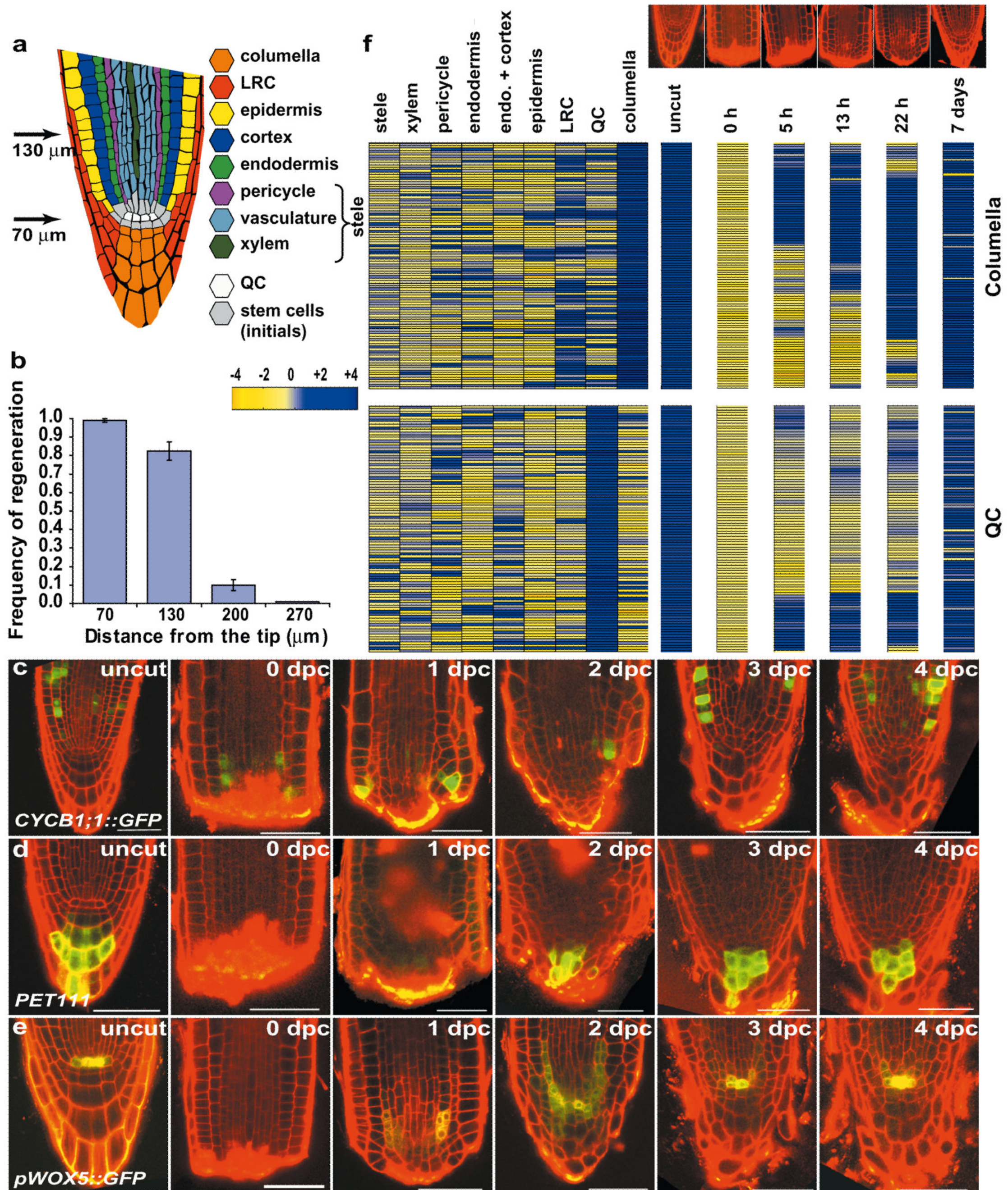

Figure 1. Root-tip regeneration and cell fate re-specification in wild type

a, Schematic of *Arabidopsis* root apical meristem with QC (70 μm) and standard excision point (130 μm) positions. **b**, Regeneration frequency in wild type (Col-0); n = 102 (70 μm), 57 (130 μm), 111 (200 μm), 32 (270 μm); error bars, standard error of the proportion (s.e.p.). **c–e**, Confocal time-lapse of single regenerating roots in *CYCB1;1::GFP* (**c**), the columella marker *PET111* (**d**) and the QC marker *pWOX5::GFP(ER)* (**e**), at consecutive days post cut (dpc); bars, 50 μm. **f**, Expression levels of columella-enriched (n=103, top panels) and QC-enriched (n=95, bottom panels) transcripts during regeneration; at left, expression in cell

types of uncut roots[9–11]; at right, expression in uncut tips or regenerating stumps at the time points indicated.

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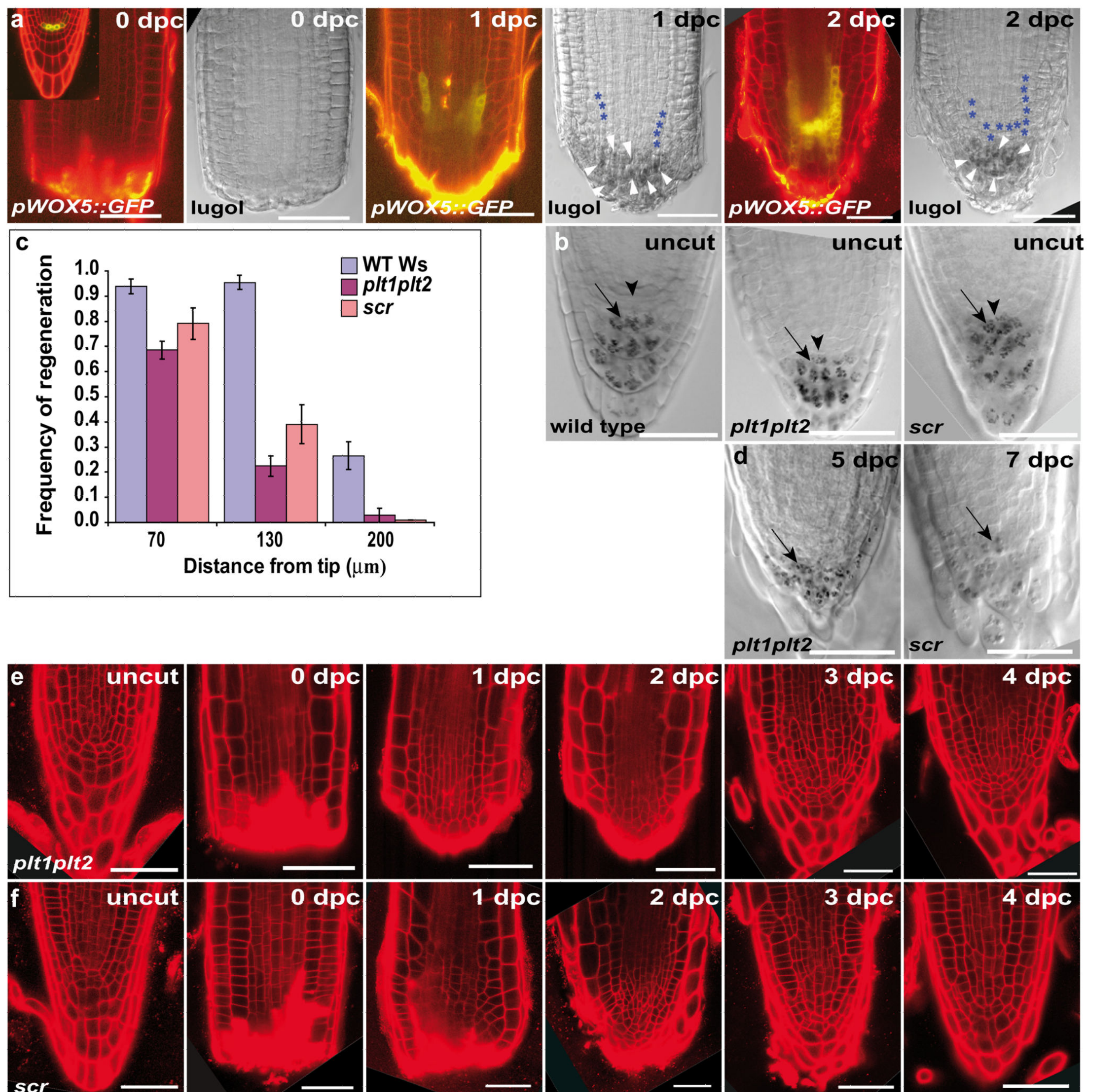

Figure 2. Columella starch staining and root-tip regeneration in stem cell mutants

a, Simultaneous confocal/DIC imaging of regenerating roots expressing *pWOX5::GFP(ER)*; inset, uncut root tip; blue asterisks locate GFP-expressing cells as shown in the corresponding confocal image; white arrowheads indicate starch staining cells; bars, 50 µm. **b**,**d**, Lugol staining of uncut (**b**) and regenerating roots (**d**) in wild type, *plt1plt2* and *scr*; arrowheads indicate position of QC, arrows indicate starch staining cells; bars, 50 µm. **c**, Frequency of regeneration in wild type (Ws), *scr* and *plt1plt2* mutants; wild type, n = 66, 65 64; *scr,* n = 43, 41, 23; *plt1plt2*, n = 162, 107, 35, at 70,130 and 200 µm, respectively; error

bars, s.e.p. **e**,**f**, Confocal time-lapse of regenerating roots in *plt1plt2* (**e**) and *scr* (**f**); bars, 50 μm.

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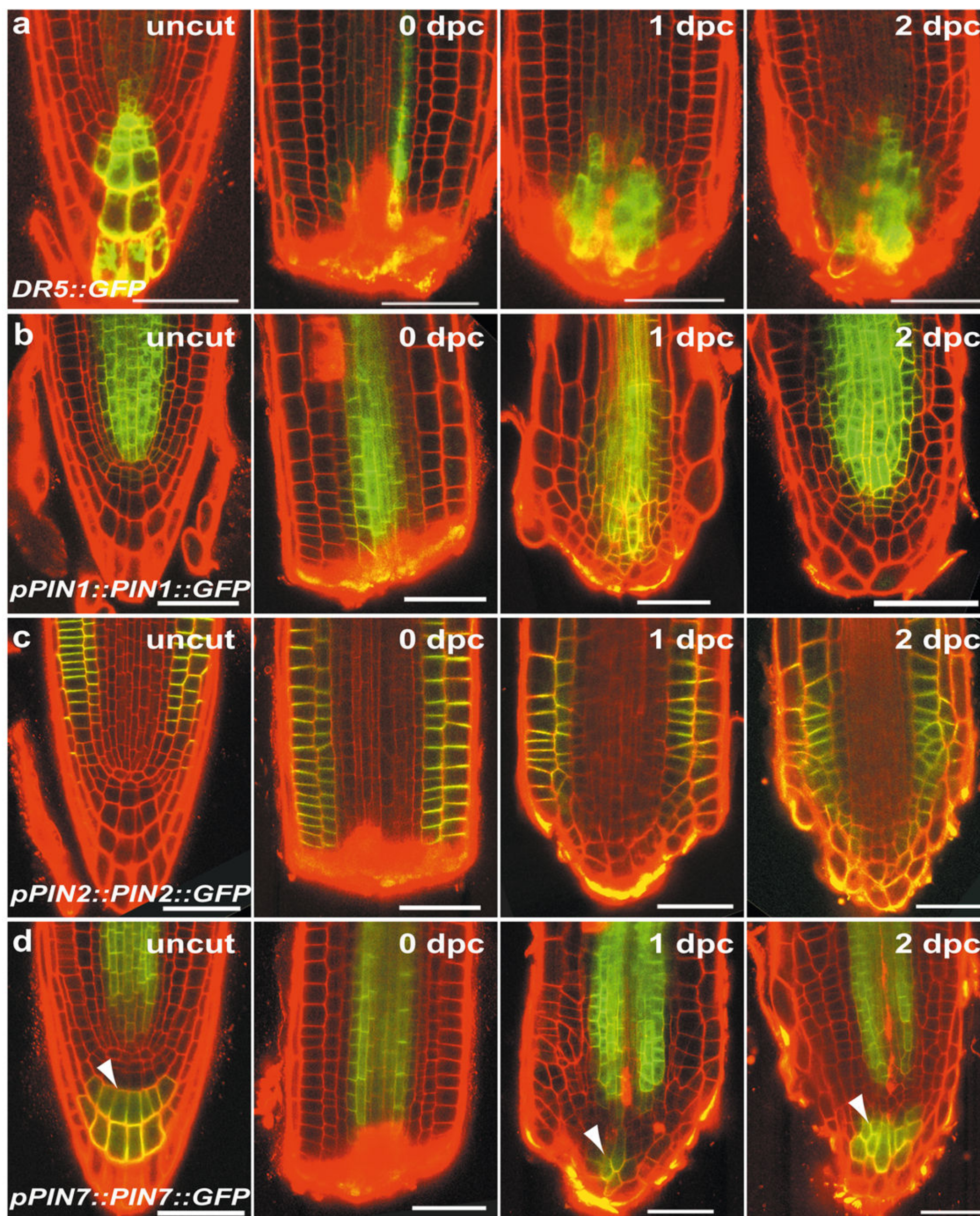

Figure 3. Early auxin distribution in the regenerating root tip

a–d, Confocal time-lapse of single regenerating roots expressing the auxin-responsive reporter *DR5::GFP(ER)* (**a**) and translational fusions of the auxin efflux carriers *pPIN1::PIN1::GFP* (**b**), *pPIN2::PIN2::GFP* (**c**), *pPIN7::PIN7::GFP* (**d**), at consecutive dpc; arrowheads in (**d**) indicate the restoration of distal expression domains, bars, 50 μm.

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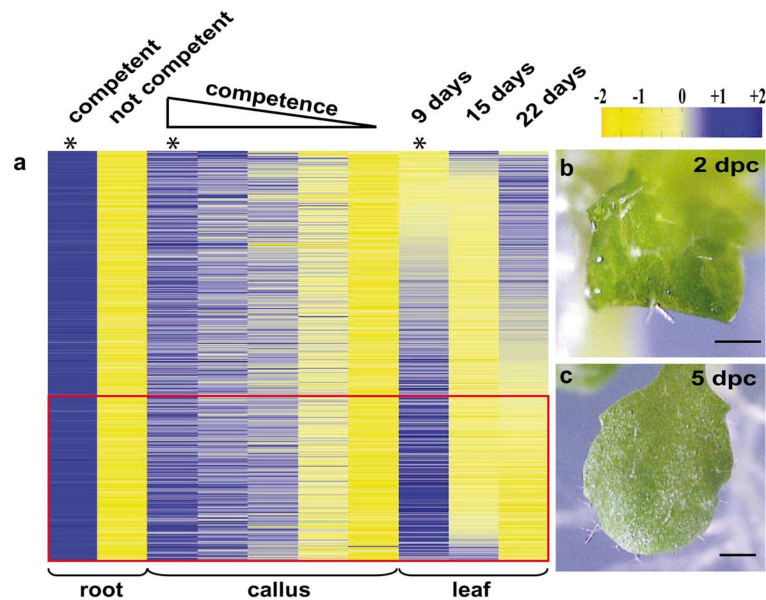

Figure 4. Regeneration competence markers and leaf regeneration

a, Expression levels of regeneration competence markers identified by enrichment in competent tissue in root and callus21 (0, 2, 4, 7 and 10-day-old tissue treated with auxin, n=647, see Methods), showing high expression of competence in young but not older leaf tissue. The red box indicates genes in the root/callus competent marker set that also matched a putative competence profile for young leaves (209 genes, Supplementary Table 1 and Methods)22. Asterisks indicate states of highest competence. **b**,**c** Regenerating single 4 day-old leaf, at 2 (**b**) and 5 (**c**) dpc; bars 50 μm.