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LETTERS

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

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Plants rely on the maintenance of stem cell niches at their apices for the continuous growth of roots and shoots. However, although the developmental plasticity of plant cells has been demonstrated[1], 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 thaliana* 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 seem to be 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 animals[2,3].

The indeterminate growth of plant organs arises from the activity of a localized stem cell niche, a micro-environment that supports stem cells[4,5]. 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, a group of cells with low mitotic activity (Fig. 1a). A newly formed quiescent centre is detected early after root-tip excision in pea and maize, and after quiescent centre laser ablation in *Arabidopsis*, which is consistent with the role of the niche as a pattern reorganizer in regeneration[6–8]. However, is the reconstitution of the stem cell niche the basis for the plant's high capacity to regenerate? Alternatively, can a wider population of cells have 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 used in maize and pea[6,7] for use in *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 previously[9–11] 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 quiescent centre, 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 markers[9]. No hormones or exogenous treatments were applied. Competence to regenerate extended to at least 200 μm from the root tip, with the frequency of regeneration dropping sharply at the proximal end of the meristematic zone, indicating an extended region of regeneration competence in the root tip (Fig. 1b).

Cell divisions during regeneration occurred in all major tissues constituting 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 Figs 1 and 2). Cell division was required, because inhibition of the cell cycle prevented regeneration (Supplementary Fig. 3). However, re-patterning during regeneration did not seem to follow a stereotypical sequence of cell divisions, as in embryogenesis or lateral root formation. Taken together, these observations indicate 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 profiles[9–11]. 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 analysed their activity during regeneration (Supplementary Table 2 and Methods). This technique permitted a highly sensitive measure of cell identity because early and late differentiation stage markers could be tracked using 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 percentage 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 (false discovery rate, $q < 5\%$, Methods), with demonstrated columella differentiation regulators, such as *AUXIN RESPONSE FACTOR 10*, induced at these early stages[12]. About 22% of quiescent centre identity recovered by 5 h ($q < 5\%$) without any further increase at 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 quiescent-centre-specific genes may have a critical role in early regeneration, but they raise the question of whether differentiated cell types can be restored before the stem cell 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

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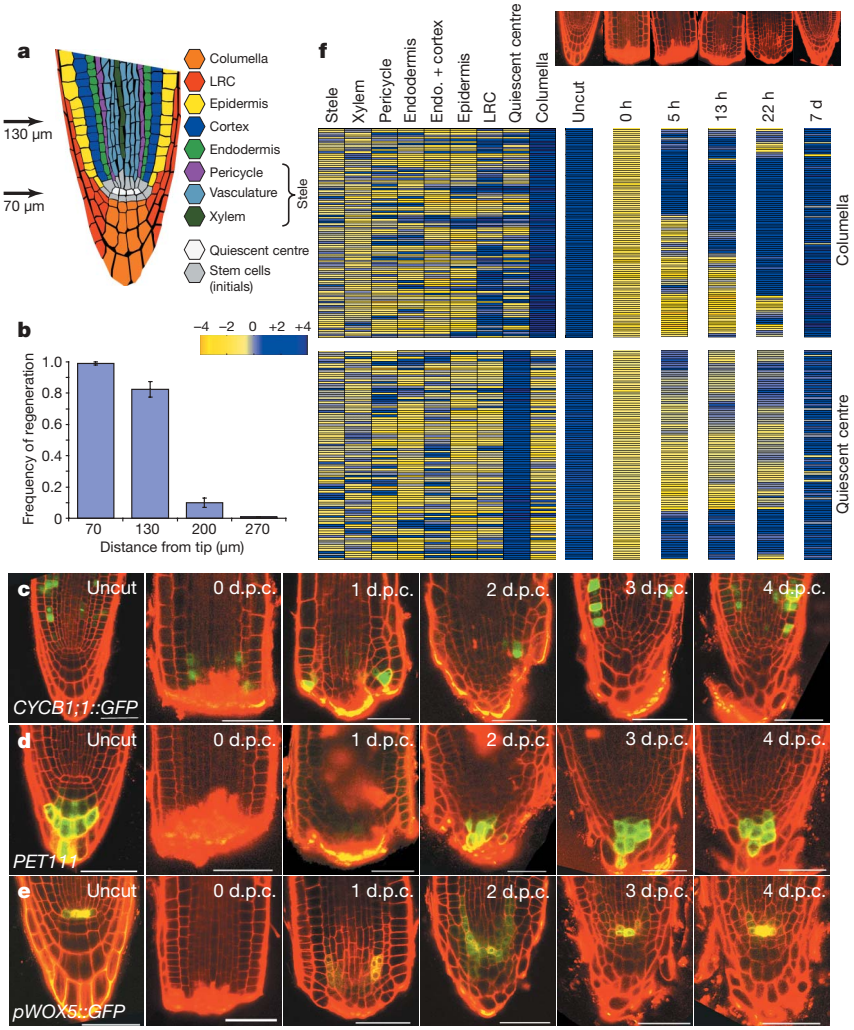

Figure 1 | Root-tip regeneration and cell fate re-specification in wild type. **a**, Schematic of *Arabidopsis* root apical meristem with quiescent centre (70 µm) and standard excision point (130 µm) positions; LRC, lateral root cap. **b**, Regeneration frequency in wild type (Col-0); $n = 102$ (70 µm), 57 (130 µm), 111 (200 µm), 32 (270 µm); error bars, standard error of the estimate of the proportion (Methods). **c–e**, Confocal time-lapse of single regenerating roots in *CYCB1;1::GFP* (**c**), the columella marker *PET111* (**d**) and the quiescent-centre-specific promoter fusion *pWOX5::GFP(ER)* (**e**), at consecutive days post cut (d.p.c.); scale bars, 50 µm. **f**, Expression levels of columella-enriched ($n = 103$, top panels) and quiescent-centre-enriched ($n = 95$, bottom panels) transcripts during regeneration; left, expression in cell types of uncut roots[9–11]; right, expression in uncut tips or regenerating stumps at the time points indicated.

for root gravitropism[13,14]. By one day post cut, Lugol staining confirmed *de novo* starch accumulation above the cut site (Fig. 2a and Supplementary Fig. 4). More intense staining was observed two days post cut (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 the response over time. All wild-type roots showed a clear gravitropic response within 12 h. Although cut roots did not respond to gravity in the first 12 h after excision when cut at 130 µm, 13.8% of the cut roots exhibited a clear gravitropic response at 1 day post cut, 55.4% at 2 days post cut and 89.2% at 3 days post cut ($n = 65$, for all time points). However, the quiescent-centre-specific marker *WUSCHEL RELATED HOMEOBOX 5* (*WOX5*) was either ectopically expressed in the endodermal file or, at times, expressed in differentiated columella cells at one day post cut (Fig. 2a and Supplementary Fig. 4). Thus, as early as one day after complete columella excision, a new set of cells expressed columella markers and performed 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 formation[15], with the double mutant *plt1plt2* showing differentiation of stem cells at three days post germination[5], as verified under our conditions (Fig. 2b, note the lack of stem cell layer between the quiescent centre and the starch-stained columella). The uncut double mutant root has abnormal tip and stem cell niche morphology but normal gravitropism and convergent longitudinal cell files[5]. Surprisingly, *plt1plt2* roots cut at four days post germination 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 the 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* (refs 5, 16, 17), were also able to restore their pre-cut pattern, starch staining and gravitropism (Fig. 2b–d, f and Supplementary Figs 5b and 6). *PLT1* and *PLT2* 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*- and *PLT2*-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), 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 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 *N*-1-naphthylphthalamic acid (NPA; Supplementary Fig. 9). Second, auxin efflux carriers and an auxin-responsive reporter

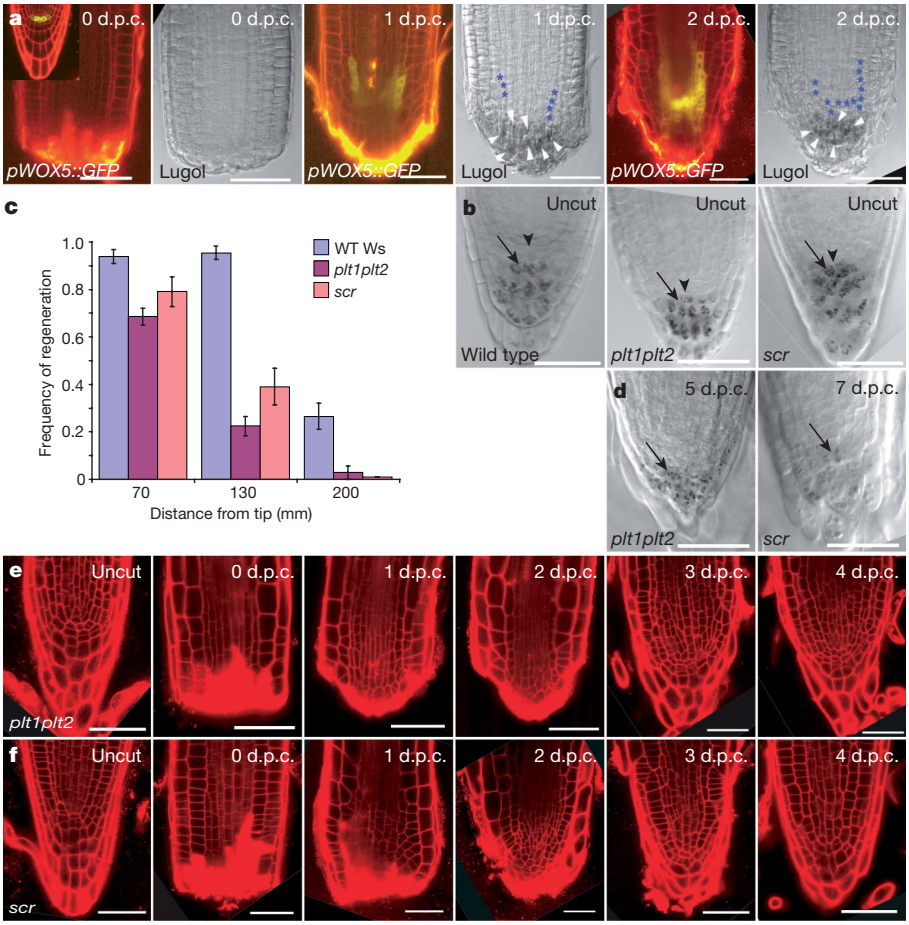

Figure 2 | Columella starch staining and root-tip regeneration in stem cell mutants. **a**, Simultaneous confocal/differential interference contrast imaging of regenerating roots expressing *pWOX5::GFP(ER)*; inset, uncut root tip; blue asterisks indicate GFP-expressing cells as shown in the corresponding confocal image; white arrowheads indicate starch-stained cells; scale bars, 50 μm. **b**, **d**, Lugol staining of uncut (**b**) and regenerating (**d**) roots in wild type, *plt1plt2* and *scr*. Arrowheads indicate the position of quiescent centre; arrows indicate starch-stained cells; scale bars, 50 μm. **c**, Frequency of regeneration in wild type (WT Ws), *scr* and *plt1plt2* mutants; wild type, $n = 66$, 65 and 64 at 70 μm, 130 μm and 200 μm, respectively; *scr*, $n = 43$, 41 and 23; *plt1plt2*, $n = 162$, 107 and 35; error bars, standard error of the estimate of the proportion. **e**, **f**, Confocal time-lapse of regenerating roots in *plt1plt2* (**e**) and *scr* (**f**); scale bars, 50 μm.

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 versus non-competent tissue using transcriptional data on root developmental zones and a time-course induction of pluripotent callus from mature tissue[21] (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 indicates that continuous growth may be a unique feature of the stem cell niche whereas 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

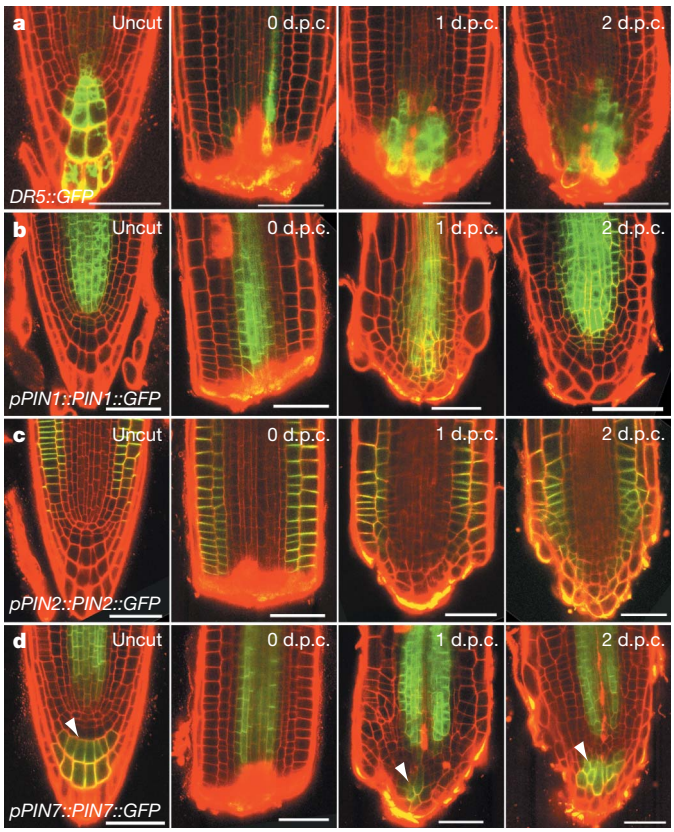

Figure 3 | Early auxin distribution in the regenerating root tip. **a**–**d**, Confocal time-lapse of single regenerating roots expressing the auxin-responsive reporter *DR5::GFP(ER)* (**a**) and translational fusions of the auxin efflux carriers *pPIN1::PIN1::GFP* (**b**), *pPIN2::PIN2::GFP* (**c**) and *pPIN7::PIN7::GFP* (**d**) at consecutive days post cut. Arrowheads in **d** indicate distal expression domains; scale bars, 50 μm.

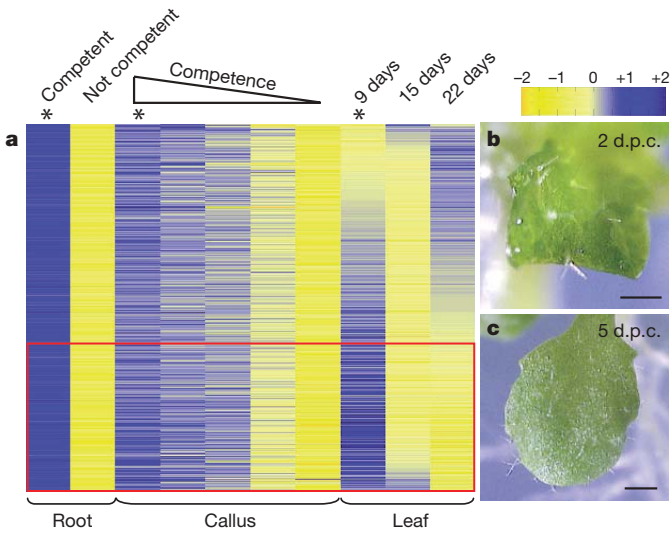

Figure 4 | Regeneration competence markers and leaf regeneration. **a**, Expression levels of regeneration-competence markers identified by enrichment in competent tissue in root and callus[21] (0-, 2-, 4-, 7- and 10-day-old tissue treated with auxin, $n = 647$, see Methods), showing high expression of competence markers in young but not in 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 four-day-old leaf, at two (**b**) and five (**c**) days post cut; scale bars, 50 μm.

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 in 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 seem 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.

METHODS SUMMARY

Mutant alleles used were *plt1-4 plt2-2* in the ecotype Wassilewskija (Ws) and *scr-4* (Ws). Seedlings at four days post germination 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 six days post cut, 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 the indicated time points.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions G.S. performed all experiments with help from X.W. and H.Y.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.

Author Information Microarray data have been submitted to GEO (NCBI) under the accession number GSE9996. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to K.D.B. (ken.birnbaum@nyu.edu)

METHODS

Microarray and statistical analysis. Microarray profiles were normalized using the MAS 5.0 method with a target intensity of 250. Cell-type-specific marker sets were generated by identifying transcripts for which the 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) cutoff $<5\%$[24] and a twofold enrichment cutoff. To increase stringency for cell specificity and assure no overlap between columella and quiescent centre markers, we also required a twofold 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 twofold enriched over quiescent centre and lateral root cap, respectively). The same procedure was followed for quiescent centre markers, ensuring a twofold enrichment over columella and lateral root cap. In addition, the root-tip-specific cell types also needed to show a twofold enrichment in root tip over proximal meristem expression.

For analysis of percentage columella and quiescent centre 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 h, 13 h 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 quiescent centre markers significantly downregulated in the *plt1plt2* mutant tips compared to wild-type tips (termed the *PLT1/2*-dependent set). Subsequent analysis tested whether any members of the *PLT1/2*-dependent set were significantly upregulated in wild-type stumps at 24 h versus wild-type stumps at 0 h (testing for early regulation of the *PLT1/2*-dependent set in wild type) or wild-type stumps at 24 h versus *plt1plt2* stumps at 0 h (testing for potential regulation of the *PLT1/2*-dependent set in the *plt1plt2* mutant during regeneration, that is, 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 upregulated in tissue freshly collected at 130–200 μm (competent zone) versus tissue freshly collected 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 work[21] using the rank method so that replicates for each time point were labelled: 1, 2, 3, 4 and 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 upregulated in 9-day-old leaves versus 22-day-old leaves[22] 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 five hours after tip-cutting, we used a two-class unpaired test in SAM ($q < 5\%$) comparing replicates in regenerating tips at 0 h versus 5 h. We found the intersection of that list and the list of auxin-induced genes[25] 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 auxin[25].

The standard error of the estimate of the proportion is the standard deviation of the population of all possible values of the proportion computed from samples of a given size n. Given P, the estimate of the true proportion as calculated from one random sample of size n, the standard error of the estimate of the proportion is estimated as the square root of $P^*(1 - P)/n$.

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