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Built to rebuild: in search of organizing principles in plant regeneration

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Abstract

Plants are under constant attack from insects, microbes, and other physical assaults that damage or remove body parts. Regeneration is one common strategy among plants to repair their body plan. How do organisms that are proficient at regeneration adapt their developmental programs for repatterning tissues? A new body of research employing high resolution imaging together with cell-fate markers has led to new insights into the tissues competent to regenerate and the mechanisms that re-establish pattern. In a parallel to new findings in metazoan systems, recent work in plants shows that regeneration programs commonly thought to rely on dedifferentiated cells do not need to reprogram to a ground state. Imaging studies that track the expression of regulators of the plant's proliferative centers, meristems, in conjunction with mutant analysis have shed new light on the earliest organizational cues during regenerative organ formation. One promise of plant regeneration studies is to reveal the common design attributes of programs that pattern similar organs in different developmental contexts.

Introduction

More than 50 years ago, plant researchers reared single differentiated cells into entire plants, demonstrating the totipotency of some adult plant cells [1]. Gurdon's classic nuclear transplantation experiments in frog showed the pluripotent potential of some differentiated metazoan cells [2]. By 2006, researchers achieved the long-sought goal of inducing an adult mammalian cell into a pluripotent state [3-4]. If important strides have been made in manipulating pluripotency, one immediate challenge in regeneration research is a better understanding of how specific developmental mechanisms are invoked during repatterning by pluripotent cells.

Few developmental pathways involved in patterning are likely to be shared across kingdoms [5]. However, the task of repatterning in plants and animals raises a set of parallel questions in regeneration, which is defined here as the replacement of lost or damaged parts. How are stereotypical developmental pathways adapted during regeneration? And, which cells are typically recruited and what triggers the activation of specific programs in them? The broadest possible comparisons may yield insights into common constraints and alternate solutions to regeneration.

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Plants and animals demonstrate examples of *in situ* restoration of a damaged organ or appendage, such as in salamander limb or plant root tip regeneration (Fig. 1). A second mode of repair is *de novo* regeneration of organs or whole organisms often in an ectopic location. Examples include the appearance of shoots or roots from stem cuttings in plants or re-establishment of the complete body plan from tissue fragments in *Planaria*. In plants, one critical feature of regeneration is the reformation of meristems, the proliferative tissues that contain stem cells and ultimately lead to adult growth. Although we distinguish the activation of dormant axillary meristems and other existing growth centers from regeneration, one intriguing question is what is the role of embryonic and post-embryonic growth mechanisms in regeneration?

Moreover, plants produce similar organs in very different developmental contexts. For example, plants produce roots in the embryo, in the adult body as continuously emerging lateral roots, and often as adventitious roots [6]. The initial morphogenesis of these organs is dramatically different but the developmental outcome is largely the same, if not identical in some cases. How do these different programs, which utilize the same genomic toolbox, reveal common organizing principles in plant organogenesis? Are any of these principles utilized during regeneration as well?

Potent reserves

After the demonstration of the totipotency of carrot phloem cells [1], a body of experiments repeated these results using other differentiated cell types, suggesting that virtually any plant cell could be a starting point for regeneration [7]. Recently, reliable microscopic imaging has begun to distinguish which cell types are typically recruited during regeneration from mixed tissue samples. In *Arabidopsis*, a regenerative tissue mass called callus can be induced in culture by treating tissue cuttings with the phytohormone auxin. Organ regeneration can then be induced from callus tissue by modulating the ratio between auxin and another phytohormone, cytokinin, in the culture medium. Using this system in combination with confocal imaging of cell-type specific markers, it was shown that callus typically arose from either proliferating and relatively undifferentiated meristematic cells or from the differentiated cell type pericycle, from which lateral roots naturally initiate [8**,9*,10*]. When pericycle cells were genetically ablated using cell-specific expression of diphtheria toxin, callus could no longer be induced from mature root zones [9]. Thus, despite widespread totipotency, certain cell types appear to be more likely recruits for callus formation, at least in *Arabidopsis*. More high-resolution cell tracking is needed in other species but previous work includes reports of “natural” callus emerging *in planta* from other tissues associated with post-embryonic growth, such as lateral meristems, which contribute to girth growth in adult plants [11,12].

If callus is treated with a high ratio of cytokinin-to-auxin concentration, it will develop shoots. The inverse ratio will induce roots [13]. This organ-specification principle, first reported in 1957, has become an important tool for regeneration studies. In one recent study, Che et al. [9] showed that auxin pre-conditioning made explants competent to express hundreds of genes after the shoot-forming treatment, including meristem determinants like *WUSCHEL* (*WUS*). These studies reveal the role of auxin treatment in making cells competent to express patterning genes. Indeed, it has long been thought that the auxin treatment enables callus to reach a dedifferentiated state.

The route to cellular plasticity

Recent studies have challenged the role of dedifferentiation in regeneration. In experiments where the *Arabidopsis* root tip is cut to initiate regeneration, the global identity of the cells in the remnant stump was tracked in time, showing that many cell-type specific markers for lost cell types were expressed within 5 hours [14**]. The analysis also showed that newly restored

columella cells, which sense gravity in the root, were functional within about 24 hours (Fig. 1). The speed of marker and cell-type recovery and lack of apparent cell dedifferentiation at the morphological and transcriptional level suggested the intriguing possibility that these cells traversed fates directly without entering a dedifferentiated state. Similarly, in cultured root explants, it was found that lateral roots initiating from the pericycle tissue could be re-specified directly into shoots without callus induction after transfer to cytokinin-rich media [10].

Two recent studies draw into question assumptions about the dedifferentiated state of callus. Surprisingly, it was shown that callus is not a homogenous mass but retains root meristem identity [10*,15**]. Even callus generated *in vitro* from the pericycle of aerial organs, a layer that does not produce lateral roots *in planta*, passed through cellular states reminiscent of those found in the root meristem, as judged by marker expression and genomic profiling [15]. Moreover, there appears to be a functional requirement for such a root-like phase as even shoot-derived tissues from mutants in lateral root initiation, such as *alf4*, could not effectively form callus [15]. Thus, callus does not appear to be a dedifferentiated tissue after all.

In a parallel finding in metazoans, researchers probed the nature of the blastema, a regenerative tissue mass analogous to callus from which, for example, a limb can regenerate in salamander (Fig. 1) [16**]. Remarkably, cell lineage tracking showed that many blastema cells appear to retain a memory of their original identity and are restricted to their own lineage during regeneration [16]. Thus, in taxonomically diverse organisms, structures thought to represent dedifferentiated tissue that regenerate entire organs or limbs contain many cells that do not need to dedifferentiate to a ground state. In axolotls, the lineage memory of blastema cells may be one mechanism to help guide the appropriate limb program during regeneration. In contrast, meristematic *Arabidopsis* cells do not show evidence of being committed to their fate [17]. Indeed, these young but differentiated cells appear to be widely pluripotent, competent to translate positional cues directly into a wide variety of cell fates.

Rebooting: which program is executed?

Within root and shoot meristems, a canonical stem cell niche continuously generates new cells, which continue to divide until they differentiate [17]. In principle, regeneration could be orchestrated by the existence of a local patterning organizer such as the niche, or emerge as the consequence of global self-organizing properties of the meristem as a whole. In the first scenario, a central organizer like the niche would be necessary for tissue reorganization and would have to be established early in the process, while in the second case its appearance would be a mere consequence of repatterning. Experiments that have determined the order of cell type recovery in the meristem are providing important clues about the nature of reorganization mechanisms during regeneration.

Within the root stem cell niche, quiescent center (QC) cells are required for maintaining surrounding stem cells in an undifferentiated state [18]. The QC, upon which the cell files of the root physically converge, has long been thought to be an organizer of root development. Xu et al. [19**] showed that genes required for QC maintenance in adult roots, including *PLETHORA1*/*PLETHORA2* (*PLT1/2*) and *SCARECROW* (*SCR*), were also critical in regenerating the QC and distal tip organization after QC ablation. These results are consistent with the QC and the functional stem cell niche acting as a central organizer. However, Sena et al. [14], who used a regeneration system in which the entire root tip is severed, found that *plt1/2* and *scr* mutants could regenerate the root tip pattern, including re-specification of excised cell types, such as the columella. It is not clear why genes known to maintain the niche are necessary in the recovery from one type of injury and not the other, although it is possible that tip excision invokes different patterning mechanisms than those triggered by QC ablation. The *scr* and *plt1/2* mutants do not completely eliminate QC identity [14], which may still play

a role in repatterning. Nonetheless, the results from the complete root tip excision showed that a functional stem cell niche was not necessary for repatterning, opening the potential for a QC-independent patterning mechanism.

Similarly, in *de novo* regeneration of the shoot apical meristem (SAM) from callus, time-lapse confocal imaging of key regulators showed an early spatial partitioning of factors expressed in the central and peripheral regions of the shoot meristem, such as a *REVOLUTA* (*REV*) domain flanked by a *FILAMENTOUS FLOWER* (*FIL*) domain [8]. Evidence from both imaging analysis and global microarray studies, showed that broad regions of the meristem were specified early and many factors important for stem cell establishment and maintenance, such as *CLAVATA3*, were expressed later [8,20**]. Thus, temporally, it seems that domains that span the entire shoot meristem are patterned prior to the establishment of an active stem cell niche, as in the root tip excision experiments. Neither the shoot nor the root tip regeneration experiments rule out the existence of a central organizer but they suggest that at least part of meristem repatterning can be independent from the establishment of an active stem cell niche in plant apical meristems.

Embryonic programs would seem to be a good source of whole meristem repatterning. In both shoot and root regeneration studies, regulators that play critical roles during embryogenesis appear early in regeneration [8-10,14,19,20]. However, the changes in gene expression during regeneration appear to be a gradual refinement to adult expression patterns more than a recapitulation of embryogenesis [8,14]. For example, in the embryo, *WOX5* is normally expressed in the lens-shaped cell, which eventually gives rise to the QC [21]. In contrast, early in root regeneration *WOX5* expressed in a u-shaped pattern, similar to other QC markers in adult roots treated with auxin [22]. Ultimately, its expression pattern refines to a group of cells with an adult QC morphology with no intervening lens-shaped stage [14,19]. In fact, many other morphogenesis events in root regeneration differ from embryogenesis or lateral root formation but it is not clear if these differences represent a truly distinct developmental program or the adaptation of stereotypical organogenesis mechanisms onto the disorganized morphologies of regenerating tissues. Parallel questions have arisen in axolotl limb regeneration, as expression of *HOX* genes do not always follow the patterns of early development. The aberrant patterns have raised the question of whether they reflect a novel dedifferentiation stage, embryogenesis, late limb development, or something entirely unique to regeneration [23].

Interestingly, in *Kalanchoe*, vegetative plantlets form on the margins of leaves in a type of regeneration incorporated into normal development. Recent studies suggest that both adult organogenesis and embryonic developmental programs are co-opted into the process [24*]. This shows that plants can use a mix-and-match strategy to patterning.

Order from disorder

Unlike most normal development, regeneration often proceeds through unpredictable and seemingly disorganized morphologies. However, recurring patterns common to different realizations of regeneration could help identify critical spatial organizers, such as adjacent domains of key regulators that could set up tissue boundaries. For example, Gordon et al. [8] frequently observed that shoot inducing treatments (a high ratio of cytokinin-to-auxin concentration) led to the first signs of regulatory organization in callus, initiating the partition of mutually exclusive auxin and cytokinin response domains. These domains appeared to subsequently induce spatially distinct expression patterns for the meristem initiation genes *CUP-SHAPED COTYLEDON2 (CUC2)* and *WUS*. The results help explain the mechanisms behind the organ-specification principle in which hormone ratios induced organ identity [13]. The authors speculate that, after the establishment of hormone response and gene expression

domains, interactions between hormones and regulatory cues progressively establish more complex patterning typical of the shoot meristem. In this view, the primary difference between *in vitro* regeneration and meristem initiation *in planta* is merely how the initial distribution of auxin and cytokinin is set up [8,25].

What triggers early events in regeneration when no exogenous hormones are applied? The question is important because the cues that re-establish order can reveal insights into the nature of the patterning mechanism. In the QC ablation experiments, Xu et al. [19] present a model in which QC ablation caused a disruption of the auxin flow that triggered regeneration. The model was supported by computational simulations showing that, given a specific distribution and polarity of PINs, an auxin maximum could be re-established a few cells proximal to its original position [26*]. The model is based on the fact that QC and a few neighboring cell types express several members of the auxin efflux carrier family, named after the *PIN-FORMED 1* (*PIN1*) mutant. The cellular polarity and expression domain of PINs results in the directional transport of auxin within the tissue. The most distal PINs of the root move auxin laterally and contribute to a circulation system that establishes a concentration maximum at the tip (Fig. 1 [27]). Auxin is known to be a critical cue for root organization, because auxin flux has been shown to be necessary for root regeneration and perturbations in auxin distribution that change the location of the auxin maximum are sufficient to switch the polarity of the root [19,14,22].

Other studies have shown that auxin can influence its own movement by inducing expression of *PIN* genes [28]. Such canalization models posit that auxin flux can feedback on PIN polarity to reinforce the direction of auxin flow, such that auxin can self-organize its own movement [29-31]. Indeed, it has been shown that, in wound healing, auxin flux reinforces polarization of PINs leading to vascular strand formation [32*] and the model was similarly corroborated recently in leaf vein formation [33].

One of the missing pieces of the puzzle is what triggers regeneration. For example, how is an auxin maximum re-established once it is removed by the excision of the root tip? One possibility is that a net downward flux of auxin is present in the root stump, which retains its proximal-distal orientation of auxin efflux carriers [14]. These remaining PINs would transport auxin toward the root tip, where auxin would induce new PIN expression that stabilizes the auxin flux. Indeed, the expression pattern of PIN7 (lateral distribution of auxin), whose distal domain was completely lost after tip excision, was detected in the root stump only 24 hours after the cut along with evidence of a new auxin maximum [14].

Alternatively, other signals could initiate regeneration by triggering cell fate or other local changes. In animal systems, apoptotic cells at wound sites release Wnt3 in Hydra, which is necessary to induce head regeneration [34]. In a parallel scenario, early regeneration events may be independent of auxin. For example, Xu et al. [19] found that cell fate and QC identity preceded re-orientation of PINs during regeneration. It will be interesting to see if potential signals induced by dying cells or new edge cells can trigger repatterning either by inducing cell fate re-specification or by helping re-establish the appropriate auxin flux.

The triggers described above may establish a local organizer. Alternatively, auxin flux in the meristem may act as a non-localized, self-repairing system where initial positional cues are specified by the remnant flow of auxin in the stump. This auxin circulation system could be both independent from a local organizer and necessary to instruct further patterning. Yet another possibility is that the reorganization of a vast field of differentiated cells in plants could in fact be obtained independently of auxin as an emergent property of the whole system, simply based on other kinds of cell-cell interactions acting on intrinsic cell fate plasticity. Thus, in root tip regeneration, it is not yet clear if auxin alone can re-organize its own flow, if it requires other inputs, or if it is simply a necessary intermediate signaling event during re-organization.

Alternatively, one possible model is that auxin flux in the meristem resembles a non-localized, self-repairing system that specifies cellular identities and patterning throughout the meristem. Another possibility is that the reorganization of a vast field of differentiated cells in plants can be obtained independently of auxin as an emergent property of the whole system, simply based on other kinds of cell-cell interactions acting on intrinsic cell fate plasticity. Thus, in root tip regeneration, it is not yet clear if auxin alone can re-organize its own flow, if it requires other inputs, or if it is simply a necessary intermediate signaling event during re-organization.

Conclusion

The plant's ability to initiate growth into adulthood from many cell types means that highly potent cells are dispersed throughout the plant body. During regeneration, the plant appears to preferentially recruit what are often partially differentiated but uncommitted cells, such as pericycle. Repatterning of these cells during regeneration can be remarkably rapid without an obvious de-differentiation phase. In addition, distinguishing causal factors that initiate patterning in regeneration has profound implications for understanding how the known patterning mechanisms operate within an organizing system. Does a signaling center like the niche, or another region of the meristem, act as a necessary and sufficient local signaling source instructing root patterning? Recent work that shows a functional niche is not necessary for patterning together with results on the timing of *de novo* shoot regeneration open the possibility that critical patterning cues are positioned independently of a local organizer. Is the source of meristem patterning a local organizer such the QC, a canalizing auxin flux, or a self-organizing system depending on other kinds of local cell interactions. Further modeling at these early stages may help generate hypotheses about how organization is established. These important questions about the fundamental organization of meristems in plants are still open and need to be resolved.

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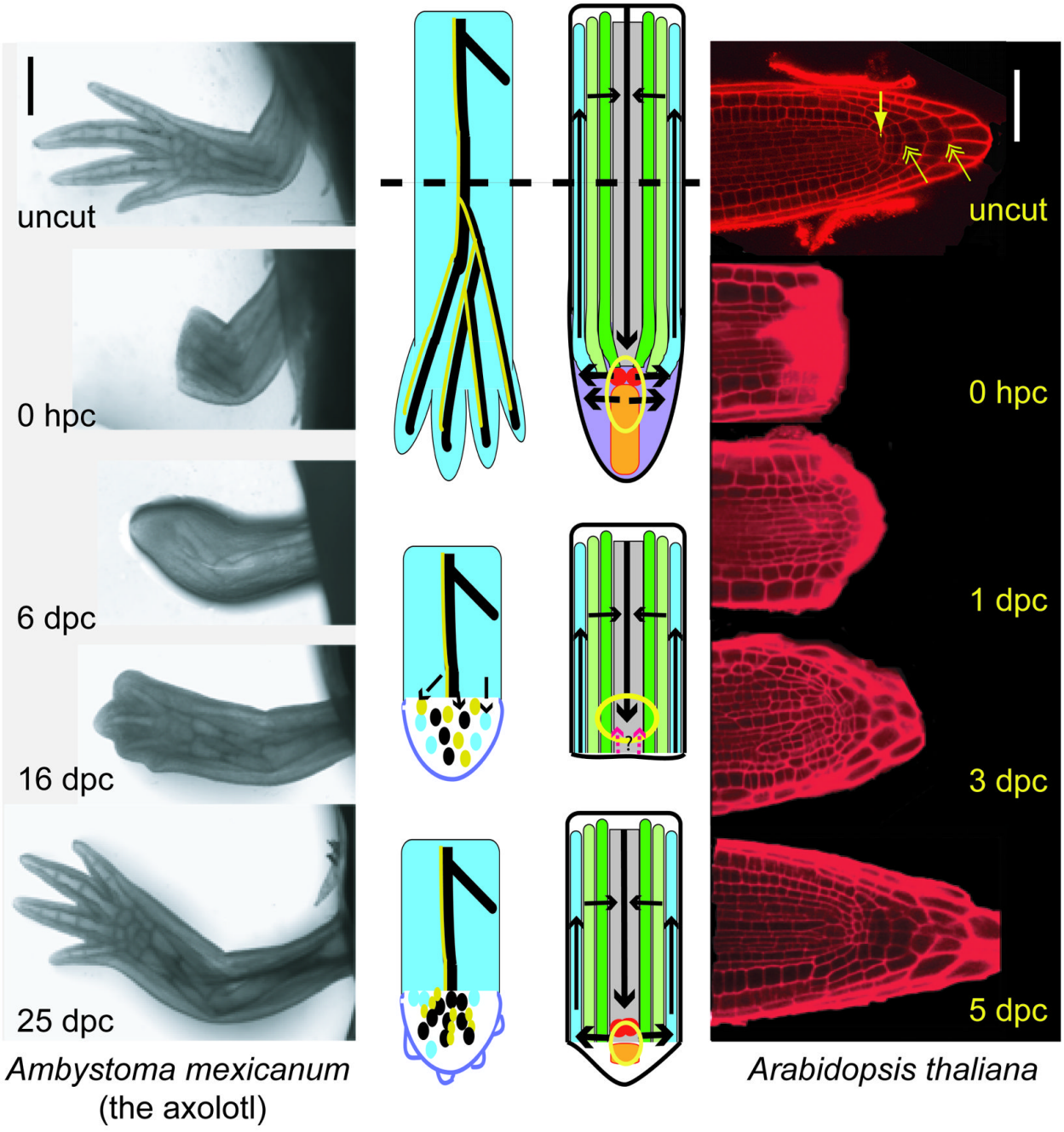

Figure 1. Two strategies for regeneration

On outside panels, axolotl limb regeneration through blastema formation and *Arabidopsis* root meristem regeneration without formation of callus (hpc, hours post cut; dpc, days post cut). In the diagram of axolotl, cells migrate from different tissues in the stump to form a heterogeneous blastema. Colors in diagram represent cells from different lineages that retain a memory of the tissue of origin. The blastema regenerates the limb over 25 days with new tissues populated by cells descendent from the same lineage in many cases. In the confocal image of *Arabidopsis*, the arrow shows the position of the QC cells and the double arrow shows the position of the columella cells. In the diagram of *Arabidopsis*, distal (toward tip) cell identities, PIN domains for lateral auxin distribution, and the auxin maximum (arrows) are removed by

the excision (red=QC; orange=columella, yellow circle=auxin maximum). In a potential model, auxin accumulates at the tip and induces PINs localized to redistribute auxin laterally. The auxin maximum determines the position of QC and then induces further patterning through a yet unknown mechanism. Arrows coming up from the cut site indicate the potential for other signals that induce cell identities or mediate auxin flux. Scale bars; axolotl=1mm; *Arabidopsis*=50μm.