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Molecular Mechanisms of Plant Regeneration

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Abstract

Plants reprogram somatic cells following injury and regenerate new tissues and organs. Upon perception of inductive cues, somatic cells often dedifferentiate, proliferate, and acquire new fates to repair damaged tissues or develop new organs from wound sites. Wound stress activates transcriptional cascades to promote cell fate reprogramming and initiate new developmental programs. Wounding also modulates endogenous hormonal responses by triggering their biosynthesis and/or directional transport. Auxin and cytokinin play pivotal roles in determining cell fates in regenerating tissues and organs. Exogenous application of these plant hormones enhances regenerative responses in vitro by facilitating the activation of specific developmental programs. Many reprogramming regulators are epigenetically silenced during normal development but are activated by wound stress and/or hormonal cues.

Contents

INTRODUCTION

Meristem: a tissue containing stem cells and transit-amplifying cells

De novo organogenesis: formation of new organs via establishment of meristems

Callus: an unorganized mass of cells typically formed in response to external stimuli

Totipotency: cellular potential to generate all cell types within a multicellular organism, enabling single cells, like zygotes, to produce whole bodies

Plants display various modes of regeneration upon loss or injury of body parts (8, 57). Analogous to regeneration of the mammalian digit tip upon amputation, plants reconstruct the apical meristem within a root or shoot when local damage occurs (105, 110). For instance, following amputation of the distal half of a root meristem, cells in the proximal half of the meristem proliferate and reconstruct the original structure (110). Similar to mammalian skin, plants repair damaged stem surfaces and reconnect interrupted vasculature (117). Different tree limbs can naturally connect with one another, owing to their intrinsic ability to regenerate vasculature, and this feature is utilized for grafting in agriculture and horticulture (**Figure 1*a–c***). Characteristic of plant regeneration is the formation of new shoots and/or roots after loss of a large portion of the body through a process called de novo organogenesis (**Figure 1*d–f***). Cells near wound sites proliferate and establish new shoot or root apical meristems, which in turn give rise to new organs. These newly formed meristems may arise directly from parental plants or indirectly from callus formed at wound sites (59). Many plant species regenerate roots de novo, and this feature enables clonal propagation from stem cuttings (42). Strikingly, in some plant species, a whole plant can be reconstructed via de novo organogenesis from just a small piece of mature tissue (98) (**Figure 1*f***).

Although only a limited number of species naturally regenerate whole plantlets upon cutting, many other species regenerate plantlets in vitro when explants are incubated on a nutrient-rich medium supplemented with auxin and cytokinin (38, 63, 115). The balance between auxin and cytokinin determines the fate of regenerating organs: A high ratio of auxin to cytokinin leads to root regeneration, but lower auxin/cytokinin ratios trigger shoot regeneration (**Figure 2*a*,*b***). A routinely used protocol for in vitro regeneration involves preculture of explants on an auxin-rich medium to generate callus that is competent for organ regeneration (130). Under in vitro culture conditions, protoplasts isolated from fully differentiated leaf mesophyll cells can undergo callus formation and subsequent plantlet regeneration, clearly demonstrating totipotency of somatic cells (125) (**Figure 2*c***). Activation of an embryonic developmental program

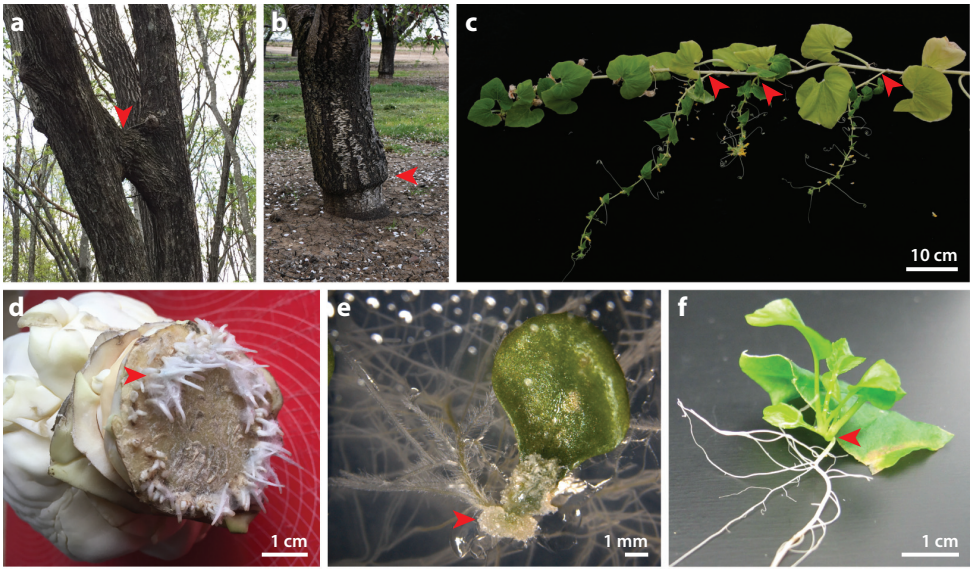

Figure 1

Different types of plant regeneration. (*a*) Inosculation, a naturally occurring process that resembles grafting, between two trunks of oak tree (*Quercus serrata*). (*b*) Artificially grafted trees consisting of a scion from almond (*Prunus dulcis*) grafted onto a rootstock from peach (*Prunus persica*). (*c*) Cucumber (*Cucumis sativus*) scions grafted onto a kabocha squash (*Cucurbita maxima*) rootstock. (*d*) Root regeneration occurring from the base of a head of cabbage (*Brassica oleracea*). (*e*) Callus formation and root regeneration from *Arabidopsis* leaf explants. (*f*) A plantlet regenerating from a leaf of the amphibious plant (*Rorippa aquatica*). Arrowheads mark graft junctions (*a–c*) and regenerating organs (*d–f*).

also enables whole-plant regeneration from somatic tissues. Embryogenesis from nonzygotic cells is referred to as somatic embryogenesis, and transient exposure to a high concentration of auxin is used to trigger this mode of regeneration (32). Various types of abiotic stress treatments, such as exposure to osmotic stress, heavy metal ions, or high temperature, are often combined with auxin treatment to facilitate somatic embryogenesis (53, 69). Somatic embryos may be generated from embryonic tissues, seedlings, cultured cells, or protoplasts either directly or indirectly, i.e., following an intermediate callus stage.

The innate regenerative capacity of plants is the fundamental basis for various horticultural and biotechnological procedures. Grafting is a widely used technique to generate composite plants possessing beneficial traits from two different individuals (**Figure 1*b***); for example, a high-yielding shoot stock, or scion, can be combined with a stress-resistant rootstock (93). Tissue culture techniques enable clonal propagation of many useful species from various types of source organs, including leaves, roots, petals, stamens, and pistils. Regeneration from haploid cells, like pollen, is particularly useful in plant breeding, as it directly gives rise to haploid plants, which can be chemically converted into diploid plants with a homozygous genome (87). Furthermore, de novo organogenesis is often critical for the production of transgenic plants, because inoculation of explants or callus with *Agrobacterium* and subsequent regeneration of plantlets is the standard procedure for generating stable transgenic lines in many species. Although tissue culture techniques have been developed and optimized for a variety of crops, many important crop cultivars still exhibit recalcitrance, and this issue is one of the major bottlenecks facing modern biotechnology (100). Cereals, in particular, are notorious for performing poorly in regeneration procedures (see the sidebar titled Regeneration in Monocot Plants).

Somatic embryogenesis: embryogenesis from somatic cells

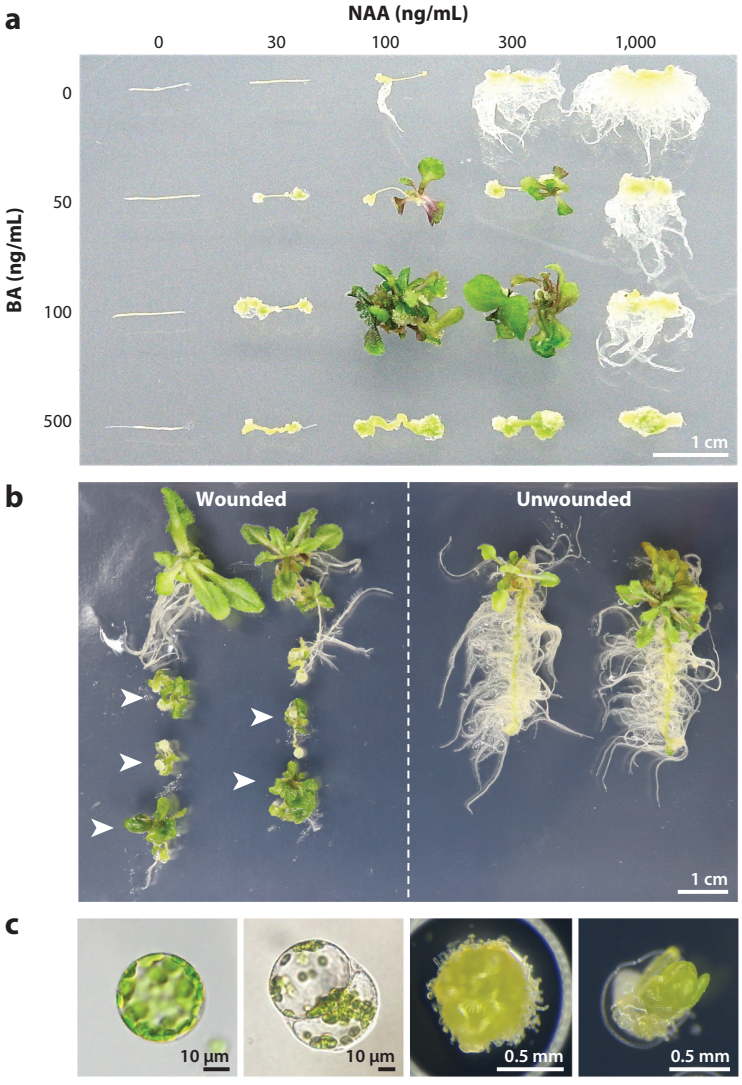

Figure 2

Organ regeneration in vitro. (*a*) Shoot and root regeneration from *Arabidopsis* hypocotyl explants cultured on media containing the indicated concentrations of auxin [1-naphthaleneacetic acid (NAA)] and cytokinin [6-benzyladenine (BA)]. Figure adapted from Iwase et al. (63). (*b*) Shoot regeneration occurs in wounded (*left*) but not in unwounded (*right*) *Arabidopsis* seedlings cultured on callus-inducing medium and subsequently on shoot-inducing medium. Arrowheads mark regenerating shoots. (*c*) An isolated protoplast initiates division and develops callus on callus-inducing medium, and eventually regenerates a shoot after incubation on shoot-inducing medium.

Understanding the molecular mechanisms of plant regeneration is important because it tackles many fundamental questions in cell and developmental biology. Over the past few decades, numerous experimental systems have been developed to study various modes of regeneration in a genetically tractable model plant, *Arabidopsis thaliana* (*Arabidopsis*), allowing key unanswered questions to be addressed. We have gained a substantial mechanistic understanding of how cells perceive and respond to inductive cues, such as wounding and hormones, as well as how these stimuli modify

REGENERATION IN MONOCOT PLANTS

Cereals tend to be recalcitrant to plant regeneration, and many important food crops, such as rice and maize, regenerate poorly from somatic organs. This is partly because regeneration-competent cells in leaf explants are restricted to the base of immature leaf blades, as opposed to *Arabidopsis* where competent cells are found throughout the leaf along the vasculature (50). Explants commonly used for regeneration in monocots are immature embryos, presenting a major bottleneck in monocot transformation because these embryos need to be dissected out individually from developing seeds. A recently reported technological advance employing *Agrobacterium*-mediated introduction of genes encoding BBM and WUSCHEL2 transcription factors during transformation greatly improves regeneration efficiency from mature seeds as well as leaf segments of recalcitrant maize varieties (86). Ectopic expression of these transcription factors also induces somatic embryogenesis in dicots, suggesting that molecular functions of these genes may be at least partially conserved between monocots and dicots (25).

ongoing developmental programs to reform tissues and organs. In addition, recent studies have revealed that regenerative processes need to be repressed in the absence of these inductive cues to maintain functional integrity and enable normal development to proceed. In this review, we summarize our latest understanding of the mechanisms governing plant regeneration and discuss key issues that remain unsolved. Several excellent review articles have been published on grafting (90), root regeneration (140), shoot regeneration (104), and somatic embryogenesis (48); thus, we highlight common cellular and physiological features among these regenerative processes and discuss how similar sets of inductive cues bring about diverse developmental outputs in different regeneration contexts.

CELLULAR BASIS OF PLANT REGENERATION

Regeneration is a complex phenomenon whereby multiple cellular processes, including cell cycle reactivation and cellular reprogramming, proceed in overlapping windows of time and space within a multicellular structure. In this section, we provide an overview of common cellular events underlying various types of plant regeneration.

Cellular Origins

Organ regeneration often entails activation of regenerative responses in a subset of cell populations that have relatively high organogenic potential. Upon local amputation of a portion of the root apical meristem, for instance, remaining meristem cells are reprogrammed and reconstruct the meristem, while differentiated cells outside the meristem do not participate in this process (29, 110) (**Figure 3*a***). Within the meristem, virtually all cells change their identity and collectively reproduce the lost distal meristem cells (29). In de novo organ regeneration and somatic embryogenesis from mature organs, usually only a subset of cells within explants are responsive to inductive cues. *Arabidopsis* root or hypocotyl explants, for instance, regenerate shoots when incubated on auxin-rich callus-inducing medium (CIM) and subsequently on cytokinin-rich shoot-inducing medium (SIM) (**Figure 2*b***). Xylem-pole pericycle cells are the source of regenerating organs in this case (2) because root explants fail to regenerate shoots upon chemical ablation of this cell population (14) (**Figure 4*a***). Xylem-pole pericycle cells possess intrinsic organogenic potential to produce root apical meristems; thus, this feature likely enables cell fate reprogramming to occur specifically in these cells. Similarly, cambium cells in the vasculature serve as the

Cell fate: specification of a future identity for a given cell

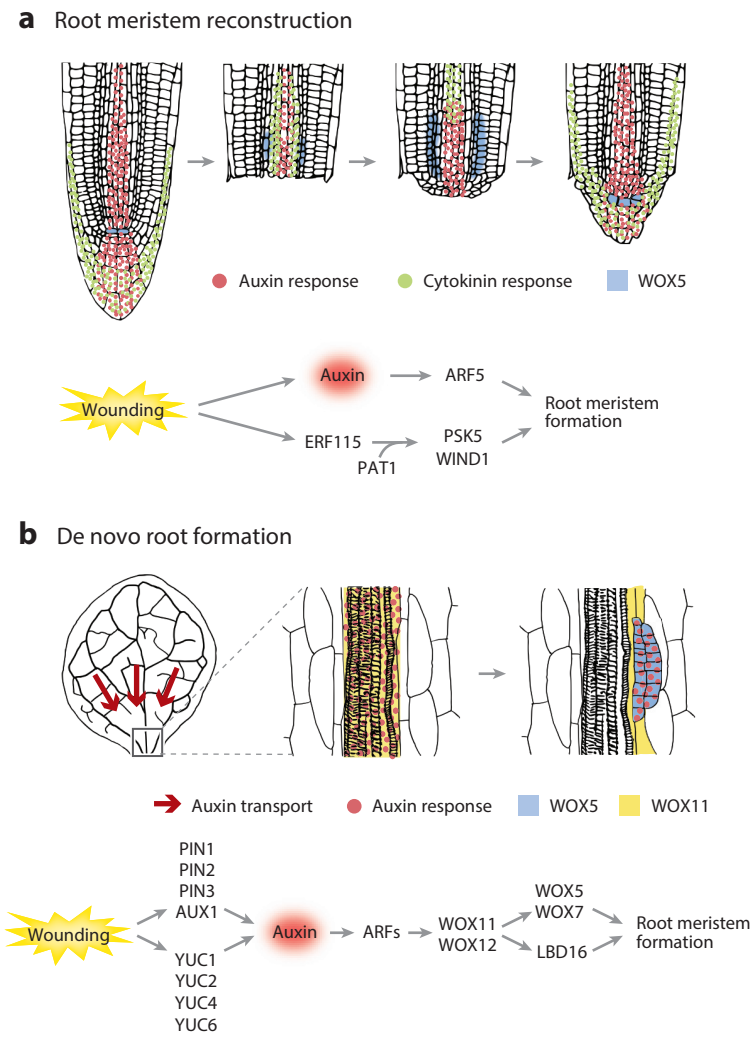

Figure 3

Cellular and molecular basis of root regeneration. (*a*) Schematic depicting root meristem reconstruction. Root tip excision results in the loss of cells with high auxin and cytokinin responses and the WOX5-expressing quiescent center. Root meristem reformation involves redistribution and eventual reestablishment of these hormone-response and expression domains. (*b*) Schematic depicting de novo root formation from leaf explants. Wounding provokes redistribution of endogenous auxin and YUCCA-mediated auxin biosynthesis. Auxin response is strongly induced near wound sites, where WOX11 and WOX12 convert regeneration-competent cells in the vasculature to root founder cells by inducing the expression of WOX5, WOX7, and LBD16. These factors, in turn, direct meristem formation.

cellular origin of roots regenerated from *Arabidopsis* leaf explants (16, 84) (**Figure 3*b***) and of somatic embryos from *Daucus carota* hypocotyl explants (40).

Although organ regeneration from vasculature and pericycle cells is commonly observed and has been extensively studied, organ regeneration can also initiate from other types of somatic cells. For instance, endodermal cells give rise to regenerating roots when thin layers of *Arabidopsis* floral stem explants lacking vasculature and pericycle cells are incubated in tissue culture (30). This

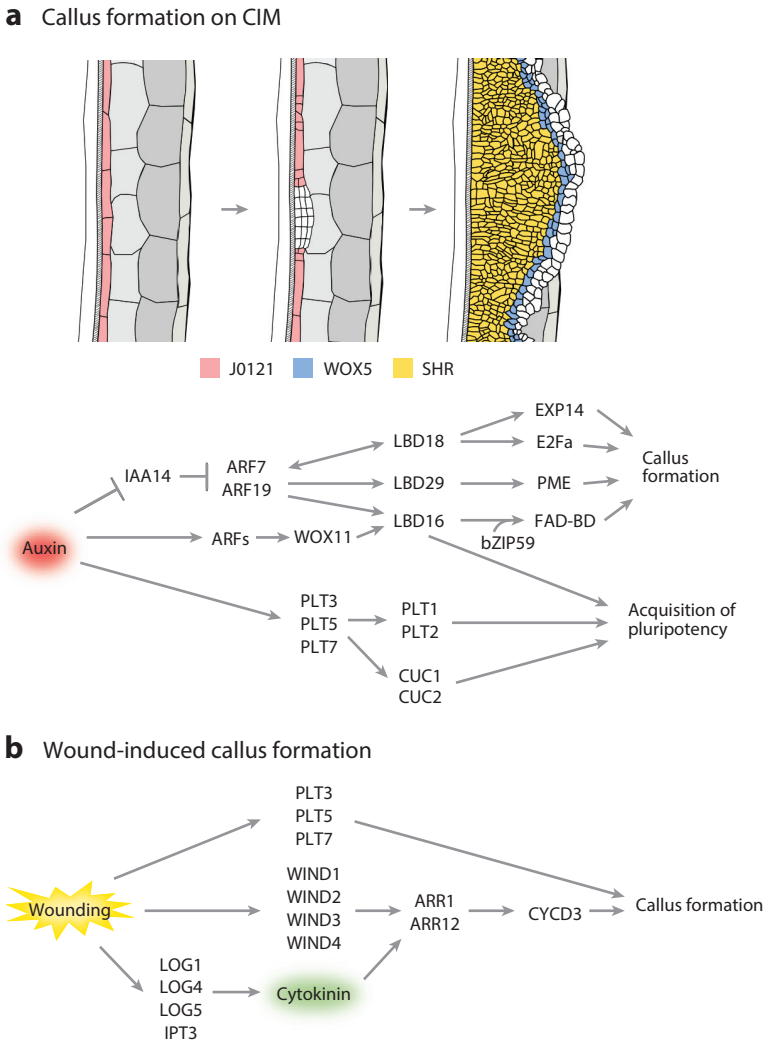

Figure 4

Cellular and molecular basis of callus formation and acquisition of pluripotency. (*a*) Upon incubation of explants on callus-inducing medium (CIM), xylem-pole pericycle cells lose expression of the J0121 marker as they dedifferentiate and proliferate to form callus. Callus is marked by the expression of root meristem markers, such as WOX5 and SHR. Auxin promotes callus formation via ARF-mediated activation of LATERAL ORGAN BOUNDARIES DOMAIN proteins (LBDs). LBDs in turn activate the expression of a suite of genes that promote cell proliferation and modify cell wall properties. Besides callus formation, auxin also promotes the acquisition of cellular pluripotency via two different pathways, one mediated by WOX11 and LBD16 and the other involving PLTs and CUC2. (*b*) Wounding activates cytokinin signaling via both induction of biosynthesis and a WIND-mediated pathway. ARR-mediated cytokinin signaling promotes cell cycle reentry through activation of CYCD3. PLTs are also induced by wounding and are important for callus formation in this context.

REGENERATION IN MOSS

Molecular mechanisms of moss regeneration are best studied in *Physcomitrella patens*. Fully differentiated leaf cells can be reprogrammed into protonema (thread-like chains of cells normally produced from spores) stem cells within 2 days after injury, a process that involves cell identity change, cell cycle reentry, cell elongation, and establishment of new protonema (61). A homolog of *Lin28*, one of the key regulators used to induce pluripotent stem cell formation in mammals, also plays a central role in cellular reprogramming in *Physcomitrella* (78). *Lin28* and its homolog encode a type of cold shock domain protein that has nucleic acid binding activity. Both proteins likely function as RNA chaperones under cold stress. WUSCHEL-RELATED HOMEOBOX 13-LIKE (WOX13L) is another class of regulators of cellular reprogramming that is induced by wounding in *Physcomitrella* (109). WOX13Ls are not required to induce cell division or mediate identity transition into protonema, but they regulate cell elongation through transcriptional induction of cell wall–loosening enzymes.

finding suggests that stem endodermal cells also possess regenerative potential, although it is likely masked during normal regeneration where vascular and pericycle cells display higher organogenic activity. Notably, cellular regenerative potential differs dramatically among taxa in seed plants, as demonstrated, for example, by *Torenia fournieri*, in which shoots regenerate from epidermal cells of stems (19). Additionally, somatic embryos develop from leaf mesophyll cells in *Medicago truncatula* (134), and isolated mesophyll protoplast cells can give rise to entire plantlets in *Nicotiana tabacum* (125), further demonstrating the regenerative potential of normally nonorganogenic cells in mature organs from certain plant species. It is also worth noting that basal land plants with simpler body structures, such as the bryophyte *Physcomitrella patens*, can regenerate stem cells from virtually all cells in mature organs upon injury (61) (see the sidebar titled Regeneration in Moss). Therefore, many seed plants whose cells differentiate to form various tissues have acquired mechanisms to limit regenerative potential in most somatic cells, particularly those located in mature organs.

Cellular Dedifferentiation and Acquisition of Competency

During the initial stage of regeneration, somatic cells likely undergo some level of cellular dedifferentiation and at least partially lose their existing identities. When root explants are incubated on CIM, for instance, expression of a xylem-pole pericycle marker, J0121, is lost after onset of callus formation (14) (**Figure 4*a***), suggesting that these cells no longer retain their pericycle identity. Similarly, the expression of a proximal root meristem–specific gene, *ARABIDOPSIS HISTIDINE KINASE 4* (*AHK4*)/*WOODEN LEG* (*WOL*), disappears during meristem restoration after root tip excision (29). In contrast to pericycle or meristem cells, which possess relatively high organogenic potential, fully differentiated leaf mesophyll cells likely undergo more extensive cellular reprogramming before committing to organ regeneration. Previous microscopic observations demonstrated that isolated mesophyll protoplasts undergo dynamic decondensation of chromatin, potentially reflecting dedifferentiation of these cells (147). Similarly, chromocenter dispersion and nucleolar disruption have been identified in freshly isolated protoplasts (126, 135), although the functional relevance of these chromatin rearrangements to cellular reprogramming is yet to be established. Upon loss of existing cell identity through dedifferentiation, cells often acquire regeneration competency. For instance, while losing pericycle cell identity during incubation on CIM and SIM, xylem-pole pericycle cells in *Arabidopsis* roots acquire competency to regenerate shoots (67, 142) (**Figure 4*a***), which we discuss in more detail below.

Stem cell: an undifferentiated cell that can divide to self-renew and generate other cell types

Cellular dedifferentiation: a process in which cells lose their distinct cellular state and take on a less specialized, often more flexible status

Cell Proliferation

Organ regeneration is often accompanied by activation of mitosis, and several lines of experimental evidence show that cell proliferation is required for successful organ regeneration. Blocking cell cycle progression hinders root meristem restoration, demonstrating that the production of new cells is pivotal for the restoration of lost meristems (110). During de novo shoot regeneration, cell proliferation is required for both the generation of a new cell mass and cell fate transition. Explants incubated on CIM containing a cell cycle inhibitor turn green when transferred to SIM but fail to establish visible shoots (112), suggesting that cell proliferation on CIM is necessary for acquisition of shoot regeneration competency (14). Subsequently, cells also need to go through a couple of rounds of cell cycles on SIM to be fully competent for shoot regeneration (146) (discussed below). Progression through the mitotic cycle likely provides an opportunity for cellular status to change dynamically, thereby facilitating cell fate transition.

Acquisition of New Cell Fates

To complete a regenerative process, cells need to acquire new fates, and this often proceeds in a stepwise fashion (28). Accumulating evidence suggests that cells exhibit mixed hormonal responses during intermediate steps: Auxin and cytokinin responses initially occur in the same cells and then gradually become partitioned into different populations of cells during root meristem restoration, shoot regeneration, and somatic embryogenesis (18, 29, 68, 97) (**Figures 3*a***, **5**, and **6**). Expression profiling of cell type–specific genes during root meristem restoration and somatic embryogenesis further suggests that cells take on mixed cellular identities as an intermediate step (29, 97). A marker gene for root quiescent center cells, *WUSCHEL-RELATED HOMEOBOX 5* (*WOX5*), for instance, is broadly expressed at an intermediate stage of meristem restoration, when its expression overlaps with those of other cell type–specific markers, before it finally becomes confined to the root stem cell niche (**Figure 3*a***). Interestingly, Efroni et al. (29) pointed out that the spatial signature of hormonal response and cell type–specific gene expression observed during root meristem restoration is reminiscent of the developmental events that occur during zygotic embryogenesis. Therefore, cells may acquire discrete cell fates and establish new developmental patterns by exploiting patterning mechanisms usually at play during zygotic embryogenesis.

MOLECULAR BASIS OF PLANT REGENERATION

We proceed to discuss regulatory mechanisms of how plants sense wound stress and initiate various types of organ regeneration. These processes are mainly mediated by modification of hormonal homeostasis and signaling as well as transcriptional modulation of key meristem or embryonic regulators. Specific regulatory components differ from one type of regenerative process to another; therefore, we discuss our current understanding of various molecular pathways that lead to specific types of regeneration. Because loci of genes that play key roles in organ regeneration are often subjected to epigenetic modifications, we also discuss how epigenetic mechanisms modify the chromatin environment to control cellular regenerative capacity. Key regulators discussed in this section are summarized in **Table 1**.

Early Wound Response and Signaling

Wounding often induces cellular reprograming and subsequent organ regeneration (57). Cutting is sufficient to trigger de novo organ regeneration in some cases, whereas wounding strongly

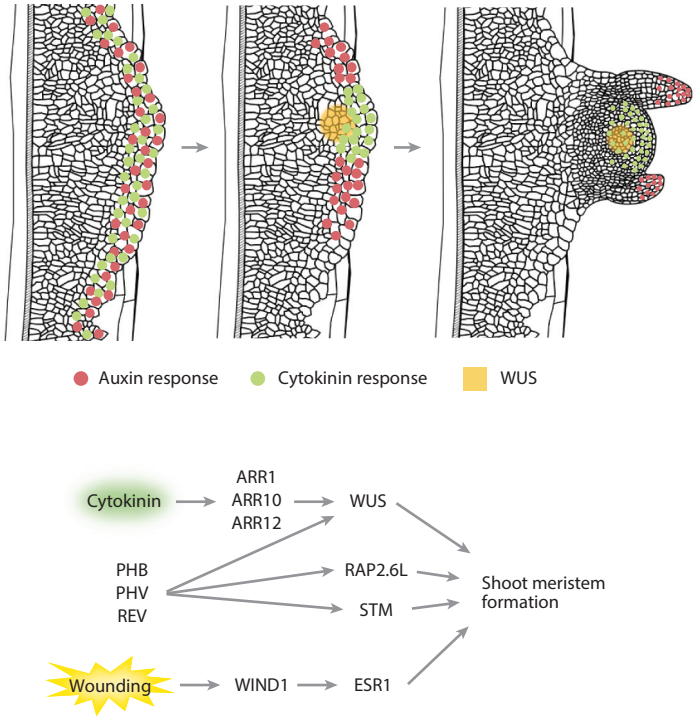

Figure 5

Cellular and molecular basis of shoot regeneration on shoot-inducing medium. Callus in explants transferred to this medium exhibits broad auxin and cytokinin responses that subsequently become localized to separate domains. WUS is eventually expressed in select cells located in the vicinity of the cytokinin response domain. Cytokinin promotes shoot meristem formation via ARR-mediated activation of WUS expression. PHB, PHV, and REV play critical roles in shoot formation through induction of WUS, STM, and RAP2.6L. Wounding promotes shoot regeneration via WIND1-dependent activation of ESR1.

promotes hormone-induced organ regeneration in others. *Arabidopsis* root explants, which experience wound stress in response to dissection, regenerate shoots after incubation on CIM followed by SIM (**Figure 2*b***). Unwounded seedlings, by contrast, develop numerous lateral roots but no shoots in the same procedure, clearly demonstrating the need for wound stress in the root-to-shoot fate conversion (65) (**Figure 2*b***). Similarly, cutting dramatically enhances the efficiency of somatic embryogenesis after exposure to auxin, further corroborating the tight link between wound stress and hormone-induced regeneration (97). Wounding causes a myriad of physiological responses, ranging from local stress responses to disruption of the transmission of long-distance signals (20, 77), but how these early responses promote regeneration remains unclear. In both animals and plants, wounding triggers a rapid influx of calcium ions into cells and a subsequent increase of reactive oxygen species (ROS), thereby activating downstream signaling cascades and transcriptional outputs (96). Calcium and ROS-dependent signaling pathways are involved in wound healing and organ regeneration in animals (22, 99, 143). Their roles in organ regeneration are poorly established in plants, but several reports imply their potential relevance. For example, hydrogen peroxide (H_2O_2) is an ROS that is elevated upon cutting in cucumber (*Cucumis sativus*) roots, and this increase in H_2O_2 contributes to root regeneration (80). However, the effect of exogenous H_2O_2 application seems context and/or dosage dependent: It promotes root regeneration in cucumber (80) and somatic embryogenesis in wheat (124) but inhibits shoot regeneration in

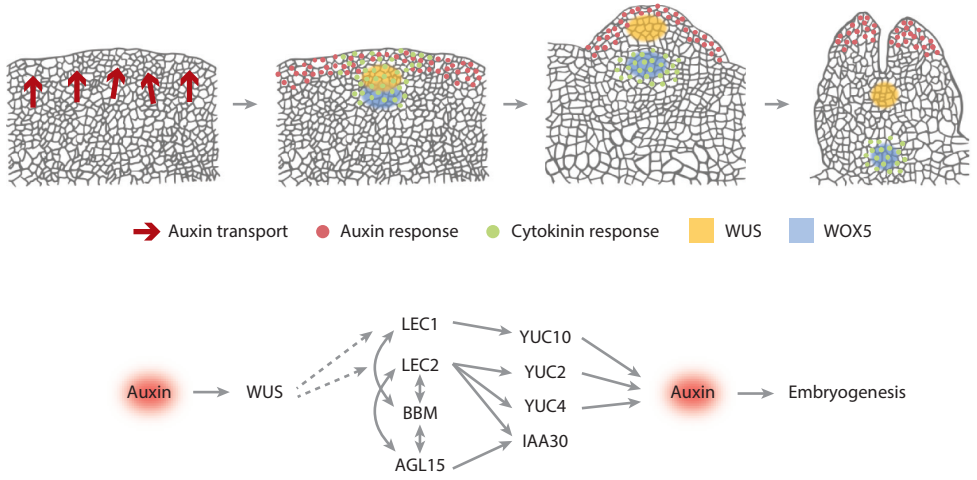

Figure 6

Cellular and molecular basis of somatic embryogenesis. Following transfer of embryonic callus to auxin-free medium, polar auxin transport leads to formation of auxin maxima near the surface of the callus. Cytokinin response domains initially overlap with auxin response domains. WUS- and WOX5-expression domains are initially located adjacent to each other, but they eventually separate to opposite poles of the developing somatic embryo. Auxin activates the expression of *WUS*, which is required for activation of the embryonic regulators LEC1 and LEC2. These factors, together with BBM and AGL15, form highly interconnected transcriptional networks that contain multiple positive feedback loops and collectively promote somatic embryogenesis. These embryonic regulators promote the expression of YUCs, TAA1, and IAA30 to modulate auxin biosynthesis and signaling.

Arabidopsis (145). Another typical response to injury is accumulation of the phytohormone jasmonic acid (JA) (72). Similar to H_2O_2, the contribution of JA in organ regeneration is context dependent. For instance, JA promotes somatic embryogenesis in *Arabidopsis* (97) and root regeneration in pistachio (*Pistacia vera*) (27). However, it is dispensable for grafting (89) and is even inhibitory for callus formation in wounded *Arabidopsis* hypocotyls (55).

Although our understanding of the molecular details of early signaling cascades invoked by wound stress in plants remains obscure, transcriptomic studies have demonstrated that plants rapidly respond to wounding and begin to modify gene expression within the first hour after injury (55, 106). Importantly, early upregulated genes are not limited to those implicated in typical defense or stress responses, e.g., genes required for JA biosynthesis and signaling, but also include genes that participate in regeneration (55). It is thus likely that plants commit to regeneration soon after they detect injury signals and quickly initiate reprograming of cell fates by modifying the transcriptional landscape. Specific effects of wounding on each regeneration response are discussed in the following sections.

Root Regeneration

Auxin plays pivotal roles in both root meristem restoration and de novo root formation, although its cellular basis appears largely distinct for each process. In intact root tips, auxin response is maximal at the quiescent center and columella root cap, and following loss of the distal part of the meristem by injury, the auxin maximum is respecified proximally (29) (**Figure 3*a***). Treatment with the auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) blocks this mode of root regeneration, suggesting that reestablishment of the auxin maximum via its polar transport is instructive

Table 1 Regulators of plant regeneration

AGI	Name	Gene description	Function in regeneration					Reference(s)
			Root	Callus	Shoot	Embryogenesis	Grafting	
Transcriptional regulation								
AT3G03660	WOX11	Homeobox	■		■			51, 83, 84, 113
AT5G17810	WOX12	Homeobox	■					51, 84, 113
AT3G11260	WOX5	Homeobox	■		■	■		51, 71, 120
AT5G05770	WOX7	Homeobox	■		■			51, 71
AT1G20700	WOX14	Homeobox			■			71
AT2G17950	WUS	Homeobox		■	■	■		36, 39, 95, 121, 146
AT5G13790	AGL15	MADS box				■		41, 49
AT1G62360	STM	KNOX			■			146
AT3G15170	CUC1	NAC domain			■			23
AT5G53950	CUC2	NAC domain			■			23, 70, 146
AT1G56010	NAC1	NAC domain	■					17
AT4G17980	ANAC071	NAC domain					■	1, 103
AT1G78080	WIND1	AP2/ERF	■	■	■	■		44, 62, 64–66
AT1G22190	WIND2	AP2/ERF		■				66
AT1G36060	WIND3	AP2/ERF		■				66
AT5G65130	WIND4	AP2/ERF		■				66
AT5G07310	ERF115	AP2/ERF	■	■				44, 55
AT3G20840	PLT1	AP2/ERF	■			■		12, 70, 110
AT1G51190	PLT2	AP2/ERF	■			■		12, 70, 110, 120
AT5G10510	PLT3	AP2/ERF			■			55, 70
AT5G57390	PLT5	AP2/ERF		■	■	■		55, 70, 128
AT5G65510	PLT7	AP2/ERF			■			55, 70
AT1G12980	ESR1	AP2/ERF		■	■			5, 62
AT1G24590	ESR2	AP2/ERF		■	■			52, 53
AT5G13330	RAP2.6L	AP2/ERF			■		■	1, 15, 141
AT5G17430	BBM	AP2/ERF				■		49
AT1G28300	LEC2	B3				■		11, 49, 118, 119
AT2G33860	ARF3	B3, ARF			■			18
AT1G19850	ARF5	B3, ARF	■					29
AT5G20730	ARF7	B3, ARF	■	■				31, 74
AT1G19220	ARF19	B3, ARF	■	■				31, 74
AT4G14550	SLR/IAA14	Aux/IAA		■				66, 111
AT3G62100	IAA30	Aux/IAA				■		149
AT3G16857	ARR1	Type-B ARR	■	■	■			12, 55, 66, 95, 108, 146, 150
AT4G31920	ARR10	Type-B ARR	■	■	■			12, 95, 146, 150
AT2G25180	ARR12	Type-B ARR	■	■	■			12, 66, 95, 146, 150
AT2G42430	LBD16	LOB	■	■	■			31, 74, 75, 83, 139
AT2G42440	LBD17	LOB		■				31
AT2G45420	LBD18	LOB		■				31
AT3G58190	LBD29	LOB	■	■				31, 74, 75, 101, 138

(*Continued*)

Table 1 (*Continued*)

AGI	Name	Gene description	Function in regeneration					Reference(s)
			Root	Callus	Shoot	Embryogenesis	Grafting	
AT2G34710	PHB	HD ZIP III			■			146
AT1G30490	PHV	HD ZIP III			■			146
AT5G60690	REV	HD ZIP III			■			146
AT3G54220	SCR	GRAS			■			71
AT4G37650	SHR	GRAS	■					12
AT5G48150	PAT1	GRAS	■	■				44
AT2G31370	bZIP59	bHLH		■				139
AT5G62940	HCA2	Dof					■	92
AT2G36010	E2Fa	E2F			■			82
AT1G21970	LEC1	NF-Y				■		49, 67, 85
AT5G53040	RKD4	RWP-RK				■		131
Epigenetic regulation								
AT2G23380	CLF	PRC2 subunit		■	■	■		13, 43, 54, 146
AT4G02020	SWN	PRC2 subunit		■	■	■		13, 43, 54, 146
AT4G16845	VRN2	PRC2 subunit		■		■		54
AT5G51230	EMF2	PRC2 subunit		■		■		54
AT3G20740	FIE	PRC2 subunit		■		■		9, 54
AT3G21820	ATXR2	SET domain		■				74
AT3G20810	JMJ30	Jumonji-C		■				75
AT3G54610	HAG1/GCN5	GNAT/MYST		■	■			71
AT5G49160	MET1/DDM2	DNA methylation			■			81, 82
Others								
AT4G32540	YUC1	Auxin synthesis	■		■	■		4, 16, 18
AT4G13260	YUC2	Auxin synthesis	■					16
AT5G11320	YUC4	Auxin synthesis	■		■	■		4, 16, 18
AT5G25620	YUC6	Auxin synthesis	■					16
AT1G48910	YUC10	Auxin synthesis				■		4
AT1G21430	YUC11	Auxin synthesis				■		4
AT5G11030	ALF4	Auxin signaling		■			■	21, 94, 111, 122
AT2G01830	AHK4/WOL	Cytokinin signaling			■	■		46, 120
AT3G63110	IPT3	Cytokinin synthesis		■	■			18, 55
AT5G19040	IPT5	Cytokinin synthesis			■			18
AT2G28305	LOG1	Cytokinin synthesis		■				55
AT3G53450	LOG4	Cytokinin synthesis		■				55
AT4G35190	LOG5	Cytokinin synthesis		■				55

(*Continued*)

Table 1 (*Continued*)

AGI	Name	Gene description	Function in regeneration					Reference(s)
			Root	Callus	Shoot	Embryogenesis	Grafting	
AT4G30290	XTH19	Xyloglucan transglucosylase/hydrolase					■	1, 103
AT5G48070	XTH20	Xyloglucan transglucosylase/hydrolase					■	1, 103
AT1G30760	FAD-BD	BBE-like enzyme		■				139
AT1G53830	PME2	Pectin methylesterase		■				138
AT1G01120	KCS1	VLCFA synthase		■				111
AT5G50260	CEP1	Cysteine peptidase	■					17
AT3G48340	CEP2	Cysteine peptidase	■					17
AT4G34160	CYCD3;1	Cyclin		■	■			55, 82
AT5G67260	CYCD3;2	Cyclin		■	■			55, 82
AT3G50070	CYCD3;3	Cyclin		■	■			55, 82
AT5G10490	MSL2	MscS-like protein		■				136
AT1G58200	MSL3	MscS-like protein		■				136
AT1G73590	PIN1	Auxin transporter	■		■	■		39, 12, 121
AT5G57090	PIN2	Auxin transporter	■					12
AT1G70940	PIN3	Auxin transporter	■					12
AT2G38120	AUX1	Auxin transporter	■					12
AT5G65870	PSK5	Peptide hormone	■					44

for meristem reconstruction (110). Genetic evidence also shows that AUXIN RESPONSE FACTOR 5 (ARF5) mediates this process (29). In addition to auxin-dependent positional cues, local induction of reprogramming regulators at wound sites is also important for meristem reconstruction (**Figure 3*a***). *ERF115*, which encodes an APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF)-type transcription factor, is induced upon amputation of the root apical meristem, and together with its interacting partner PHYTOCHROME A SIGNAL TRANSDUCTION 1 (PAT1), ERF115 promotes reconstruction of the meristem (44). Cells adjacent to those damaged during root tip excision express ERF115, which in turn induces expression of *PHYTOSULFOKINE 5* (*PSK5*), subsequently inducing cell proliferation (44, 45). In addition, ERF115 likely promotes acquisition of cellular competency via transcriptional induction of *WOUND-INDUCED DEDIFFERENTIATION 1* (*WIND1*) (44), which encodes another AP2/ERF transcription factor, this one functioning as a key regulator of cellular reprogramming as discussed in detail below. These observations suggest that global spatial information provided by auxin distribution, in combination with local activation of mitosis induced by reprogramming regulators, enables spatially controlled cell proliferation and patterning during meristem reconstruction. Intriguingly, two key regulators of stem cell specification and maintenance during root development, PLETHORA 1 (PLT1) and PLT2, are dispensable for meristem restoration,

although they are required for the initiation of new root meristems during de novo root formation (12, 110). This reinforces the idea that the meristem repair process is mechanistically different from the generation of new root meristems.

Auxin also plays pivotal roles in de novo root formation (16, 84, 123). In explants competent for root regeneration, cellular auxin response, as visualized by a synthetic reporter, *DR5::GUS*, becomes detectable by 12–18 h after cutting near wound sites, particularly in the vasculature, from which de novo roots eventually emerge (12, 16, 17, 84, 123) (**Figure 3*b***). Polar auxin transport is critical for auxin-mediated promotion of de novo root formation, because inhibition of auxin transport by NPA blocks both *DR5::GUS* expression near wound sites and subsequent root regeneration (12, 17, 84, 123). Genetic evidence suggests that auxin transporters, such as PIN-FORMED 1 (PIN1), PIN2, PIN3 and AUXIN RESISTANT 1 (AUX1), play roles in root regeneration from leaf explants (10). Auxin biosynthesis also contributes to root regeneration from leaf explants, as rooting is suppressed by chemical inhibition of auxin biosynthesis or in *yucca1* (*yuc1*) *yuc2 yuc4 yuc6* quadruple mutants defective in auxin production (16). *YUC4* gene expression is enhanced within 4 h after excision in mesophyll cells distant from wound sites, and within 2 days, it also becomes elevated in approximately the same region where the auxin response maximum is observed (16). Early activation of auxin biosynthesis likely contributes to increasing the pool of auxin available for polar transport toward wound sites, whereas late activation of auxin synthesis near wound sites is likely important for maintaining the auxin maximum preceding meristem initiation.

Auxin accumulation near wound sites promotes the activity of ARFs, which directly activate *WOX11* to initiate cell fate transition (84, 129) (**Figure 3*b***). WOX11, together with its close homolog WOX12, promotes the conversion of regeneration-competent vasculature cells near wound sites to root founder cells, a process known as first-step cell fate transition during root regeneration (84, 113). In turn, WOX11 and WOX12 activate another pair of WOX family members, *WOX5* and *WOX7*, to convert root founder cells into root primordia (16, 51). WOX11, in addition, directly upregulates the expression of *LATERAL ORGAN BOUNDARIES DOMAIN 16* (*LBD16*), which encodes a transcriptional regulator of root meristem initiation (84, 113). Interestingly, LBD16 participates in both root regeneration and lateral root formation, suggesting that this factor is a general downstream effector of auxin-mediated root meristem formation (84, 101, 113). WOX11, on the other hand, is involved exclusively in root regeneration (113), implying that it acts only in a specific developmental or environmental context.

The final step of root regeneration from leaf explants involves outgrowth of roots through the surrounding cell layers of explants, and this process is regulated independently of auxin. A NAC domain transcription factor, NAC1, is activated within 2 days after wounding, and it is required for efficient root regeneration (17). Interestingly, NAC1 is expressed in leaf mesophyll cells surrounding emerging root primordia, and it appears to facilitate the modification of cell wall properties through transcriptional regulation of the Cys endopeptidase-encoding genes *CEP1* and *CEP2* (17). How these auxin-dependent and auxin-independent cellular events are coordinated to complete root regeneration remains unknown.

Callus Formation

Callus formation can be induced by application of auxin and cytokinin or in response to wounding and other types of severe stress (59). CIM-induced callus formation proceeds via a root meristem-associated pathway (122). Calli that develop on CIM have histological features resembling the root meristem, with organized spatial expression of root meristem regulator genes such as *WOX5* and *SHORT ROOT* (*SHR*) (122) (**Figure 4*a***). Furthermore, the *solitary root* (*slr*) mutant,

which contains a gain-of-function mutation in IAA14, an Aux/IAA repressor of auxin signaling, is defective in both lateral root formation and callus formation on CIM (34, 66). Similarly, an *aberrant lateral root formation 4* (*alf4*) mutant, in which auxin signaling is compromised (3), fails to form both lateral roots and callus (26, 122). As occurs in lateral root development, auxin leads to degradation of IAA14 and subsequent activation of ARF7 and ARF19 (33), which in turn directly activate expression of *LBD16*, *LBD17*, *LBD18*, and *LBD29* to promote callus formation (31, 101) (**Figure 4*a***). LBD18 further reinforces auxin-induced callus induction through positive feedback regulation on *ARF7* and *ARF19* transcription (102). In addition, these LBDs regulate cellular processes associated with root initiation or callus formation. For instance, LBD18, together with LBD33, promotes cell proliferation through transcriptional activation of the core cell cycle regulator *E2 PROMOTER BINDING FACTOR a* (*E2Fa*) (6). LBD18 also modulates cell wall properties through direct transcriptional activation of *EXPANSIN 14* (*EXP14*) (73). A recent study further revealed that LBD16 forms a heterodimer complex with the transcription factor basic region/leucine zipper motif 59 (bZIP59) and directly activates *FAD-BD*, which encodes a FAD-binding Berberine enzyme that catalyzes oxidation of monolignols in the cell wall (139). Additionally, LBD29 seems to be involved in the modification of cell walls, given that a cell wall–related process is a highly enriched gene ontological category among LBD29 targets identified via transcriptome and chromatin immunoprecipitation analyses. Finally, the cell wall modifier *PECTIN METHYLESTERASE 2* (*PME2*) is among the LBD29 targets that can recapitulate the LBD29 overexpression phenotype when expressed at high levels (138).

As opposed to auxin, which promotes callus formation from pericycle cells, very-long-chain fatty acids (VLCFAs) or their derivatives negatively regulate callus formation on CIM (111). VLCFA synthesis is catalyzed by the enzyme 3-KETOACYL-COA SYNTHASE 1 (KCS1), whose mutation enhances callus formation from pericycle cells. VLCFA-derived signals appear to repress callus growth at least in part by repressing the expression of *ALF4*. Interestingly, KCS1 is expressed in cell layers surrounding the pericycle and also in emerging lateral root primordia, yet only its expression in cortex cells is sufficient to complement the *kcs1* mutant (111). Therefore, it is likely that VLCFAs or their derivatives from surrounding cell layers suppress organogenic activity of pericycle cells non-cell autonomously. Further elucidation of VLCFA-derived molecules and their signaling cascades should uncover novel aspects of callus regulation.

Callus formation at wound sites, by contrast, does not seem to rely on auxin signaling or a root initiation pathway, because the efficiency of callus formation at wound sites is not affected in *slr* (66). Wounding, instead, promotes cytokinin biosynthesis via transcriptional activation of genes encoding its biosynthetic enzymes, including *ISOPENTENYL TRANSFERASE 3* (*IPT3*), *LONELY GUY 1* (*LOG1*), *LOG4*, and *LOG5* (**Figure 4*b***). Accordingly, mutants for these biosynthetic enzymes display defects in wound-induced callus formation (55). Wound stress, in addition, induces expression of reprogramming regulators, such as *WIND1* and its homologs *WIND2*, *WIND3*, and *WIND4* (66) (**Figure 4*b***). WINDs play key roles in callus formation at wound sites: Plants overexpressing one of the *WIND* genes develop callus in the absence of exogenous hormones, and dominant repression of WIND1 strongly represses wound-induced callus formation. WIND1 likely participates in the activation of cytokinin signaling at wound sites because expression of a cytokinin response marker is diminished in *WIND1-SRDX* plants (66). Cytokinin synthesis and WIND-dependent pathways converge on the activation of cytokinin signaling mediated by type-B ARABIDOPSIS RESPONSE REGULATOR 1 (ARR1) and ARR12 (55, 66), which leads to cell cycle reentry via upregulation of *CYCLIN D3;1* (*CYCD3;1*) (55). In addition to the *WIND* genes, wounding upregulates genes encoding other AP2/ERF transcription factors, including *ERF115*, *PLT3*, *PLT5*, and *PLT7*, which are all involved in wound-induced callus formation (**Figure 4*b***) (55). Given that none of the corresponding mutants completely lacks callus

formation, concurrent upregulation of multiple reprogramming regulators likely ensures robust induction of wound-induced callus formation. A recent genetic study suggests that abiotic stress beyond wounding also triggers callus induction in a WIND- and cytokinin-dependent manner. The *msl2 msl3* double mutant affected in two mechanosensitive channel of small conductance (MscS)-like plastid mechanosensors suffers from constitutive osmotic stress and consequently develops callus that resembles wound-induced callus with increased cytokinin levels and elevated *WIND1* and *WIND3* expression (136).

Pluripotency: cellular potential to give rise to various cell types

Acquisition of Competency for Shoot Regeneration

Explants incubated on CIM undergo rounds of cell proliferation to produce a mass of callus cells. A recent study by Kareem et al. (70) uncovered that an additional key process beyond propagation of the cell population must be activated during CIM incubation to confer pluripotency to callus cells. Upon incubation on CIM, auxin induces PLT3, PLT5, and PLT7 expression, which confers pluripotency to callus cells (70) (**Figure 4*a***). PLT3, PLT5, and PLT7 induce root meristem regulators PLT1 and PLT2 to convey competency as well as shoot-promoting transcription factors CUP-SHAPED COTYLEDON 1 (CUC1) and CUC2 (23). Accordingly, overexpressing PLT1, together with CUC2, overcomes shoot regeneration defects in the *plt3 plt5 plt7* triple mutant (70). Another line of evidence indicates that cells competent for shoot regeneration are embedded in the root meristem. Using a method to induce shoot regeneration directly from lateral root primordia without incubation on CIM, Rosspopoff et al. (107) showed that emerging root primordia at a specific developmental window, namely when *WOX5* begins to be expressed, are competent for transformation into shoot apical meristems. This observation is consistent with the genetic requirement of root meristem regulators, such as PLTs, for the acquisition of shoot regeneration competency (**Figure 4*a***). Similarly, other regulators of root meristem formation such as WOX11 and LBD16 (83) as well as WOX5, WOX7, WOX14, and SCARECROW (SCR) (71) are involved in the acquisition of competency for shoot regeneration.

Shoot Regeneration

Cytokinin induces shoot regeneration from competent cells, and molecular components associated with its perception and signaling mediate this process. The cytokinin receptor AHK4/WOL plays a major role in shoot regeneration (46), and plants overexpressing a downstream signaling component, such as *ARR1*, regenerate shoots in the absence of exogenous cytokinin application (108). The role of auxin during shoot regeneration is less well established, but *YUC1* and *YUC4* are induced on SIM, and YUC-mediated auxin biosynthesis is required for efficient shoot regeneration (18). Imaging analyses revealed that cells either with high cytokinin responses or with high auxin responses undergo spatial compartmentalization prior to meristem initiation (**Figure 5**) (18). Reciprocal suppression of cytokinin and auxin signaling appears to govern these mutually exclusive hormonal response patterns. Specifically, *ARF3* is transcriptionally activated by auxin, and the repressor-type ARF encoded by this gene in turn represses *IPT5*, which encodes an enzyme involved in cytokinin biosynthesis (18). Conversely, type-B ARRs, including ARR1, ARR10, and ARR12, directly suppress *YUC4* expression to prevent auxin biosynthesis in cytokinin response domains (95).

A key molecular event underlying cytokinin-induced shoot regeneration is the transcriptional activation of *WUSCHEL* (*WUS*) (**Figure 5**). Increasing the level of *WUS* is sufficient to trigger shoot formation ectopically (36), and a loss-of-function mutant for *WUS* fails to regenerate shoots on SIM (39). Cytokinin activates *WUS* expression, and multiple recent studies have demonstrated that ARR1, ARR10, and ARR12 directly upregulate *WUS* expression (95, 132, 146, 150).

Accordingly, *WUS* overexpression rescues shoot regeneration defects in *arr1 arr12*, supporting the hypothesis that transcriptional activation of *WUS* is a core event downstream of cytokinin-induced shoot regeneration (95). Intriguingly, however, only select cells start to express *WUS* in callus cultured on SIM, despite activation of cytokinin response throughout the tissue, indicating that activation of cytokinin signaling alone is not sufficient to induce *WUS* (146). The class III homeodomain-leucine zipper (HD Zip III) transcription factors PHABULOSA (PHB), PHAVOLUTA (PHV), and REVOLUTA (REV) are additional regulators required for cytokinin-dependent *WUS* induction and subsequent shoot regeneration (**Figure 5**) (146). These HD Zip III proteins directly interact with type-B ARRs to activate *WUS* expression (146). Interestingly, HD Zip III proteins upregulate other core regulatory factors for shoot meristem formation including the homeobox transcription factor *SHOOT MERISTEMLESS* (*STM*) (114) as well as the AP2/ERF-type transcription factor *RAP2.6L* (15, 141), further demonstrating that HD Zip III proteins are key developmental regulators of shoot regeneration.

As mentioned above, cutting is another inductive cue for shoot regeneration (**Figure 2*b***). WIND1 functions in wound-induced signaling to promote shoot regeneration, as overexpression of *WIND1* is sufficient to promote shoot regeneration in uncut *Arabidopsis* seedlings (65). Conversely, *WIND1-SRDX* explants are severely defective in shoot regeneration on SIM. A recent study has shown that WIND1 regulates shoot regeneration via direct transcriptional activation of *ENHANCER OF SHOOT REGENERATION 1* (*ESR1*) (**Figure 5**) (62). ESR1 and its paralog ESR2 are key regulators of shoot formation. Ectopic expression of either *ESR1* or *ESR2* is sufficient to trigger shoot formation, and the *esr1 esr2* double mutant is severely defective in shoot regeneration (5, 52, 62). Importantly, increasing the level of *ESR1* expression permits shoot regeneration from *Arabidopsis* explants that do not normally develop shoots from wound sites (62); thus, insufficient *ESR1* expression might be one important limiting factor that underlies recalcitrance for shoot regeneration.

Protoplast Regeneration

Regeneration from protoplasts differs from the processes described above in that it occurs from a single, isolated cell (**Figure 2*c***). Transcriptomic studies investigating *Arabidopsis* protoplast regeneration have shown that protoplasts undergoing dedifferentiation display gene expression profiles resembling those associated with stress responses and stress-associated cellular senescence (21, 24). Similar trends are also observed in *Physcomitrella patens* protoplasts (133, 137). Cellular oxidative stress is transiently elevated upon isolation of protoplasts, and ROS levels are several-fold higher within 1 day after protoplast isolation (127). Subsequent recovery from acute oxidative stress may be important for successful regeneration from protoplasts, because mutation of the gene encoding CATALASE 3, which is involved in hydrogen peroxide degradation, abolishes initial division of isolated protoplasts (127). Stress-mediated pathways may also promote cellular pluripotency; for example, some reprogramming regulators, including *WIND1*, are stably induced after protoplast isolation (21). Given that protoplast-derived callus and CIM-induced callus have distinct cellular origins, the question of whether these two processes are governed by similar regulatory mechanisms arises. Genetic analyses have demonstrated that *ALF4* is required for both callus induction on CIM and protoplast division (21, 122), indicating at least some level of similarity between the regulatory pathways that govern these two different modes of callus formation.

Somatic Embryogenesis

Developmental events underlying somatic embryogenesis have been best characterized using immature zygotic embryos of *Arabidopsis*, from which somatic embryos can be induced indirectly

following an intermediate callus stage (121) (**Figure 6**). In this procedure, zygotic embryos are precultured with the synthetic auxin 2,4-dichlorophenoxyacetic acid, which causes embryonic callus formation. Upon transfer of the embryonic calli to auxin-free medium, auxin response maxima are established at peripheral regions within the callus via PIN1-dependent directional auxin transport (**Figure 6**). The level of endogenous auxin also increases within 12 h after the transfer, and YUC-dependent de novo synthesis of auxin consistently contributes to efficient somatic embryogenesis (4). Within 24 h after media transfer, *WUS* and *WOX5* start to be expressed in subsets of cells near the auxin response maxima. Early on, *WUS*- and *WOX5*-expressing domains nearly overlap, but these domains gradually separate to specify the shoot and root poles, respectively (120, 121) (**Figure 6**). The molecular link between auxin response and *WUS* expression is not clear, but pharmacological evidence shows that auxin transport is required for *WUS* expression and subsequent somatic embryogenesis (121), suggesting that generation of auxin maxima by PIN1 is required for *WUS* expression. Cellular cytokinin response is also established upon transfer to auxin-free medium, and cytokinin signaling is also required for somatic embryogenesis (120). The cytokinin response domains initially overlap with those of auxin response at the edge of embryonic callus, with the cytokinin response domains later becoming internalized and restricted to the domain of *WOX5* expression (120) (**Figure 6**).

How somatic cells gain an embryonic fate under this culture condition is an important but unanswered question (40). The CCAAT-binding NF-Y transcription factor LEAFY COTYLEDON 1 (LEC1) and the B3 domain transcription factor LEC2 are embryo-specific regulators that are both necessary and sufficient for somatic embryogenesis (10, 35). A clear sign of embryonic transition is found when these genes are transcriptionally activated on auxin-free medium, and, interestingly, this appears to follow the establishment of *WUS* and *WOX5* expression domains (120, 121). In addition to *LEC1* and *LEC2*, several other genes encoding transcriptional regulators that function in zygotic embryogenesis, such as the MADS-box transcription factor AGAMOUS-LIKE 15 (AGL15), the AP2/ERF transcription factor BABY BOOM (BBM), and RWP-RK DOMAIN-CONTAINING 4 (RKD4), also induce somatic embryogenesis when overexpressed (32, 41, 48, 85, 119, 131). Similarly, overexpression of several other AP2/ERF-encoding genes, such as *WIND1*, *PLT1*, *PLT2*, *PLT3*, *PLT5*, or *PLT*7, induces embryogenesis in *Arabidopsis* seedlings (48, 59, 128), although a functional requirement for auxin-induced somatic embryogenesis has been shown only for PLT2 (120). Several genome-wide transcriptome analyses have uncovered a highly interconnected transcriptional network among these embryonic transcriptional regulators (**Figure 6**). LEC2, for instance, forms a positive feedback loop with AGL15 (11, 149). A recent study also demonstrated that BBM directly activates *LEC1*, *LEC2*, and *AGL15*, and, conversely, LEC1, LEC2, and AGL15 are required for *BBM* expression, further corroborating the mutual regulatory relationships among these embryonic regulators (49).

Many of these embryonic regulators appear to promote embryogenesis by modulating auxin biosynthesis and signaling. Both LEC2 and AGL15, for example, activate expression of *IAA30*, and *iaa30* loss-of-function mutants show defects in AGL15-mediated somatic embryogenesis (149), demonstrating that IAA30 functions downstream of AGL15 (**Figure 6**). LEC1 induces auxin biosynthesis via induction of *YUC10*, and LEC2 activates *YUC2* and *YUC4* (67, 118, 148). Similarly, BBM directly binds loci encoding auxin biosynthesis enzymes including YUC3 and YUC8, implying that it may also regulate auxin homeostasis (49). Given that establishment of auxin maxima is essential for embryonic gene expression in vitro (121), regulatory relationships between these transcriptional regulators and auxin response are also likely mutually dependent.

CONNECTION OF VASCULATURE DURING HAUSTORIUM FORMATION IN PARASITIC PLANTS

Upon invasion of host plants, parasitic plants, such as *Striga* and other members of the Orobanchaceae family, develop specialized structures known as haustorium (144). Parasitic plants use haustoria to establish vascular connections with host plants, through which they draw nutrients and water. Therefore, in some sense, haustorium formation resembles grafting because both processes involve the formation of new vascular connections between two plants (91). Similar to grafting, auxin promotes vascular connection during haustorium formation in *Phtheirospermum japonicum* (60). Upon perception of a host-derived chemical compound such as 2,6-dimethoxy-*p*-benzoquinone, *P. japonicum* roots activate *PjYUC3* expression, leading to the formation of an auxin maximum at the position of future haustorium formation.

Stem Repair and Grafting

Molecular mechanisms of tissue repair are studied using incised *Arabidopsis* floral stems. Cells neighboring incision sites transiently proliferate until they are sealed. Both ethylene and apically derived auxin promote transcription of *NAC DOMAIN CONTAINING PROTEIN 71* (*ANAC071*), particularly above incision sites (1). ANAC071 is a key regulator of tissue repair and directly upregulates the *XYLOGLUCAN ENDOTRANSGLUCOSYLASES/HYDROLASE 19* (*XTH19*) and *XTH20* genes, which promote pith cell proliferation through as-yet-unknown mechanisms (103). Regulatory pathways acting below incision sites are also important for successful healing because *RAP2.6L* is expressed below the incision and is required for complete tissue repair (1).

Arabidopsis hypocotyls are mainly used to study mechanisms of grafting, where vascular connections between rootstocks and scions can be established within a few days (94, 142). Auxin signaling in rootstocks is important for phloem reconnection (94) because mutants for *ALF4* are defective in this process. Basipetal auxin flow across graft sites results in accumulation of auxin in rootstocks, which promotes vascular reconnection at least partly via the DNA-binding with one zinc finger (Dof) transcription factor HIGH CAMBIAL ACTIVITY 2 (HCA2) (92). It is interesting to note that parasitic plants also utilize an auxin-dependent mechanism to establish vascular connections with host plants (see the sidebar titled Connection of Vasculature During Haustorium Formation in Parasitic Plants). Another plant hormone, gibberellin, also promotes the ability of cortex cells at graft junctions to expand and fill in the gap between rootstocks and scions (88), although its contribution to the successful reestablishment of vasculature connections remains unclear.

Epigenetic Control of Regeneration

As discussed in the above sections, regeneration often involves activation of key transcriptional regulators, causing dynamic changes in gene expression and subsequently leading to changes in cell fate. Accumulating evidence suggests that the transcription of many reprogramming genes is epigenetically regulated, permitting them to be induced or repressed in the right cells at the correct developmental window of time (56). Chromatin environment is influenced by various covalent modifications of histones, and acetylation and methylation of lysine or arginine residues in their N-terminal tails are associated with activation or repression of gene expression. Histone H3 lysine 27 trimethylation (H3K27me3) maintains the repressive status of target genes, and this methylation is mediated by an evolutionarily conserved protein complex, POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) (47). A well-characterized developmental role of PRC2 is to repress the expression of genes encoding embryonic regulators, including *LEC2* and *BBM*, and

reprogramming regulators, like *WIND3*, to prevent ectopic onset of embryogenesis and callus formation. In PRC2 mutants, these PRC2-targeted genes are ectopically expressed, leading to spontaneous somatic cellular dedifferentiation, callus formation, and embryoid development (9, 13, 54). PRC2-mediated histone modification can also have a positive effect on organ regeneration in tissue culture conditions; it seems to be involved in transcriptional repression of cell identity in explants undergoing cell fate change (43).

An important question is how epigenetically repressed loci acquire permissive chromatin states that facilitate activation of gene expression to initiate regeneration. A recent study revealed that dilution of repressive histone marks through rounds of cell division is required for transcriptional activation of *WUS* on SIM (146). Wild-type plants experience a couple days of lag between cytokinin application and onset of *WUS* expression, whereas inhibition of cell cycle progression by olomoucine further delays *WUS* induction after cytokinin application. PRC2 mutants, by contrast, express *WUS* rapidly after cytokinin application, indicating that passive loss of H3K27me3 during cell division permits activation of PRC2-target genes (146). Strongly suggesting a cell cycle–independent mechanism for overcoming repression, transcription of PRC2-targeted loci, such as *PLT3*, *PLT5*, *PLT*7, and *ERF115*, is activated upon wounding a few days prior to initiation of cell proliferation (55). In addition, H3K27me3 levels on genes involved in auxin homeostasis, *GH3.2* and *IAA2*, dramatically decline well before the activation of cell proliferation on CIM (43), implying that H3K27me3 is actively removed at these loci. Consistent with these findings, the histone demethylase JUMONJI 30 (JMJ30) promotes callus growth via removal of repressive histone marks. JMJ30 has biochemical activity that removes H3K27me3 and H3K9me3 (37, 75), and during callus formation JMJ30 reduces H3K9me3 on *LBD16* and *LBD29* (75). However, it remains unclear if JMJ30 counteracts PRC2-dependent repression.

Transcriptional activation is often associated with histone modifications like H3K36me3 and H3 acetylation. Deposition of H3K36me3 by ARABIDOPSIS TRITHORAX RELATED 2 (ATXR2) promotes callus formation on CIM via transcriptional upregulation of *LBD16* and *LBD29* (74). Interestingly, ATXR2 and JMJ30 act synergistically to induce and maintain these target genes (75). Both ATXR2 and JMJ30 physically interact with ARF7 and ARF19, through which they may be recruited to target loci (74, 75). HISTONE ACETYLTRANSFERASE OF THE GNAT/MYST SUPERFAMILY 1 (HAG1), also known as GENERAL CONTROL NONREPRESSED 5, plays a pivotal role in the acquisition of shoot regeneration competency (71). Although severely compromised in shoot regeneration on SIM, *hag1* mutants form callus normally on CIM. The shoot regeneration defects of *hag1* mutants are associated with reduced levels of histone acetylation at loci such as *WOX5*, *SCR*, *PLT1*, and *PLT2* and can be overcome by ectopic expression of *WOX5* and *SCR* (71). These results thus indicate that HAG1 is responsible for histone acetylation and subsequent transcriptional induction of *WOX5* and *SCR* to confer cellular pluripotency.

DNA methylation can serve as another epigenetic mechanism to repress target gene expression. Several lines of evidence show that reduced levels of DNA methylation promote organ regeneration in several plant species. Short-term application of the DNA methyltransferase inhibitor 5-azacytidine, for instance, promotes microspore embryogenesis in *Brassica napus* and *Hordeum vulgare* (7, 116). Similarly, an *Arabidopsis* loss-of-function mutant for the DNA methyltransferase *MET1* displays improved shoot regeneration on SIM with precocious *WUS* expression (81). This MET1-dependent mechanism appears to prevent *WUS* expression downstream of cytokinin signaling and E2Fa-dependent cell cycle regulation (82). After prolonged incubation on SIM, *MET1* expression becomes restricted to the outer cell layer of callus, thus allowing ARR-mediated activation of *WUS* expression in inner cell layers.

CONCLUSIONS AND PERSPECTIVES

We have made unprecedented progress in recent years in our molecular understanding of how stress- and hormone-mediated pathways promote various modes of regeneration. It is now apparent that initiating a regenerative program often involves transcriptional activation of meristematic and/or embryonic regulators and that auxin and cytokinin play pivotal roles in such control. What remains unsolved is how these factors promote such diverse arrays of regenerative processes, i.e., from tissue repair to generation of new shoots, roots, or embryos, depending on which tissues or organs are used and how they are cultured in vitro. Gradual compartmentation of auxin and/or cytokinin response domains plays a central role in determining future root or shoot meristem formation, and further elucidation of their molecular details will be essential for working out exactly how new cell fates are specified in different regeneration contexts. The interplay between hormonal signaling and stress signaling is key for defining cell fate, and a recent large-scale analysis unveiled the gene regulatory network that underlies this crosstalk (58). Providing an important discovery, a study employing single-cell transcriptome analysis showed that cells transit through a mixed identity state before taking on a defined cell fate during meristem restoration (29). It will be interesting to explore whether other types of regeneration also involve similar intermediate states during cell fate transitions. The finding that root meristem regulators define pluripotency under in vitro culture conditions (70, 71, 107) is another exciting breakthrough, because it provides the first molecular insight into this enigmatic concept. Uncovering exactly how these regulators confer pluripotency and how root meristem cells, or more precisely their stem cells, acquire shoot fate when exposed to cytokinin will be important goals in future studies. In addition, wounding likely confers pluripotency through different mechanisms, and exploring how wound-induced reprogramming regulators such as WINDs and other AP2/ERF transcription factors participate in this control should help further identify the molecular signature of pluripotency. Identification of epigenetic mutants displaying ectopic callus formation and somatic embryogenesis has highlighted the need for a mechanism to prevent unscheduled cellular reprogramming during normal development. Key unanswered questions include how hormonal and/or stress signals modify the chromatin state at specific loci under given conditions. Several epigenetic regulators are recruited to target loci by binding to a specific set of transcriptional regulators (76). Testing how widespread this regulatory phenomenon is in the context of regeneration will be an interesting future task.

Having identified a number of key regulators in *Arabidopsis*, we can now start exploring the molecular basis behind other diverse modes of regeneration in additional plant species. It may also be possible to develop a diagnostic tool to investigate why many crop cultivars are recalcitrant and design a protocol to overcome this long-standing problem (57). Several pioneering studies have developed new genetic tools to boost regeneration efficiencies in both dicot and monocot plants (64, 65, 86) (see the sidebar titled Regeneration in Monocot Plants). Pharmacological approaches for generating accessible chromatin present an alternative strategy for overcoming recalcitrance as demonstrated in *Arabidopsis* and Brassica (7, 79). Further refinement of these approaches, for instance, by locus-specific modification of histone marks and DNA methylation status by CRISPR-Cas9 technology, should facilitate more precisely targeted improvement of regeneration efficiencies.

SUMMARY POINTS

1. Tissue or organ regeneration usually involves multiple intermediate steps of cellular reprogramming and is often accompanied by cell cycle progression.

2. Wound stress promotes callus formation and organ regeneration by activating a set of reprogramming regulators including *ERF115*, *WIND1–WIND4*, *PLT3*, *PLT5*, and *PLT7*.
3. Wounding also promotes biosynthesis and signaling of auxin and cytokinin in a context-dependent manner to induce callus formation and organ regeneration.
4. Auxin and cytokinin generally play key roles in promoting cell proliferation and reprogramming.
5. Production of auxin maxima via its directional transport is instructive for generating and patterning new root meristems during root regeneration and somatic embryogenesis.
6. Several regulators of root meristem formation, such as *PLTs*, *WOXs*, *LBD16*, and *SCR*, are required to acquire cellular competency for shoot regeneration.
7. Type-B ARR-mediated cytokinin signaling induces *WUS* expression to establish shoot meristems during shoot regeneration.
8. Many genes encoding reprogramming regulators, including *WOX5*, *WUS*, *PLT*s, and *WIND3*, are epigenetically repressed in normal development and need to be activated by stress and/or hormonal signaling.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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146. Shows that dilution of repressive histone marks via cell proliferation is a prerequisite for cytokinin-mediated WUS activation during shoot regeneration.