

REVIEW

Plant regeneration: cellular origins and molecular mechanisms

Momoko Ikeuchi, Yoichi Ogawa, Akira Iwase and Keiko Sugimoto*

ABSTRACT

Compared with animals, plants generally possess a high degree of developmental plasticity and display various types of tissue or organ regeneration. This regenerative capacity can be enhanced by exogenously supplied plant hormones *in vitro*, wherein the balance between auxin and cytokinin determines the developmental fate of regenerating organs. Accumulating evidence suggests that some forms of plant regeneration involve reprogramming of differentiated somatic cells, whereas others are induced through the activation of relatively undifferentiated cells in somatic tissues. We summarize the current understanding of how plants control various types of regeneration and discuss how developmental and environmental constraints influence these regulatory mechanisms.

KEY WORDS: Cellular reprogramming, *De novo* organogenesis, Somatic embryogenesis

Introduction

When living organisms injure or lose part of their bodies, many are able to regenerate new tissues or organs to minimize the impact of local damage. Regeneration is a widely conserved physiological response in both animals and plants (Pulianmackal et al., 2014). What is collectively referred to as regeneration can range from the repair of a small amputation to the formation of new organs or individuals, and the mode of regeneration varies markedly among taxa (Birnbaum and Sánchez Alvarado, 2008). Plants possess a high capacity to regenerate, which has long been utilized for clonal propagation in the form of cutting and grafting (Hartmann et al., 2010; Melnyk and Meyerowitz, 2015). The attempts to regenerate whole individual plants from small tissues or single cells *in vitro* started in the early 20th century when Haberlandt (1902) proposed the concept of tissue culture. A landmark breakthrough in the history of tissue culture was the discovery that the balance of two exogenously applied plant hormones, namely auxin and cytokinin, could determine the fate of regenerating tissue: high ratios of auxin to cytokinin generally led to root regeneration and high ratios of cytokinin to auxin tended to promote shoot regeneration (Skoog and Miller, 1957). Steward et al. (1958) further demonstrated that even single cells from carrot vascular phloem retain totipotency – the capacity to regenerate whole plants – thus highlighting the astonishing regenerative potential of plant somatic cells.

A common mode of plant regeneration both in nature and *in vitro* is *de novo* organogenesis, in which plant cuttings or explants first form ectopic apical meristems and subsequently develop shoots and roots. Meristems are specialized plant tissues where new cells, tissues and organs are generated through cell division and differentiation. Plants can also regenerate through somatic embryogenesis *in vitro*, whereby isolated protoplasts or cells first

develop cellular structures similar to zygotic embryos and subsequently generate whole plant bodies. Both of these regeneration processes occur either directly from parental tissues or indirectly via the formation of a callus. Over recent decades, various culture conditions have been established for plant regeneration and utilized for clonal propagation and genetic transformation in diverse plant species. In this Review, we first describe the diverse forms of regeneration that occur in various plant species and compare the underlying cellular basis. We then summarize our current understanding of how plants control regeneration at the molecular level and discuss how developmental and environmental contexts influence the efficiency of plant regeneration.

Regeneration in green algae, liverworts and mosses

Many unicellular green algae have macroscopic bodies, and when these cells are injured they need to repair the damage quickly for survival. Regenerating the whole body directly from damaged cells is unique to these unicellular organisms, as multicellular organisms usually abandon damaged cells and use the remaining intact cells as a source of regeneration. Although most green algae simply seal cut sites by reconnecting the plasma membrane, some algae display astonishing regenerative responses. In the unicellular multinucleated alga *Bryopsis plumosa*, for example, nuclei squeezed out of cut sites aggregate in the seawater and construct the primary membrane *de novo* (Kim et al., 2001) (Fig. 1). These new protoplasts subsequently regenerate the second membrane and eventually rebuild the complete body.

Bryophytes, a group of basal land plants comprising liverworts, hornworts and mosses, have relatively simple body structures and they generally display high regenerative capacities (Necker et al., 1774). Explants of the liverwort *Marchantia polymorpha*, for example, regenerate new apical meristems within 60 h after wounding (Fig. 1) (Nishihama et al., 2015). Regenerating tissues often derive from the ventral midrib near cut ends (Vöchting, 1885), but other cell types also seem to contribute to regeneration (Nishihama et al., 2015). The moss *Physcomitrella patens* regenerates sporelings called protonema from leaf explants (Fig. 1). Protonema are thread-like chains of cells normally produced from spores, but can also be produced within the first 48 h after wounding when leaf cells facing the cut sites change their cell fate into protonema stem cells (Ishikawa et al., 2011). These stem cells subsequently start to elongate and proliferate, leading to the generation of new plant bodies.

Diverse forms of regeneration in seed plants

Compared with basal land plants, seed plants possess more complex body structures and display more diverse modes of regeneration depending on the developmental and/or environmental context (Fig. 2). Seed plants harbor apical meristems at both the top and bottom end of their bodies, known as the shoot and root apical meristem, respectively. The meristems are responsible for repair after injury: when the central zone of the shoot apical meristem is locally ablated, surrounding cells in the peripheral zone reconstruct

RIKEN Center for Sustainable Resource Science, 1-7-22 Suehiro-cho, Tsurumi, Yokohama, Kanagawa 230-0045, Japan.

*Author for correspondence (keiko.sugimoto@riken.jp)

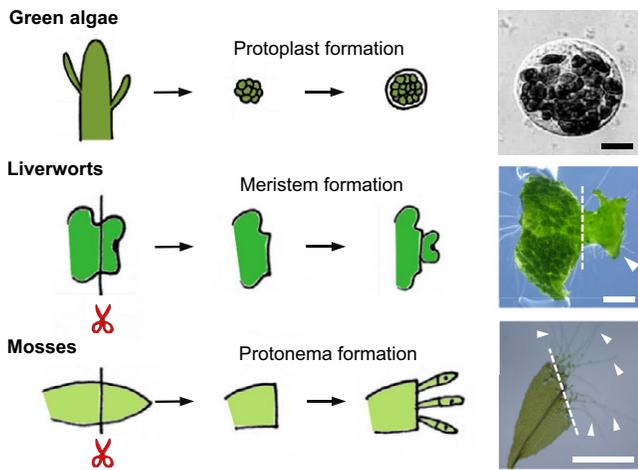


Fig. 1. Diverse forms of regeneration in green algae, liverworts and mosses. The unicellular green alga *Bryopsis plumosa* can regenerate complete bodies through the *de novo* formation of protoplasts. The liverwort *Marchantia polymorpha* can regenerate new meristems (arrowhead) from the cut site. The moss *Physcomitrella patens* can regenerate new protonema cells (arrowheads) from leaf cuttings. Scissors indicate the cut site. Scale bars (from top to bottom): 10 μ m, 1 mm, 500 μ m. The top photograph is reprinted from Kim et al. (2001) with permission. The middle and bottom photographs were provided by Ryuichi Nishihama and Masaki Ishikawa, respectively.

the functional meristem (Reinhardt et al., 2003) (Fig. 2). Similarly, when part of the root meristems is removed, remaining cells in the meristem undergo additional division and rebuild a complete meristem (Sena et al., 2009) (Fig. 2). Plants adopt alternative strategies when entire meristems are excised by macroscopic injuries. In shoots, meristems in axillary buds are kept dormant by

the presence of apical meristems. Upon loss of these apical meristems, apical dormancy is broken and axillary buds begin to grow (Shimizu-Sato and Mori, 2001) (Fig. 2). Similarly, when the whole root meristem is removed, new lateral and/or adventitious roots are formed from remaining roots and stems, respectively (Aloni et al., 2006; Bellini et al., 2014) (Fig. 2). In addition to these apical meristems, plant stems also display several types of tissue regeneration, including vascular reformation after debarking (Stobbe et al., 2002), tissue repair after partial incisions (Asahina et al., 2011) (Fig. 2) and vascular reconnection during grafting (Melnik et al., 2015).

A unique feature of plant regeneration is the formation of new organs from cut sites (Hartmann et al., 2010) (Fig. 2), with the regeneration of roots from shoot cuttings seen in many different plant species. Some limited but phylogenetically diverse plant species, such as those in Crassulaceae (for example *Crassula* spp., *Echeveria* spp., *Kalanchoe* spp. and *Sedum* spp.), Gesneriaceae (for example *Saintpaulia ionantha*, *Sinningia speciosa* and *Streptocarpus xhybridus*) and others (for example *Begonia* spp., *Peperomia* spp. and *Sansevieria trifasciata*), regenerate both shoots and roots from leaf cuttings (Fig. 2). In some bulbous plants, such as *Hippeastrum* spp., *Hyacinthus* spp. and *Lilium* spp., detached bulb scales regenerate shoots and roots from cut sites (Fig. 2). Some plants, including the perennial herbaceous plants *Papaver orientale*, *Primula sieboldii* and *Taraxacum officinale*, are capable of regenerating shoots from root cuttings (Fig. 2).

The regenerative capacity of plant cells can be enhanced *in vitro* when explants are cultured on nutrient media supplemented with plant hormones (Skoog and Miller, 1957; Murashige, 1974; George et al., 2008) (Fig. 3). Shoot explants of many ornamental plants are used for clonal propagation because multiple shoots can be formed from a shoot tip or stem node carrying a single bud (Fig. 3). Shoot or

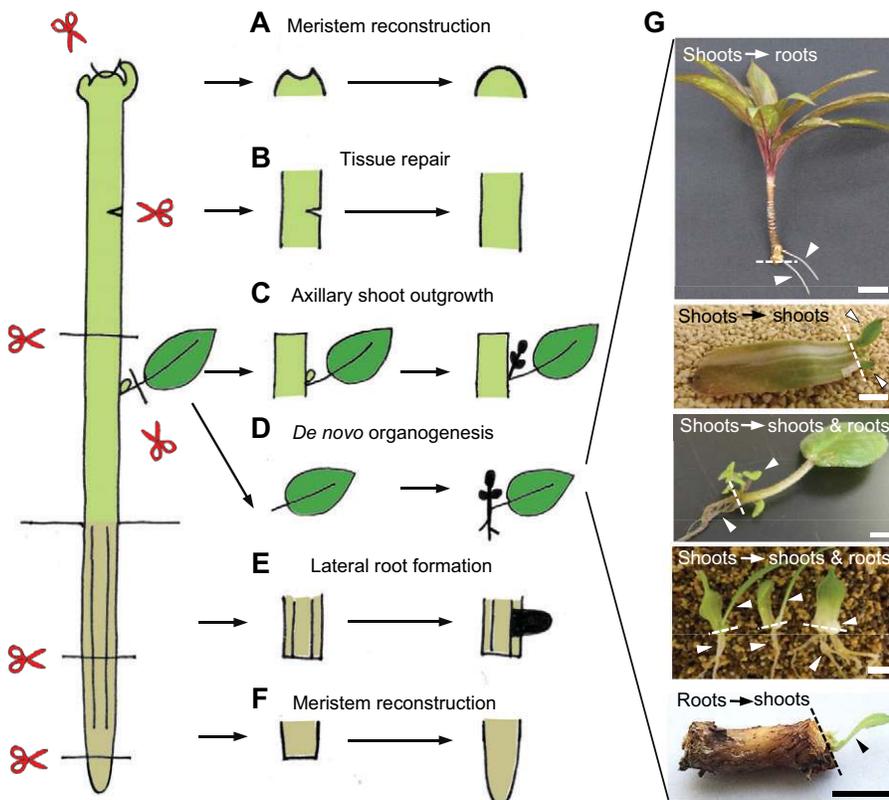


Fig. 2. Diverse forms of regeneration in seed plants. Shoots and roots restore functional apical meristems when part of these meristems is removed (A,F). When they cannot repair existing meristems – for example when the whole meristems are excised – they develop new organs such as axillary shoots and lateral roots (C,E). Plant stems also repair tissues after partial incisions (B). Some plants undergo *de novo* organogenesis and develop new organs from cut sites (D,G). Photographs (G) show examples of *de novo* organogenesis, from top to bottom: root regeneration from a shoot cutting of *Dracaena* spp.; shoot regeneration from a leaf cutting of *Haworthia* spp.; shoot and root regeneration from a petiole of African violet *Saintpaulia ionantha*; shoot and root regeneration from detached bulb scales of lily *Lilium longiflorum*; and shoot regeneration from a root cutting of dandelion *Taraxacum officinale*. Scissors indicate the cut site and arrowheads mark regenerating organs. Scale bars: 5 mm, except 5 cm in the top photograph.

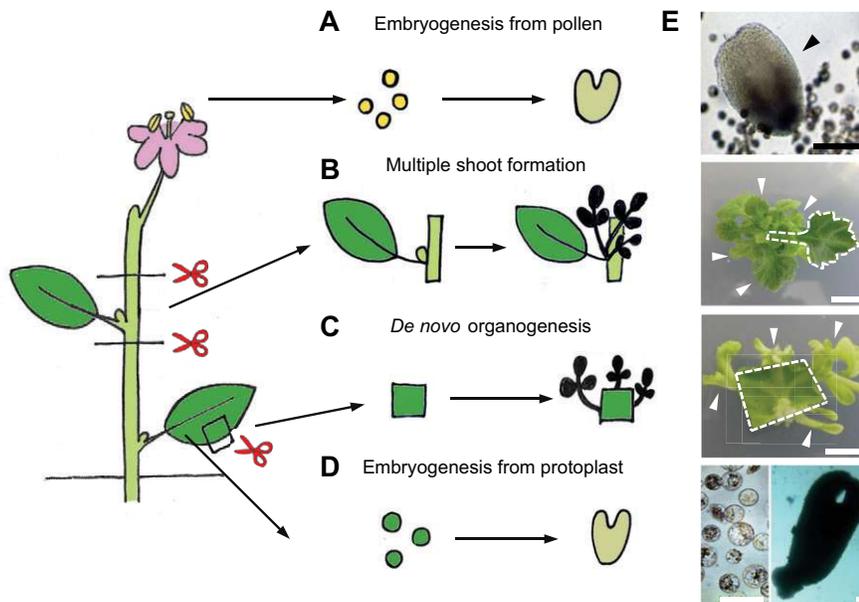


Fig. 3. Diverse forms of regeneration in the *in vitro* tissue culture environment. Regeneration *in vitro* can be induced from (A) pollen, (B) cut stems, (C) leaf cuttings and (D) protoplasts. (E) Photographs show, from top to bottom: regeneration of a somatic embryo (arrowhead) from isolated microspores of Chinese cabbage *Brassica rapa* var. *pekinensis*; regeneration of multiple shoots (arrowheads) from a node explant (dashed outline) carrying a single axillary bud of chrysanthemum *Chrysanthemum morifolium*; regeneration of calli and shoots (arrowheads) from a leaf explant of tobacco *Nicotiana tabacum* (dashed outline); and regeneration of a somatic embryo from protoplasts isolated from calli of grapevine *Vitis vinifera*. Scissors indicate the cut site. Scale bars: 5 mm, except 100 μ m in the top photograph.

root regeneration can also be induced *de novo* from various mature somatic tissues, and whole plants can be regenerated even from single protoplasts through *de novo* organogenesis or somatic embryogenesis (Takebe et al., 1971; Zhu et al., 1997; Chupeau et al., 2013) (Fig. 3). The regenerative capacity of pollen in some species, such as *Brassica napus*, *Nicotiana tabacum* and *Hordeum vulgare*, is utilized to develop haploid plants via somatic embryogenesis (Maraschin et al., 2005) (Fig. 3), from which homozygous diploid plants can be chemically induced in a single generation. In many monocotyledonous cereals, including *Oryza sativa* and *Zea mays*, mature somatic tissues are less regenerative, and thus zygotic embryos are commonly used for *in vitro* regeneration (Green and Phillips, 1975). For the remainder of this article we focus our attention on *de novo* organogenesis and somatic embryogenesis in seed plants, from which we have gained substantial mechanistic insights into regeneration over the last few decades.

Cellular origins of regeneration

Plants possess at least two distinct cellular strategies to begin the process of regeneration. One is through the reactivation of relatively undifferentiated cells, and the other through the reprogramming of differentiated somatic cells. In both cases, regeneration relies on the phenomenon of cellular plasticity, which can be broadly defined as the ability to respecify cell fate. Plant cells in immature or juvenile bodies tend to have a high regenerative potential and, accordingly, those in zygotic embryos readily undergo somatic embryogenesis. A study by Kim et al. (2007) showed that even trichome initial cells on the embryonic hypocotyls of *Tilia amurensis* regenerate new somatic embryos (Fig. 4A), demonstrating that cellular fate can be redirected at an early stage of plant development.

During post-embryonic development, most somatic cells become differentiated and only a limited set of cell types remains competent for new tissue and organ formation. Plant roots, for example, have a cylinder of pericycle cells situated between the endodermis and stele, and they have the potential to produce new lateral roots (Beeckman and De Smet, 2014). These pericycle cells, together with neighboring vascular parenchyma and/or procambium cells, are often the source of root regeneration (Bellini et al., 2014) and in many cases they also serve as a primary source for shoot

regeneration and somatic embryogenesis *in vitro* (Atta et al., 2009; Che et al., 2007; Tarré et al., 2004) (Fig. 4B). On the other hand, plants can regenerate whole bodies from protoplasts or pollen (Fig. 3), and there are many other examples showing regeneration from differentiated cells. Shoot regeneration, for instance, initiates from mature leaf epidermal cells in *Chirita flavimaculata* (Nakano et al., 2009) (Fig. 4C) or from stem cortex cells in *Chrysanthemum morifolium* (Kaul et al., 1990) (Fig. 4D). Likewise, calli that give rise to somatic embryos have a cellular origin clearly distinct from pericycle or vascular cells in *Medicago truncatula* (Wang et al., 2011). It was reported that epidermal cell fate in developing leaves of *Arabidopsis thaliana* (*Arabidopsis*) can also be overwritten by overexpression of the RWP-RK protein RKD4 in order to initiate embryogenesis (Waki et al., 2011) (Fig. 4E). These observations indicate that ‘youth’ is not the prerequisite for plant regeneration and, at least under *in vitro* culture conditions, fully mature somatic cells can initiate regeneration.

Wound stress as a trigger for regeneration

Given that most naturally occurring regeneration starts at cut sites (Fig. 2), wound stimuli may in fact provide a primary inductive trigger for this phenomenon (Birbaum and Sánchez Alvarado, 2008; Ikeuchi et al., 2013; Sugiyama, 2015). In *Arabidopsis* tissue culture where explants incubated on auxin-rich callus-inducing medium (CIM) were subsequently transferred on to cytokinin-rich shoot-inducing medium (SIM) (Valvekens et al., 1988), intact, uncut plants hardly regenerated shoots at all (Iwase et al., 2015), thus demonstrating a requirement for wound stimuli in initiating regeneration. Wounding induces numerous cellular responses, including the production of plant hormones (Ahkami et al., 2009), loss of cell-to-cell communication and disruption of long-distance signaling (Melnik et al., 2015).

What exactly plants perceive as a wound signal and how they start regeneration are not well understood, but recent studies in *Arabidopsis* showed that the AP2/ERF-type transcriptional regulator WOUND-INDUCED DEDIFFERENTIATION1 (WIND1) and its homologs WIND2, WIND3 and WIND4 are induced upon wounding and promote callus formation at cut sites (Iwase et al., 2011a,b). Importantly, callus induced by transient overexpression of

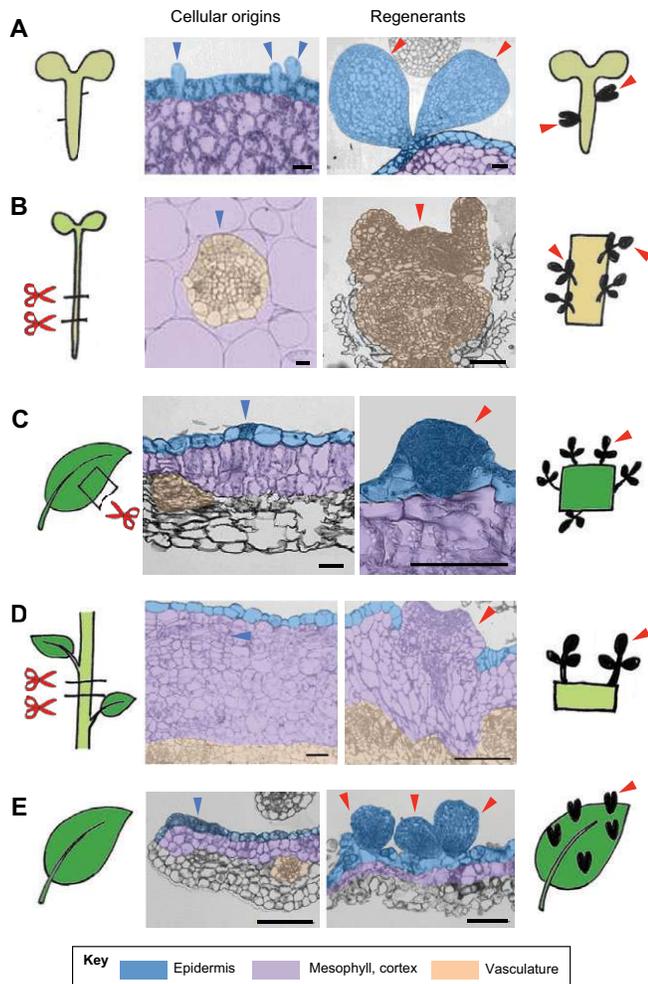


Fig. 4. Cellular basis of plant regeneration. (A) Somatic embryogenesis from embryonic hypocotyls of *Tilia amurensis*. Transverse sections (middle panels) of hypocotyls show that epidermal trichome initial cells (blue arrowheads) give rise to new embryos (red arrowheads). (B) *In vitro* shoot regeneration from root or hypocotyl explants of *Arabidopsis thaliana*. Transverse sections of explants show that pericycle cells (blue arrowhead) give rise to regenerating shoots (red arrowheads). (C) *In vitro* shoot regeneration from leaf explants of *Chirita flavimaculata*. Transverse sections of leaf explants show that epidermal cells (blue arrowhead) give rise to regenerating shoots (red arrowhead). (D) *In vitro* shoot regeneration from stem explants of *Chrysanthemum morifolium*. Transverse sections of stem explants show that cortex cells (blue arrowhead) give rise to regenerating shoots (red arrowhead). (E) Somatic embryogenesis in *Arabidopsis* leaves reprogrammed by overexpression of the embryonic regulator RKD4. Transverse sections of reprogrammed leaves show that epidermal cells (blue arrowhead) give rise to new embryos (red arrowheads). Scissors indicate the cut site. Scale bars: 100 μ m. Photographs in A are modified from Kim et al. (2007) with permission from Oxford University Press; in B are modified from Atta et al. (2009) with permission from Blackwell Publishing; in C are modified from Nakano et al. (2009) with permission from Salvia Press; in D are modified from Kaul et al. (1990) with permission from Springer; in E are provided by Keiji Nakajima.

WIND1 regenerates shoots and roots when transferred to non-inducible media (Iwase et al., 2011a), suggesting that *WIND1* can reprogram somatic cells to confer pluripotency. Intact *Arabidopsis* plants ectopically expressing *WIND1* regenerate shoots without wounding, and plants expressing the dominant-negative form of *WIND1* display reduced efficiency of *in vitro* shoot regeneration (Iwase et al., 2015). These data support a role for WINDs as key

mediators of wound-induced cellular reprogramming (Fig. 5A). Consistent with this, sequential activation of *WIND1* and an embryonic regulator, *LEAFY COTYLEDON2 (LEC2)*, induces somatic embryogenesis at both cut and non-cut sites, whereas single activation of *LEC2* permits embryogenesis only at cut sites (Iwase et al., 2015). Downstream genes regulated by WINDs are currently unknown, although WINDs have been implicated in the control of cytokinin signaling based on the observation that the repression of WIND activity abolishes the wound-induced cytokinin response (Iwase et al., 2011a,b). Further investigation of how wounding activates WIND gene expression and how, in turn, WINDs promote cellular reprogramming will be crucial to advance our molecular understanding of wound-induced regeneration. Precisely which cell types contribute to naturally occurring regeneration is also not fully established, and thus future studies should clarify the origin of cells in natural regeneration and determine whether it involves fate conversion of fully differentiated somatic cells and/or the activation of existing competent cells.

Molecular basis of *de novo* shoot organogenesis

As in many other plant species, *Arabidopsis* explants do not readily regenerate shoots, but incubation on CIM and SIM strongly enhances shoot regeneration from pericycle cells (Valvekens et al., 1988; Che et al., 2007; Atta et al., 2009). Recent histological and transcriptome analyses have revealed that CIM-induced callus resembles lateral root meristem, which is competent to regenerate shoots upon transfer to SIM (Che et al., 2007; Atta et al., 2009; Sugimoto et al., 2010; Duclercq et al., 2011). Induction of the AP2/ERF transcription factors PLETHORA3 (PLT3), PLT5 and PLT7 is among the earliest transcriptional responses induced by CIM, which in turn leads to the activation of the key root meristem regulators *PLT1* and *PLT2* to establish a pluripotent root meristem-like callus (Kareem et al., 2015). Expression of PLT3, PLT5 and PLT7 also induces the NAC family transcription factors CUPSHAPED COTYLEDON1 (*CUC1*) and *CUC2*, which are involved in shoot meristem initiation during zygotic embryogenesis (Kareem et al., 2015; Aida et al., 1997, 1999). The *plt3 plt5 plt7* triple mutants are defective in shoot regeneration, but dual overexpression of *PLT1* and *CUC2*, but not their single expression, partially complements this phenotype, suggesting that PLT3, PLT5 and PLT7 promote both PLT1-mediated acquisition of pluripotency and *CUC2*-mediated initiation of shoot fate (Kareem et al., 2015) (Fig. 5A). Both *CUC1* and *CUC2* are uniformly expressed in CIM-induced callus (Gordon et al., 2007) and their presence is associated with cellular pluripotency in various experimental conditions (Cary et al., 2002; Daimon et al., 2003; Gordon et al., 2007; Motte et al., 2011).

Upon transfer to SIM, partitioning of the auxin and cytokinin responses in the pluripotent cell mass is thought to refine the shoot meristem fate. *CUC2* expression is restricted to the low cytokinin response domains, whereas the shoot meristem regulator *WUSCHEL (WUS)* is induced in the high cytokinin response domains (Gordon et al., 2007; Chatfield et al., 2013; Che et al., 2006) (Fig. 5A). The *CUC2*-expressing cells continue to proliferate to form dome-like structures called promeristems, in which localized upregulation of other regulators such as *PIN-FORMED1 (PIN1)* and *SHOOT MERISTEMLESS (STM)* further defines the radial patterning of newly developing meristems and primordia initiation (Gordon et al., 2007). Similar to *WUS*, other AP2/ERF transcription factors, such as ENHANCER OF SHOOT REGENERATION1/DORNRÖSCHEN (*ESR1/DRN*) and *ESR2/DRN-LIKE (DRNL)*, which control embryonic patterning and

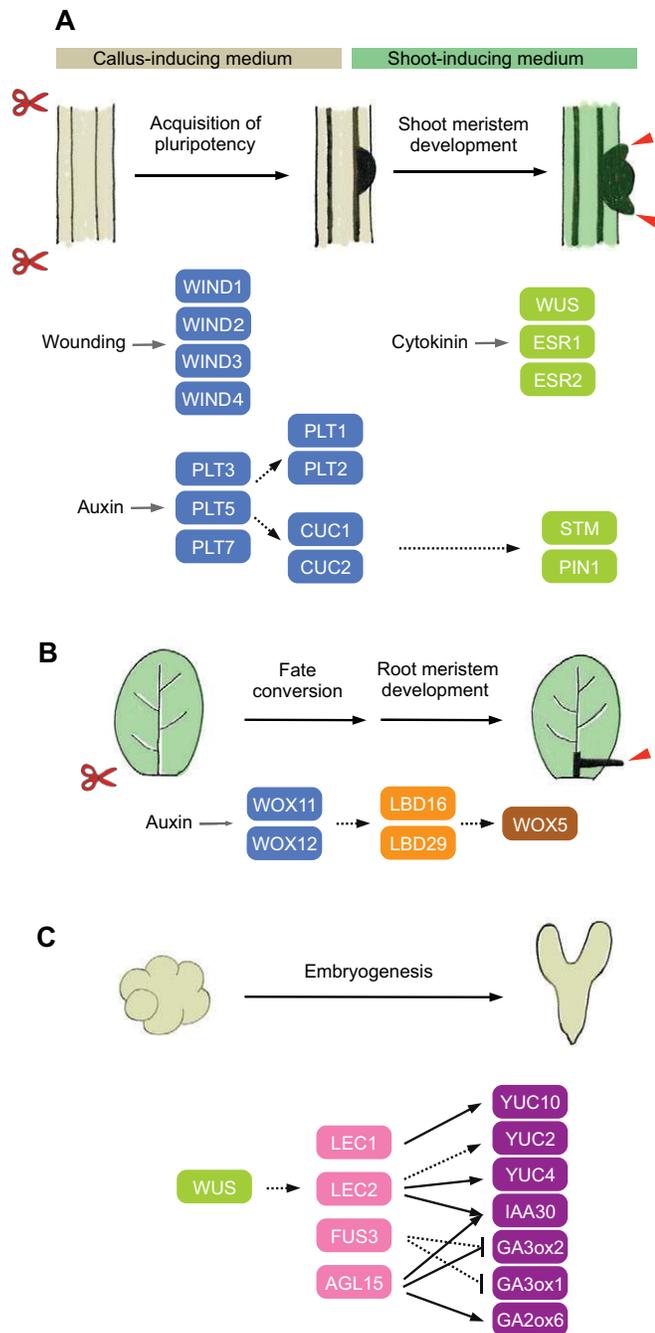


Fig. 5. A molecular framework for plant regeneration. (A) A schematic model showing how *Arabidopsis* explants regenerate shoots *in vitro*. Wounding induces *WIND1-4* expression to promote the acquisition of pluripotency at cut sites. Culturing plant explants on auxin-rich callus-inducing medium upregulates the expression of *PLT3*, *PLT5* and *PLT7*, which subsequently promotes the acquisition of pluripotency through the induction of *PLT1*, *PLT2*, *CUC1* and *CUC2*. Upon transfer to cytokinin-rich shoot-inducing medium, the *WUS*, *ESR1* and *ESR2* genes are induced, conferring cells with shoot fate. *CUC2* expression becomes spatially confined to promeristems, in which STM and PIN1 further regulate patterning and formation of the meristems (red arrowheads). (B) A schematic model showing how root regeneration is controlled in *Arabidopsis* leaf explants. Accumulation of auxin at cut sites promotes the fate conversion from leaf procambium/parenchyma cells to root founder cells by activating *WOX11* and *WOX12* expression. *WOX11* and *WOX12* subsequently induce the expression of *LBD16*, *LBD29*, and then *WOX5* to initiate root meristem formation (red arrowhead). (C) A schematic model showing how indirect somatic embryogenesis is regulated in *Arabidopsis*. A gradient of auxin in the embryonic callus specifies *WUS* expression to low auxin response domains. *WUS* subsequently induces expression of *LEC1*, *LEC2* and *FUS3*, which together with *AGL15* modulate the endogenous levels of auxin, GA and ABA to promote embryogenesis. Solid black lines indicate direct transcriptional regulation demonstrated by molecular evidence and dotted black lines indicate direct or indirect transcriptional regulation inferred from genetic evidence. Proteins that promote cellular competency are in blue; those that mediate shoot fate are in green, root fate in orange or brown, and embryonic fate in pink or purple.

regenerate roots from both cut and non-cut sites without pretreatment on CIM, whereas hypocotyl explants regenerate roots only from cut sites under these conditions (Ozawa et al., 1998). Pretreatment of hypocotyl explants on CIM allows root regeneration from non-cut sites (Ozawa et al., 1998), confirming the physiological role of CIM in endowing regenerative competence.

Some plant species naturally regenerate roots from cuttings and several plant hormones, including auxin and cytokinin, are known to control this process (da Costa et al., 2013; Bellini et al., 2014). A recent study by Liu et al. (2014) uncovered a novel molecular link connecting auxin accumulation at cut sites to the formation of new root meristems during regeneration. When *Arabidopsis* leaves are detached, accumulation of auxin at cut sites induces the expression of two homeobox transcription factors, namely WUSCHEL RELATED HOMEBOX11 (*WOX11*) and *WOX12*, in the procambium and surrounding parenchyma cells. Expression of these genes promotes the fate conversion from leaf procambium/parenchyma cells to root founder cells (Fig. 5B). Both *WOX11* and *WOX12* subsequently participate in the *de novo* establishment of root meristems, which further involves the expression of *LATERAL ORGAN BOUNDARIES DOMAIN16* (*LBD16*), *LBD29* and *WOX5* (Liu et al., 2014) (Fig. 5B). The LBDs and *WOX5* are also involved in lateral root development, in which locally accumulated auxin promotes the formation of new root meristems from pericycle cells (Goh et al., 2012; Ditengou et al., 2008). These two pathways thus share some key regulators to facilitate the auxin-mediated establishment of root meristems. Of note, the induction of *WOX11* in root regeneration requires auxin response elements (AuxREs) in its promoter, suggesting that some members of the AUXIN RESPONSE FACTOR (ARF) family directly activate *WOX11* expression in leaves (Liu et al., 2014).

shoot formation (Kirch et al., 2003; Chandler et al., 2007), are also induced on SIM and stimulate shoot regeneration at least partially by enhancing *CUC1* expression (Banno et al., 2001; Ikeda et al., 2006; Matsuo et al., 2009, 2011) (Fig. 5A).

Molecular basis of *de novo* root organogenesis

Culturing *Arabidopsis* explants on CIM and root-inducing medium (RIM) strongly enhances root regeneration from pericycle cells (Ozawa et al., 1998; Che et al., 2002). This is probably because CIM promotes the production of a root meristem-like pluripotent cell mass, which then becomes further specified by RIM to develop root meristems. Consistent with this, root explants, which already possess lateral root meristem primordia along their body axis,

Molecular basis of somatic embryogenesis

Some somatic cells in plants can restart embryogenesis *in vitro* when they are exposed to a wide range of severe abiotic stressors (Fehér, 2014). Somatic embryogenesis can be induced by salt, hypochlorite, osmotic pressure, heavy metal ions or high temperature in *Daucus carota* (Kiyosue et al., 1989, 1990;

Kamada et al., 1989, 1993, 1994), and similar stress-induced embryogenesis has also been reported in *Arabidopsis* (Ikeda-Iwai et al., 2003). Many plant species also undergo somatic embryogenesis when they are cultured on auxin-containing medium and then transferred to auxin-free medium (Wernicke and Brettell, 1980; Lu et al., 1983; Ikeda-Iwai et al., 2002). Among several synthetic auxin-like substances, 2,4-dichlorophenoxyacetic acid (2,4-D) is the most effective inducer of somatic embryos in many plants, possibly because it triggers both auxin and stress responses simultaneously (Gliwicka et al., 2013). During indirect somatic embryogenesis, by which most somatic embryos are formed, high levels of auxin in the culture medium first promote cell proliferation and embryonic callus formation (Ikeda-Iwai et al., 2002). A key physiological event after the transfer to auxin-free medium is the *de novo* establishment of auxin gradients in the embryonic callus. This initiates a developmental program similar to zygotic embryogenesis, and is also guided by polarized auxin distribution (Liu et al., 1993; Su et al., 2009). These auxin gradients subsequently lead to the localization of *WUS* expression to low auxin response domains, marking the position of future shoot meristem formation (Su et al., 2009) (Fig. 5C).

Several key regulators of zygotic embryogenesis and seed development, including the CCAAT box-binding transcription factor *LEAFY COTYLEDON1* (*LEC1*), the B3 domain transcription factors *LEC2* and *FUSCA3* (*FUS3*), and the MADS box transcription factor *AGAMOUS-LIKE15* (*AGL15*), are subsequently induced during somatic embryogenesis and control several downstream physiological responses to promote embryonic development (Braybrook and Harada, 2008). A key consequence of this transcriptional reprogramming is the further refinement of auxin production and signaling. *LEC1* induces the *YUCCA10* (*YUC10*) gene, which encodes an auxin biosynthesis enzyme, and *LEC2* activates the *YUC2* and *YUC4* genes (Junker et al., 2012; Stone et al., 2008). *LEC2* and *AGL15* promote the expression of *INDOLE ACETIC ACID INDUCIBLE30* (*IAA30*), a negative regulator of auxin signaling, to modulate the auxin-mediated signaling (Braybrook et al., 2006; Zheng et al., 2009). Previous studies also suggest that a low level of gibberellin (GA) relative to abscisic acid (ABA) favors embryogenesis. Consistent with this, *AGL15* positively regulates *GA2ox6*, which encodes a GA degrading enzyme, and negatively regulates the GA biosynthesis gene *GA3ox2*, resulting in the reduced endogenous GA level (Wang et al., 2004; Zheng et al., 2009). In addition, *FUS3* downregulates GA biosynthesis by repressing *GA3ox1* and *GA3ox2*, and induces ABA biosynthesis through as yet unknown mechanisms (Curaba et al., 2004; Gazzarrini et al., 2004; Kagaya et al., 2005).

Epigenetic control of regeneration

The regenerative capacity of plant cells is required only when they experience damage. Recent studies have shown that several epigenetic mechanisms actively suppress regenerative potential during normal development (Ikeuchi et al., 2015a). *POLYCOMB REPRESSIVE COMPLEX2* (*PRC2*) is a chromatin modifier that maintains transcriptional repression through the deposition of histone H3 lysine 27 trimethylation (H3K27me3) (Holec and Berger, 2012). A recent study showed that *PRC2* mutants initially develop wild-type-like roots with fully differentiated, endoreplicated root hair cells, but that they subsequently reprogram and develop callus and embryo-like structures (Ikeuchi et al., 2015b). The reprogramming regulator *WIND3* and the embryonic regulator *LEC2* are among the key targets repressed by *PRC2* in this context and elevated expression of these genes

contributes to cellular reprogramming in *PRC2* mutants. This study thus enforces the idea that highly differentiated cells still retain the capacity to undergo embryogenesis, and that this potential must be tightly regulated – in this case, epigenetically repressed by *PRC2* to maintain the differentiated status. Intriguingly, many other key regulators of regeneration, such as *WOX11*, *WOX5*, *WUS* and *STM*, are also under *PRC2*-mediated repression (Liu et al., 2014, 2011; He et al., 2012; Lafos et al., 2011). An important question is whether the cells carrying these repressive marks on regeneration regulators initiate regeneration in the wild-type context and, if so, how these repressions are relieved to allow regeneration to proceed in nature or *in vitro* conditions. *PRC2* is also required for root regeneration from leaves (Liu et al., 2014), suggesting that repression of original cell fate might be another important aspect of regeneration.

Histone deacetylation, which is also implicated in transcriptional repression, might serve as another safeguard to prevent the untimely onset of somatic embryogenesis. Wild-type *Arabidopsis* plants treated with an inhibitor of histone deacetylases, trichostatin A (TSA), produce embryo-like structures from true leaves (Tanaka et al., 2008). Similarly, loss-of-function mutants of two histone deacetylases, *HDA19* and *HDA6*, generate embryo-like structures in shoots (Tanaka et al., 2008). These phenotypes are associated with the ectopic expression of several key embryonic regulators, such as *LEC1* and *LEC2*, and can be suppressed by introducing the *lec1* mutation (Tanaka et al., 2008). Interestingly, TSA in combination with heat treatment greatly enhances the efficiency of somatic embryogenesis from *Brassica napus* microspores (Li et al., 2014). It is plausible, then, that heat stress and histone deacetylation converge on the upregulation of embryonic regulators to initiate the embryonic program.

Genetic studies in *Arabidopsis* also suggest the involvement of other epigenetic mechanisms in the control of organ regeneration. Mutations in *DNA METHYLTRANSFERASE1* (*MET1*) enhance shoot regeneration on SIM and this phenotype is accompanied by the elevated expression of several *MET1* targets, including *WUS* (Li et al., 2011). As with many other regeneration regulators, the *WUS* locus is marked by several other epigenetic signatures and these marks are modified when *WUS* expression is upregulated during shoot regeneration (Li et al., 2011). Uncovering the causal relationships between these epigenetic modifications and transcriptional changes will be an important task for future studies.

Natural variations that impact plant regeneration

Small genetic variations within the same species can cause dramatic differences in the regenerative response. Genetic variability has been utilized to identify novel factors that modulate the efficiency of regeneration in many plant species (Armstrong et al., 1992; Taguchi-Shiobara et al., 1997; Ben Amer et al., 1997; Flores Berrios et al., 2000; Mano and Komatsuda, 2002; Trujillo-Moya et al., 2011). A leucine-rich repeat receptor-like kinase, *RECEPTOR-LIKE PROTEIN KINASE1* (*RPK1*), for instance, was identified as a major quantitative trait locus (QTL) that affects shoot regeneration in *Arabidopsis* accessions (Motte et al., 2014). *RPK1* is implicated in ABA signaling, and although this hormone has not been studied extensively in the context of regeneration it has been reported to influence shoot regeneration in several plant species (Ghasemi Bezdi et al., 2007; Hoang and Raldugina, 2012; Huang et al., 2012). The single-nucleotide polymorphism responsible for the genetic variation lies within a putative ligand-binding domain, and thus the identification of its ligands should help to reveal its molecular functions.

In *Oryza sativa*, some varieties or even cultivars within the same variety exhibit markedly different shoot regeneration capabilities

(Nishimura et al., 2005). Map-based cloning using the low regeneration cultivar Koshihikari (*Japonica*) and high regeneration cultivar Kasalath (*Indica*) identified a ferredoxin-nitrite reductase as a major QTL causing variations in shoot regeneration. Further studies showed that the reductase activity positively correlated with regeneration capacity in several other *Japonica* cultivars as well (Nishimura et al., 2005). Furthermore, introduction of the ferredoxin-nitrite reductase gene from Kasalath improved shoot regeneration in Koshihikari (Nishimura et al., 2005). Ferredoxin-nitrite reductase is involved in the nitrogen assimilation pathway, and thus it might be that low reductase activity in Koshihikari results in an accumulation of nitrite, which might hinder shoot regeneration.

The *Regeneration1* (*Rg1*) locus in tomato was originally identified as a natural variation responsible for highly efficient shoot regeneration in the wild relative *Solanum peruvianum* (Koornneef et al., 1993). It was later shown that *Rg1* increases the competency for both root and shoot regeneration, and that this response does not involve alterations in auxin sensitivity or *CUC* expression (Lombardi-Crestana et al., 2012). Interestingly, the tomato *DELLA* mutant *procera* (*pro*), which shows a constitutive response to GA, displays a low regeneration phenotype, and *Rg1* rescues these defects in an *Rg1 pro* double mutant. These data suggest an involvement of *DELLA*-mediated GA signaling in the control of shoot regeneration (Lombardi-Crestana et al., 2012).

Developmental constraints that impact plant regeneration

The regenerative capacity of explants varies markedly with the condition of parental plants, generally declining as plants get older. Compromised root regeneration in aged trees is a serious problem in horticulture, limiting the clonal propagation of elite cultivars. Histological studies using woody species *Castanea sativa* and *Quercus* sp. showed that the exogenous application of auxin reactivates cell proliferation but not the formation of new root meristems in mature explants (Ballester et al., 1999; Vidal et al., 2003). A recent study using *Pisum sativa* suggested that the vegetative-to-reproductive transition is linked to the reduced root regenerative capacity and that this is caused by the loss of auxin responsiveness in reproductive shoots (Rasmussen et al., 2015). By contrast, application of auxin improves the root regeneration efficiency of *Arabidopsis* leaves from aged plants (Chen et al., 2014), suggesting that there are multiple physiological constraints imposed by aging.

Explants from juvenile plants regenerate shoots more effectively than those from mature plants (Dong and Jia, 1991; Baker and Bhatia, 1993; Becerra et al., 2004; Zhang et al., 2015). The decline in shoot regeneration capacity with aging is at least partly due to a reduced responsiveness to plant hormones. The microRNA miR156 has been shown to regulate the juvenile-to-adult phase transition in plants (Wu et al., 2009), and a recent study suggests that a decline of miR156 expression in old plants is responsible for reduced shoot regeneration (Zhang et al., 2015). This reduction in miR156 increases the level of its target *SQUAMOSA PROMOTER BINDING-LIKE9* (*SPL9*). *SPL9*, in turn, inhibits the transcriptional activity of B-type ARABIDOPSIS RESPONSE REGULATOR proteins (ARRs), leading to the reduced responsiveness to cytokinin and hence compromised shoot regeneration (Zhang et al., 2015).

Environmental constraints that impact plant regeneration

The regenerative capacity of plant explants is also influenced by various environmental conditions, such as nutrient composition,

gelling agents, pH, light and temperature (George et al., 2008). A well-documented environmental condition that influences plant regeneration is the exposure to light, but its impact on regeneration appears to be highly context dependent. Light is required for shoot regeneration in some plant species (Reuveni and Evenor, 2007) and can also trigger organ regeneration (Saitou et al., 1992; Sorin et al., 2006; Gutierrez et al., 2009). On the other hand, exposure to light can have an inhibitory effect on root or shoot regeneration in some contexts (Bellini et al., 2014; Nameth et al., 2013). A series of tissue culture experiments using *Arabidopsis* cotyledons showed that light exposure during the first hours after tissue excision is mostly deleterious to shoot regeneration, and keeping explants in darkness for as little as 2-6 h is sufficient to improve regeneration (Nameth et al., 2013).

Light exposure invokes several parallel signaling pathways, some of which cause oxidative damage due to the production of reactive oxygen species. At least two photoreceptors are implicated in the light response of shoot regeneration: the blue/UV-A light receptor CRYPTOCHROME1 (*CRY1*), which mediates the strong inhibition of shoot regeneration; and the far-red light receptor PHYTOCHROME A (*PHYA*), which protects explants against initial light inhibition (Nameth et al., 2013). A key regulator acting downstream of light signaling is the transcription factor ELONGATED HYPOCOTYL5 (*HY5*), which appears to protect explants against light exposure by inducing anthocyanin accumulation. Several accessions in *Arabidopsis* display different responses to light in shoot regeneration, and an interesting topic for future studies will be the cause of such genetic variations.

Conclusions and future perspectives

During regeneration, select intrinsic developmental programs are ectopically activated in response to external stimuli. These responses require context-dependent integration of developmental and environmental signals, leading to diverse strategies and efficiencies of regeneration. Given that regeneration originates from a relatively small population of cells in somatic tissues, it is important to identify these cell populations and to study how external stress can cause them to undergo changes in cell fate. During normal development, many central regulators of regeneration are epigenetically silenced to prevent inappropriate cellular reprogramming. A central challenge, therefore, is to understand how these repressions are overcome by external stimuli. Molecular genetic studies in *Arabidopsis* have provided substantial insight into how plants regenerate from relatively undifferentiated cells, but other plants that regenerate from differentiated cells may utilize distinct mechanisms. With rapid advances in next-generation sequencing and genome editing technologies, we should be able to investigate the molecular mechanisms of these currently underexplored forms of regeneration and carry out functional studies in non-model plants. The QTL analysis on accessions with differing regeneration efficiencies has proved an excellent complementary approach and the further identification of new QTLs should help us to uncover novel mechanisms of plant regeneration.

An important goal of plant regeneration research is to use our knowledge of basic biology to design new molecular tools to analyze and improve regeneration efficiencies in crops. Indeed, the ectopic expression of key regeneration regulators, such as *WUS* and *WIND1*, has already been shown to promote organ regeneration and/or somatic embryogenesis in various crops (Srinivasan et al., 2007; Arroyo-Herrera et al., 2008; Heidmann et al., 2011; Iwase et al., 2013, 2015; Florez et al., 2015). Expression profiles of key

regeneration regulators have also been used in crops to identify cultivars with high regeneration capacities (Malik et al., 2008). Further mechanistic understanding of plant regeneration should help us to advance the classic but not fully exploited field of tissue culture, with numerous downstream implications for both basic and applied biology.

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Competing interests

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