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STEM CELLS AND PLANT REGENERATION

Pablo Perez-Garcia, Miguel A. Moreno-Risueno

Departamento de Biotecnología y Biología Vegetal, Universidad Politécnica de Madrid (UPM), Madrid, Spain
Corresponding author: M.A. Moreno-Risueno (e-mail: miguelangel.moreno@upm.es)

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Abstract

Multicellular organisms show the ability to replace damage cells, tissues and even whole organs through regeneration mechanisms. Plants show a remarkable regenerative potential. While the basic principles of plant regeneration have been known for a number of decades, the molecular and cellular mechanisms underlying such principles are currently starting to emerge. Some of these mechanisms point to the existence of highly reprogrammable cells. Developmental plasticity is a hallmark for stem cells, and stem cells are responsible for the generation of distinctive cell types forming plants. In the last years, a number of players and molecular mechanism regulating stem cell maintenance have been described, and some of them have also been involved in regenerative processes. These discoveries in plant stem cell regulation and regeneration invite us to rethink several of the classical concepts in plant biology such as cell fate specification and even the actual meaning of what we consider stem cells in plants. In this review we will cover some of these discoveries, focusing on the role of the plant stem cell function and regulation during cell and organ regeneration.

1. Introduction

Regeneration is the capacity of multicellular organisms of reconstituting or developing new cells, tissues or even complete organs upon damage and/or wound (Birnbaum and Alvarado, 2008). Every species shows different regenerative capacities and each organ of the same individual may respond differently during regeneration. In animals, specific areas of the brain do not appear to show regenerative potential while the surface of the gut is renewed every 3-5 days (Alvarado and Yamanaka, 2014). In contrast, plants show higher potential to regenerate, which is thought to rely in certain tissues or types of cells more broadly located throughout the plant (Pulianmackal et al., 2014). Interestingly, different plant organs vary in their regenerative properties suggesting there might be distinctive biological mechanisms used during regeneration (Kareem et al., 2016; Pulianmackal et al., 2014). Despite high regeneration potential of plants, spatial and temporal restriction of this process is also found which highly correlates with location of stem cells, their daughter cells, meristematic cells or highly reprogrammable cells. The specific role of stem cells

during regeneration is not fully understood. Many molecular players involved in plant stem cell function have been studied in detail while first insights about molecular mechanism regulating regeneration are starting to emerge. In this review we will cover some of these discoveries highlighting the role of stem cells during regeneration.

2. Importance of regeneration and stem cells in plant development

In plants, regeneration is especially important due to their sessile condition. Plant exceptional regenerative capacities allow them to successfully face continuous biotic and abiotic stresses that may compromise their body integrity (Lup et al., 2016). While most animals complete their body axis development at their embryonic state, plants grow continuously during most or all of their life creating new branches, flowers, fruits, lateral roots (LR) and thickening of theirs bodies, being all these processes commonly designated as postembryonic development (Birnbaum and Alvarado, 2008). As plant postembryonic development allows tissue and organ formation after embryogenesis, both regeneration and postembryonic development might share common principles. In addition, new organ and tissue formation during postembryogenesis involves generation, specification and differentiation of new cells from already existing ones so it can be interpreted as a regenerative process. Plants regenerative capacity reaches high levels of complexity in comparison with animals, and thus plants are able to regenerate damaged organs, whole organs from explants (*de novo* organ regeneration) or fully restore individuals from a set of few highly regenerative cells, in the latter only upon hormonal induction through *in vitro* culture. These set of highly regenerative cells organize in a structure called callus which resembles a lateral root primordium (Perianez-Rodriguez et al., 2014; Skoog and Miller, 1957). Recently, callus has also been found to exist during *de novo* organ regeneration under non-hormonal inductive conditions (Bustillo-Avendaño et al., 2017). Callus is also formed endogenously upon wound, and although this regenerative mechanism does not appear to lead to formation of new organs (Iwase et al., 2011), these data indicate that callus is not exclusive of exogenously induced regeneration. Initial research in regeneration suggested that every plant cell could participate in the formation of a callus indicating totipotency to all plant cells at any developmental stage. More recently, it has been shown that only cells with pericycle identity (expressing the J0121 marker) appear to be reprogrammable generating callus upon hormonal supplementation or endogenous induction (Bustillo-Avendaño et al., 2017; Sugimoto et al., 2010).

Regeneration of wounded organs, *de novo* formation of organs and the development of new individuals or organs from callus involves the specification of new stem cells (Beeckman and De Smet, 2014; Bustillo-Avendaño et al., 2017; Efroni et al., 2016; Iwase et al., 2015; Kareem et al., 2015; Perianez-Rodriguez et al., 2014). Stem cells in plants are normally confined into stem cell niches (SCNs) within meristems. Meristems are proliferative zones in which tissues are generated from stem cells and grow. Because tissues are generated from stem cells, stem cells are also designated as tissue initials, although strictly tissue initials represent the origin of all distinctive tissue lineages. These tissue stem cells divide asymmetrically to regenerate themselves and form a stem cell daughter which proliferates, grow and eventually differentiates (Greb and Lohmann, 2016). Stem cell asymmetric divisions are the source of cell renewal and maintenance during

postembryonic development. In seed plants, and more particularly in *Arabidopsis thaliana*, meristems can be found at both ends of seedlings. In the aerial part, the shoot apical meristem (SAM) is the origin of above-ground part of the plant body while the root apical meristem (RAM) generates the underground half of the plant (Fisher and Sozzani, 2016; Gaillochet et al., 2015). A third meristem, the lateral/vascular, meristem is responsible of thickening of the plant (De Rybel et al., 2016; Ruonala et al., 2017). Importantly, new meristems are generated or derived postembryonically during aerial and root branching shaping adult plant body.

3. Role of root stem cells in organ self-regeneration

The RAM, which is established during embryogenesis, determines the postembryonic development of the underground part of the plant. (Drisch and Stahl, 2015; Fisher and Sozzani, 2016; Greb and Lohmann, 2016). Structurally and functionally, the SCN locates at the RAM and it is made up of the quiescent center (QC) surrounded by stem cells (Drisch and Stahl, 2015; Fisher and Sozzani, 2016; Greb and Lohmann, 2016) (Fig. 1A/ 1B/ 1C). The phytohormones auxin and cytokinins (CKs) play a major role regulating RAM maintenance and activity. A prominent effect of auxin is found at the root tip with a maximum peak of activity in QC cells which promotes the specification of the SCN (Mähönen et al., 2014). The CKs domain is located shootwards and promotes cell differentiation (Ioio et al., 2008) in a low auxin environment in which differentiation programs may operate (Di Mambro et al., 2017) (fig. 1B). In addition, brassinosteroids (BR) have been shown to regulate the SCN. Missing functional BR-signaling results in lower division rate and distal stem cell renewal, while BR gain-of-function mutants show premature cell differentiation (González-García et al., 2011). An important player of the BR signaling module at the RAM is *BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTER (BRAVO)*, a R2-R3 MYB transcription factor expressed at the QC. BRAVO counteracts BR positive effect on cell division and thus, balances division to a lower rate or a more quiescent state (Vilarrasa-Blasi et al., 2014).

At the genetic level, the highly specific and QC-expressed gene *WUSCHEL-RELATED HOMEODOMAIN 5* (*WOX5*) delineates QC identity and maintenance (Sarkar et al., 2007) (Fig. 1C). *WOX5* activity could most likely occur through direct effect on cell cycle regulators (Forzani et al., 2014). Plants with disrupted expression levels of *WOX5* show aberrant differentiation rates of the distal stem cells indicating a role of *WOX5* in preventing stem cells to differentiate (Sarkar et al., 2007). *WOX5* expression within the stem cells is tightly regulated by the ligand-receptor couple *CLAVATA3-ESR RELATED 40* (*CLE40*)/*CLAVATA1* (*CLV1*) and *ARABIDOPSIS CRINKLY4* (*ACR4*) (Stahl et al., 2013; Stahl et al., 2009). CLE40 is a small peptide produced in the columella cells and then secreted extracellularly where it may bind the leucine rich receptor (LRRs) CLV1 or its homologous ACR4 located in columella stem cells (CI) (Fig. 1C). As a consequence, *WOX5* expression and activity is mostly limited to the QC allowing stem cell differentiation outside of the SCN boundaries (Stahl et al., 2013; Stahl et al., 2009). *WOX5* expression is also regulated by stem cell regulators such as SCARECROW (SCR) and PERIANTHIA (PAN) and by epigenetic modifications (de Luis Balaguer et al., 2017; Pi et al., 2015; Sabatini et al., 2003). In addition, WOX5 protein movement to QC adjacent cells contributes to their identity (Pi et al., 2015), which demonstrates WOX5 effect is not restricted to the QC but it also affects stem cells generating root tissues. In turn, stem cells

show specific regulation such as that mediated by the transcriptional negative feedback loop formed by *SOMBRERO* and *FEZ* in the epidermis (Willemsen et al., 2008). Other example is the non-cell autonomous effect of SHORT ROOT on activating SCR and the BIRD transcription factors BLUEJAY (BLJ) and JACKDAW (JKD) in the ground tissue to promote the asymmetric division generating endodermis and cortex (Long et al., 2015; Moreno-Risueno et al., 2015) (Fig. 1C). In addition, SCR, BLJ and JKD provide stem cell identity to the ground tissue initial, indicating the existence of stemness endogenous determinants (Moreno-Risueno et al., 2015).

Rapid regeneration of the SCN can be observed upon damage. During postembryonic development cell replenishment of stem cells at the SCN is maintain by the slow division rate of the QC. However, when stem cells are stressed or the SCN damaged, the QC divides rapidly to replace dead stem cells. BR signaling at the QC promotes expression of *ETHYLENE RESPONSE FACTOR 115* (*ERF115*), a transcription factor able to transduce the BR positive effect on QC cell divisions (Heyman et al., 2013). Under normal conditions, ERF115 is degraded, while *BRAVO* inhibits division of the QC. Upon damage or stress, ERF115 is activated while *BRAVO* is repressed, which promotes QC division to ease regeneration of the SCN (Vilarrasa-Blasi et al., 2014). Thus, ERF115 and BRAVO oppose function is required for the correct balance of QC divisions under harmful or regular situations.

Upon damage of the QC, the undamaged portion of the SCN quickly adapts and divides to regenerate a fully functional SCN within hours. After ablation of the QC, a new auxin maximum re-specifies cell fate of surrounding stem cells as can be inferred from the expression of the root stem cells maintenance genes *PLETHORA* (*PLT*), *SHR* and *SCR* (Xu et al., 2006). The new positional information provided by *PLT*, *SHR* and *SCR* will thus specify a new QC just a few cells above the original QC at 72 hours post-ablation (Xu et al., 2006) (Fig. 2A). These experiments demonstrate the extremely plastic cellular state of meristematic cells, showing their ability to molecularly interchange cell fates with stem cells to form a new SCN. In agreement with plant unique regenerative capacities, the whole meristem may be regenerated after complete excision (Sena et al., 2009) Intriguingly, although a new SCN is formed, plants missing factors involved in postembryonic stem cell function still undergo regeneration to a fair extent. Thus, it has been proposed that regeneration of a root meristem does not required a fully functional SCN (Sena et al., 2009), while more recent discoveries point out to meristem root regeneration following an embryo-like program of development (Efroni et al., 2016) which culminates with the formation of a new SCN (Fig. 2B). In contrast, other SCN activity factors such as SCR and the BIRDs JACKDAW and BLUEJAY are needed for the proper re-specification of the ground tissue lineage after excision of the RAM (Moreno-Risueno et al., 2015). These results indicate that embryonic and postembryonic developmental programs are required and involve fine temporal and cell-type specific regulation. Besides, these results evidence the regenerative properties of undifferentiated meristematic cells showing that tissues may arise from non-stem cells through a reprograming process or a cell fate transition (Efroni et al., 2016). Eventually, stem cells are formed and required for proper organ function and growth.

4. Root stem cells in whole organ regeneration.

Regeneration of a whole organ may occur naturally from explants or exogenously induced from callus upon hormonal supplementation. Factors regulating root SCN maintenance and activity play an important role in whole organ regeneration. In addition, cell fate transitions and reprogramming, which are features normally associated to stem cells are crucial for these developmental processes.

4.1. Pericycle reprogramming capacities are required for organ regeneration and resemble stem cell features

Current advances suggest that callus formation is a required stage for natural regeneration of organs from explants as well as upon hormonal inductive conditions (Bustillo-Avendaño et al., 2017; Perianez-Rodriguez et al., 2014). Callus formation is derived from J0121 marked cells, which is marker for xylem pole pericycle in roots and cells adjacent to xylem in aerial tissues (Sugimoto et al., 2010). This data suggested that cells of the pericycle type (marked by J0121) were the main contributors to callus formation. Because of this wide developmental potential, pericycle and pericycle-like cells have been considered as a type (or reservoir) of stem cells during plant postembryonic development. Particularly pericycle-like cells retain a high ability to dynamically re-specify their own identity under appropriate conditions to develop a callus (Sugimoto et al., 2010) or form new organs such as lateral or adventitious roots (Beeckman and De Smet, 2014; Bustillo-Avendaño et al., 2017). One factor involved in pericycle re-specification is the transcriptional regulator *MINIYO* which is continuously excluded from the nucleus to prevent its effect on the activation of cell differentiation programs (Muñoz et al., 2017). Thus, undifferentiated state of pericycle might arise as a requirement for plasticity in cell fate re-specification. Following this idea, pericycle cell fate plasticity might require specific signaling preventing MINIYO nuclear subcellular localization. In contrast, absence of this putative signaling in cell-types different from pericycle might allow MINIYO lo localize in nuclei leading to rapid differentiation and preventing cell-fate re-specification. Future studies might unravel the possible existence of this signaling and its role in establishment of cell fate plasticity.

Pericycle also needs to be re-specified for the proper development of lateral roots. Lateral root formation involves formation of a new organ, although, it is not considered to be *per se* a regeneration process. This re-specification is periodic and regulated by oscillations in gene expression and in auxin response which are part of the so called “lateral root clock” (Moreno-Risueno et al., 2010). Periodic oscillations in auxin response appear to require intermittent pulses of auxin triggered by cell death related processes occurring at the root tip cap (Xuan et al., 2016). As a consequence of these periodic inputs, clusters of pericycle cells are re-specified as lateral root founder cells every 5-6 hours demonstrating a remarkable competence of this tissue to switch between cell identities (Fig. 3A). As callus is also initiated from pericycle-like cells but it did not appear to have a specific organization, it was surprising to learn that callus and LR resemble each other as can be inferred from tissue marker and transcriptomic analysis (Sugimoto et al., 2010; Vanneste et al., 2005; Xu et al., 2012). Genetic studies also identified common regulation between callus and LR formation. Particularly, mutants in *ABERRANT LATERAL ROOT FORMATION 4 (ALF4)*, which was a gene originally shown to be involved in promoting cell division (Celenza et al., 1995),

do not normally develop LRs nor calli (Sugimoto et al., 2010). Other relevant gene regulating LR and callus formation is *SOLITARY ROOT* (*SLR*). SLR is an auxin signaling factor of the Aux/IAA protein family. The lack of a functional SLR in the *slr-1* dominant mutant, which results in reduced auxin response, affects development of both LRs and calli (Fig. 3A/ 3B). *ALF4* and *SLR* are genetically related as *ALF4* ectopic expression in *slr-1* mutant background partially rescues *slr-1* phenotypes (Fukaki et al., 2002; Shang et al., 2016). Recent discoveries indicate that ALF4 physically interacts with subunits of the SCF^{TIR1} complex regulating degradation of Aux/IAA proteins. *alf4* mutation has been shown to destabilize the CULLIN1 subunit of the SCF complex to promote expression of Aux/IAA proteins (Bagchi et al., 2018), which suggests that increased levels of SLR in *alf4* mutant could result in reduced auxin response during LR and callus formation. As *ALF4* ectopic expression in *slr-1* background rescues callus formation but not LR formation (Fukaki et al., 2002; Shang et al., 2016) and *alf4* itself is severely impaired in LR formation, it is possible that ALF4 could regulate LR formation through other AuxIAAs different from SLR or alternatively involve other signaling pathways. IAA28 factor regulates auxin signaling required for pericycle priming or reprograming (De Rybel et al., 2010). Future studies might address if ALF4 regulates stability of IAA28 protein, proving, perhaps a role for ALF4 in regulation of pericycle reprogramming. Because IAA28 does not appear to be involved in callus formation (Bustillo-Avendaño et al., 2017), a complex scenario in which ALF4 might regulate stability of various AuxIAAs in a tissue and developmental dependent manner is plausible.

In addition, the transcription factor *OBF Binding Protein 4* (*OBP4*), which is expressed in the root pericycle, promotes callus formation without altering LR initiation. Interestingly, *OBP4* ectopic expression is able to partially rescue the callus deficient phenotype found in the *alf4* mutant plants (Ramirez-Parra et al., 2017) (Fig. 3B). OBP4 is activated upon wound and by exogenous auxin. Therefore, it appears that specific regulation might operate from outside the pericycle during its re-specification, suggesting that pericycle might miss self-organizing properties commonly found in plant stem cells or SCNs. It is therefore unclear, if pericycle cells can be considered as actual stem cells, although they clearly constitute a reservoir of dynamically reprogrammable cells required for new organ formation and regeneration.

4.2. Hormone signaling is required for regeneration and shows connections with lateral root developmental programs.

Hormonal regulation affects stem cell activity and it has been shown to play a major role in callus and LR formation, especially auxin and CKs (Skoog and Miller, 1957). Auxin is necessary to induce both callus and LRs from pericycle-like cells (Perianez-Rodriguez et al., 2014). Auxin signaling through *AUXIN RESPONSE FACTOR 7* (*ARF7*) and *ARF19,* which were genes originally involved in lateral root formation, has been shown to lead to transcriptional up-regulation of *LOB-DOMAIN 16* (*LBD16)*, *LBD17*, *LBD18* and *LBD29* genes. These *LBDs* are necessary and sufficient to start a callus (Fan et al., 2012), and have also been found to be involved in LR formation downstream of auxin signaling (Ito et al., 2016; Okushima et al., 2007; Porco et al., 2016) (Fig. 3A/ 3B). Importantly, *LBD16* regulates polarization and the first asymmetric division of lateral root

founder cells, allowing thus initiation of LRs through symmetry breaking, which is a feature typical of stem cells to specify different cell fates.

CKs have been shown to be required for callus formation, particularly CKs appear to have a predominant role during callus formation upon wounding of plant aerial parts (Iwase et al., 2011). The wounding signal is transduced by *WOUND INDUCED DIFFERENTIATION 1* (*WIND1*), an AP2/ERF transcription factor rapidly upregulated at wound sites. WIND1 promotes CKs signaling and cell de-differentiation to generate a regenerative callus in the affected area (Iwase et al., 2017; Iwase et al., 2011) (Fig. 3C). Recently, it has been shown that in order to start a callus, *WIND1* directly activates other AP2/EFR transcription factors, the *ENHANCER OF SHOOT REGENERTION 1* (*ESR1*) and *ESR2* (Iwase et al., 2017). In agreement with these findings, CKs biosynthesis genes are up-regulated at wounded areas. Moreover, mutants of the CKs signaling pathway show compromised callus growth upon wound induction, and callus inductive medium (CIM) contains CKs, which further demonstrates the role of this hormone during this process (Ikeuchi et al., 2017). The fact that CKs alone are a primary signal inducing callus formation is surprising since CKs induce differentiation and repress LR formation programs (Bielach et al., 2012). Auxin and CKs effects on plant regeneration have been studied in detail but still the cell-specific responses produced by each phytohormone or their combinatorial effect have not been elucidated. Deciphering the molecular changes produced by these hormones in individual cells from which callus and LR are initiated might help to better understand the parallelisms and differences between mechanisms regulating both regenerative processes.

4.3. Role of root stem cell regulators in pluripotency acquisition

In order to regenerate new organs from callus, callus needs to acquire competency. This competency can be understood as pluripotency to form that organ, or more broadly as stemness required to generate the SCN found in that organ. The pluripotency state of the callus to form shoots depends on root SCN maintenance genes (Kareem et al., 2015). Auxin triggers expression of the redundant genes *PLT3*, *PLT5* and *PLT7* which in turn activate *PLT1* and *PLT2* to promote a pluripotent state (Fig. 3B). Once pluripotent progenitor cells are generated within callus, new organs might arise from them according to external hormonal conditions. This transcriptional cascade by which PLT3/5/7 activate PLT1/2 during shoot pluripotency acquisition appears to be independent of the origin of the explant from which the callus was formed. In addition, this sequential activation has been shown to operate during LR formation, demonstrating again similarities between callus and LR formation (Du and Scheres, 2017; Kareem et al., 2015). Interestingly, callus formation itself is unaffected by the mis-expression of *PLT* genes although the resulting callus is unable to proceed with organ regenerative processes (Kareem et al., 2015). In contrast, *PLT3/5/7* are required for wound-induced callus formation (Ikeuchi et al., 2017). WOX11 and LBD16 have also been shown to be required for establishment of pluripotency (Liu et al., 2018). Overexpression of WOX11 promotes callus formation and activates *LBD16* expression (Hu and Xu, 2016; Liu et al., 2014). *LBD16* is also activated in callus upon CIM induction. Inhibiting the WOX11-LBD16 pathway, through overexpression of *WOX11* fused to the SRDX repression domain or in *lbd16* mutants, results in a callus which is affected in regeneration of new shoot organs.

Notably, *PLT1/2* (but not *PLT3/5/7*) expression is downregulated in *lbd16* mutants, suggesting a role for WOX11 and LBD16 in pluripotency acquisition through PLT1/2 root stem cell regulators (Liu et al., 2018).

4.4. Root regeneration and formation of new root stem cells

The generation of roots from callus might be considered as the simplest case of cellular reprograming because the callus itself resembles a LR primordia (Sugimoto et al., 2010); and in addition root formation from callus is promoted by high auxin concentration in the medium (Che et al., 2002; Skoog and Miller, 1957). However, it is unknown if despite similar tissue organization between LRs and calli, calli have SCNs or functional root stem cells. It would be interesting to observe the effect of auxin in the temporal progression leading to root regeneration from a callus. It could be expected that auxin might form maxima to establish new SCNs through *PLTs* genes similarly to the mechanism described for lateral roots (Du and Scheres, 2017). In addition, it could use the SCR pathway to specify a new QC as it does during the formation of LRs (Goh et al., 2016). Alternatively, a regulation similar to that found during RAM regeneration (Efroni et al., 2016) could take place. In this case, root primordia formation from callus may follow an embryo-like program in which auxin and CKs domains would define the origin and orientation of the primordium to subsequently re-specify a complete SCN.

De novo production of adventitious roots can be observed in sectioned aerial portions of plants, normally stem or leaf explants. This process implies the re-specification of cells from shoot to root fate. Particularly intriguing is the regeneration of roots from detached leaves, also known as leaf rooting. Local auxin response can be appreciated at injured leaf blades activating the expression of *WOX11* and *WOX12* in tissues adjacent to xylem (Liu et al., 2014). Overexpression of *WOX11* promotes the expression of *LBD* genes (Liu et al., 2014), which reminds the developmental program described during LR and callus formation. Although *de novo* root and callus formation share common regulation, no callus formation was observed when roots were regenerated from leaf blades (Liu et al., 2014) (Fig. 3C). Recent findings show formation of callus during rooting of whole leaves at the petiole base. Because this regeneration system does not use hormone supplementation and callus was formed in response to endogenous activation of auxin and CKs biosynthesis, this callus was designated as endogenous callus (Bustillo-Avendaño et al., 2017). Importantly, only few cells within this callus appear to have rooting competence. These cells were specified as root founder cells and their specification preceded *de novo* root initiation and formation. This mechanism reminds specification of shoot progenitors within hormone-induced callus (Kareem et al., 2015), indicating that pluripotency acquisition might be a tightly regulated process and not an intrinsic characteristic of callus as initially thought. It is unknown how root founder cells are specified in callus formed during whole leaf rooting, but it might require the SCN regulators *PLT1*, *PLT2* and *SHR*, as no roots were initiated from leaves in the triple mutant *shr plt1 plt2* (Bustillo-Avendaño et al., 2017). This suggests a putative role for these regulators in root pluripotency acquisition during root regeneration. Notably, *shr plt1 plt2* mutants are also impaired in lateral root formation (Bustillo-Avendaño et al., 2017).

5. Role of shoot stem cells during organ self-regeneration

The SAM is located at the apical extreme of the stem and is the source of new cells that will be part of the aerial postembryonic organs (Fig. 4A/4B). Structurally and functionally, SAM activity requires the central zone, which has 3 layers of stem cells named L1, L2 and L3 and immediately below a group of cells known as the organizing center (OC) (Greb and Lohmann, 2016) (Fig. 4B). Similarly as it is found in the RAM, a homeodomain transcription factor gives identity to the OC. This transcription factor is named *WUSCHEL* (*WUS*) and although its expression is limited to the OC, the WUS protein can move upwards to regulate stem cell activity (Daum et al., 2014; Mayer et al., 1998). Within the SAM stem cells, the peptide CLV3 is produced by the positive effect of *WUS* and it is secreted to the apoplasto, where it is recognized by CLV1, a LRR receptor. This genetic circuit makes an analog system as that described for the RAM involving *CLE40* and *ACR4*. As a consequence of *CLV3/1* signaling, *WUS* expression decreases in the OC, creating a negative feed-back loop of transcriptional regulation (Fletcher et al., 1999). SAM stem cells suffer asymmetrical division at any spatial direction oriented to the external side of the meristem pushing new cells away from the SCN. Conversely to what is observed in the RAM, CKs in the SAM favors SCN maintenance while auxin promotes cell differentiation (Besnard et al., 2014; Zhao et al., 2010).

SAM integrity is very well preserved and self-regeneration is observed. Ablation of the SAM is counteracted by regeneration of the surrounded cells demonstrating the great importance of meristems for plant survival (Reinhardt et al., 2003). This regeneration occurs rapidly and strikingly the number and positions of the new branches result unaffected (Reinhardt et al., 2003). Interestingly, not all the cells within the SAM appear to show the same regenerative capacities. While in the RAM a whole new meristem may regenerate after removal, complete removal of SAM activates dormant buds (from where new branches will develop) instead of activating a regenerative process (Reinhardt et al., 2003; Sena et al., 2009). These findings suggest a higher regenerative capacity of the root in comparison to the stem. RAM regeneration requires undifferentiated meristematic cells, and accordingly if the RAM is excised at the differentiation zone no regeneration takes place (Efroni et al., 2016; Sena et al., 2009). It is possible that given the more compact organization of the SAM, or because differentiation occurs more rapidly in the RAM than in the SAM, that regeneration in the SAM is practically confined to the SCN.

6. Role of shoot stem cells during whole organ regeneration

The regeneration of shoots from a callus is not a simple process. It is unclear if any type of stem cell is formed at early stages but the first event appears to be acquisition of pluripotency mediated by PLT3, PLT5 and PLT7 (Kareem et al., 2015) (Fig. 3B) and WOX11-LBD16 pathway (Liu et al., 2018). Subsequently, cells giving rise to new shoots need to be further specified. Particularly, PLT3, PLT5 and PLT7 activate their downstream targets *CUPSHAPED 1* (*CUC1*) and *CUC2*, which are two NAC transcription factors known to regulate SAM initiation during embryogenesis (Aida et al., 1997; Aida et al., 1999; Kareem et al., 2015). However, when *CUC2* gene is overexpressed in *plt3/5/7* mutant callus, shoot regeneration is not restored. Additional evidence shows that shoot regeneration is only restored in plants overexpressing *CUC2* when *PLT2* is simultaneously expressed under *PLT7* regulatory regions, indicating that shoot regeneration

competence also requires root stem cell regulators (Kareem et al., 2015). In addition, the factors ESR1 and ESR2*,* which are able to promote shoot regeneration (Iwase et al., 2017), are direct activators of *CUC2* expression (Ikeda et al., 2006) (Fig. 4C). Another striking indication of the future location of a regenerated shoot from a callus is the presence of *WUS* expression (Gordon et al., 2007). As *WUS* is expressed in the SCN of the SAM, its expression may identify where (and when) shoot stem cells are generated in the regenerative process. Although *wus* loss of function mutant does not show dramatic phenotypes during embryogenesis, shoot regeneration from callus is severely compromised (Gordon et al., 2007; Zhang et al., 2017). Interestingly, overexpression of *WUS* or *ESR2* in *plt3/5/7* mutant callus does not result in shoot formation, confirming that shoot regeneration competence is primarily established by PLT3, PLT5 and PLT7 (Kareem et al., 2015). Subsequently, expression of *WUS* and *CUC* genes in competent cells would give rise to new shoot stem cells, thus regenerating new SAMs. It is unclear the molecular nature of these competent cells; but since their specification requires root stem cell regulators and lateral root founder are also pluripotent cells formed postembryonically, it could be interesting to address if these two cell types resemble one another. An important regulator of SAM stem cell identity during embryogenesis is *WOX2 (*Zhang et al., 2017)*. WOX2* has been very recently shown to act in the earliest events of stem cell specification, however, its role during shoot regeneration is limited (Zhang et al., 2017). It appears that during embryogenesis *WOX2* act as a primary organizer of shoot stem cell specification (Zhang et al., 2017), while *WUS* role is mainly at postembryonic stages, being also preferred during regeneration processes.

Hormones play a role in the regeneration of shoots from callus. The shoot-inducing medium (SIM) provides a high *in vitro* CKs environment promoting shoot cellular identity and further shoot development (Skoog and Miller, 1957). Under shoot inductive conditions, CKs activity in the callus gets restricted to those areas where shoots will be regenerated (Fig. 4C). Importantly, factors involved in shoot pluripotency acquisition or shoot stem cell fate acquisition such as *CUC2, WUS* and *ESR1/2* show restricted expression within the callus to this CKs domains, (Chatfield et al., 2013; Gordon et al., 2007). Although auxin levels in SIM are proportionally lower than those of CKs, auxin also plays an important role in shoot regeneration from callus. Auxin flux is redirected by the cell specific activation of the efflux carrier *PIN-FORMED1* (*PIN1)* in the high CKs domains (Atta et al., 2009; Gordon et al., 2007) resembling endogenous SAM hormonal situation. The use of auxin transport inhibitors decreases shoot regeneration showing the relevance of the correct balance between CKs and auxins in this process (Cheng et al., 2013). Furthermore, plants expressing *miR160* knock-down *ARF10* resulting in less efficient shoot regeneration from callus, which reiterates the relevance of auxin during this process (Liu et al., 2016). It is unknown if auxin is required for expression of shoot stem cell factors in CKs domains, but current evidences point to the combination of cell fate regulators, CKs and auxin signaling converging in specific domains to specify a SAM primordium from which a complete shoot will develop.

Shoot regeneration can be also achieved from roots, promoted in the LR sites of the main root. Surprisingly, despite SAM and RAM being structures organized differently and requiring different set of regulators for their maintenance and activity, it has been recently described that the conversion from a RAM to a SAM is a relatively easy process which occurs after a few rounds

of cell division when appropriate amount of hormones are supplied (Rosspopoff et al., 2017). Interestingly, SAM-fate specification can be also achieved in pericycle cells confirming this tissue constitutes a reservoir of cells highly reprogrammable, even to shoot fate. Thus, when treated with CKs, the xylem pole pericycle experiences shoot genetic reprograming as shown by upregulation of the SAM stem cell factors *WUS* and *CLV3* (Atta et al., 2009; Chatfield et al., 2013). In fact, overexpression of *WUS* is enough to promote shoot identity in root pericycle cells (Gallois et al., 2004). *WUS* has a positive effect on CKs responses, preventing CKs inhibition by type-A *ARABIDOPSIS RESPONSE REGULATORS* (*ARRs*) (Buechel et al., 2010). In addition, *WUS* is a shoot stem cell regulating factor so likely it is the combined regulation of stemness and CKs signaling which converges in shoot or SAM identity. Wounding has been also shown to trigger shoot regeneration from roots but independently of LR initiation sites (Iwase et al., 2015). Current evidences show that *WIND1* transduces the wounding signal, favoring, in roots, a cellular environment required for shoot regeneration (Iwase et al., 2015). Root tissues from which shoots are regenerated upon *WIND1* expression are unknown. As aerial regenerative mechanisms through *WIND1* involve the epidermis (Iwase et al., 2011), it is tempting to speculate about a possible role of the epidermis in shoot regeneration from roots. Root epidermis is thought to be a tissue undergoing rapid differentiation, and thus, it would be interesting to further study if drastic cell-fate changes (such as conversion of root to shoot fate) may occur in fully differentiated tissues upon WIND1 activation.

7. Loss and gain of regenerative capacity

Regeneration capacities of plants rely on stem, meristematic and pericycle cells. These cells are able to undergo major reprogramming to acquire new fates and replace missing cells during regeneration. It has been shown that genome-wide reprogramming of histone H3 lysine 27 trimethylation (H3K27me3), which is a hallmark of a repressive chromatin state, is required for callus formation from aerial explants. Polycomb Repressive Complex 2 (PRC2) functions in establishing H3K27me3 during this process (He et al., 2012). Accordingly, mutants in *CURLY LEAF* (*CLF*) and *SWINGER* (*SWN*), which are methyltransferases of the PRC2 complex, are defective in callus formation from leaf blades and cotyledons. Interestingly, these mutants are not affected in callus formation from roots, suggesting partial redundancy in the activity of these methyltransferases at the organ level, or regulation through alternative mechanisms. It is therefore possible that epigenetic reprogramming may also associate to pericycle reprogramming, since callus formation requires reprogramming of pericycle like cells in aerial explants. Other gene involved in callus formation from leaf explants is *METHYLTRASFERASE 1* (*MET1*), which mediates direct DNA methylation at the locus of the stem cell regulator *WUS*. As a consequence, *WUS* expression is upregulated in *met1* mutants (Li et al., 2011) although the final effect of this regulation is unclear. Notably, other DNA methylation mutants promote *WUS* locus hypomethylation, which allows shoot regeneration from aerial explants without previous generation of a callus (Shemer et al., 2015). These findings indicate that missing epigenetic regulation on SCN maintenance genes of the SAM can promote direct switches in cell identity leading to regeneration, while these switches in cell fate are restricted under normal regulation along with restriction in regeneration capacities.

Mutations of some of PCR2 components also show involvement in developmental transitions. Particularly, *swn-3 clf-50* double mutant plants may generate somatic embryos upon growth in *in vitro* inductive medium for long periods of time. These results indicate that these genes repress embryonic fate and/or inhibit totipotency acquisition (Bouyer et al., 2011; Chanvivattana et al., 2004). Interestingly, somatic embryos formed in *PCR2* mutants can generate a plant, indicating that the main function of the H3K27me3 could be to stabilize developmental transitions or differentiation, providing some kind of cell memory (Birnbaum and Roudier, 2017). In agreement with this observation, it has been shown that *PRC2* member genes display complex spatiotemporal gene expression patterns and function in root meristematic cell specification, balancing cell proliferation and differentiation (de Lucas et al., 2016). However, it is unknown if PCR2 complex is required for regenerative process involving stem or meristematic cells and no phenotypes in known *PCR2* mutants have been reported during organ-self regeneration. However, it is formally possible that epigenetic regulation might allow cells to recover pluripotency or undergo certain changes in cell identity during this or other regenerative processes.

8. Confinement of regenerative capacity

It appears that in higher plants, the greater degree of cell specialization and differentiation of cells, the lower regeneration capacity which those cells show. This can be observed in root cells during RAM regeneration upon excision. Thus, cells remaining young not fully differentiated, can undergo regeneration of the whole meristem while cells in more advanced developmental stages, are devoid of regenerative capacities (Birnbaum and Alvarado, 2008; Efroni et al., 2016; Sena et al., 2009). On the contrary, most cells of plants with a gametophyte dominant phase such as algae, bryophytes and pteridophytes show high ability to regenerate (Huang and Fujita, 1997; La Farge et al., 2013; Somer et al., 2010) while only few types of cells of gymnosperms and angiosperms show high regenerative potential (Garner, 2013). Noteworthy, the gametophytic phase of angiosperms, mostly present as the germlines, shows high regenerative potential (Soriano et al., 2013), and associates with very little differentiation. In contrast, in the sporophytic phase of angiosperms, cell lineages with regenerative potential get confined to specific areas of the plant body, such as meristems or certain tissues (e.g pericycle).

It is accepted that stem cells, in many higher plants, are confined in meristems and that these meristems are found in specific areas of the plant (Greb and Lohmann, 2016). Meristems and SCNs participate in regenerative processes, but not exclusively. As previously mentioned, pericycle like cells are an important reservoir of reprogrammable cells for regeneration upon hormonal induction, and in addition during natural rooting of aerial explants (Bustillo-Avendaño et al., 2017) and lateral root formation (Perianez-Rodriguez et al., 2014). Meristematic and stem cells can be reprogrammed to other cells fates during regeneration, despite the fact they have been specified or pre-assigned an identity. However, not all switches in cell fate are possible. On the contrary, once meristematic cells undergo differentiation, cell fate transitions are stopped, along with their regenerative capacities. Interestingly, pericycle-like cells are found in most plant organs outside meristems and SCNs, and although they may be considered to have undergone differentiation they retain capacity to change fate and regenerate other cell types. Current

evidence highlights cell fate transitions as the driving force of regeneration (Efroni et al., 2016), and accordingly all cells undergoing regenerative processes have been shown to be reprogrammed to many other cell fates. These observations indicate the importance of pluri- or multipotency acquisition as one of the initial steps in regeneration while other features classically associated to stemness, such as asymmetric cell division, might be acquired subsequently during patterning or growth. This scenario suggests that what we currently consider stem cell features might be the convergence of different cell properties which do not necessarily need to occur simultaneously. On the one hand, some properties would involve the capacity of changing fate, in turn linked to remaining undifferentiated, and on the other hand, other properties would involve the capacity of dividing asymmetrically (in size or by changing the division plane) to generate a pattern. Current knowledge indicates that cell fate specification and patterning divisions are mechanisms tightly coordinated (Moreno-Risueno et al., 2015; Sozzani et al., 2010). However, we might just define stem cells as those cells capable of changing fate during developmental programs, while patterning divisions might not be necessarily intrinsic properties of all plant stem cells. By consequence, it is possible that meristematic cells might be considered as stem cells and the whole meristem as an expanded stem cell niche.

9. Concluding remarks

The basic principles of plant regeneration have been known for a number of decades but actual molecular mechanisms behind those principles have been only started to be elucidated. Current advances and developments in microscopic, molecular and computational technologies have been used to generate new data and relevant information. These advances are opening new questions and invite us to rethink classical concepts in plant biology such as pluripotency, cell fate specification and even the actual meaning of what we consider a stem cell in plants.

In the last years, remarkable advances in the knowledge of plant regeneration have been made, and a number of players and mechanism in plant regeneration have been elucidated. However, it is still unknown the reason behind the wide variety of regenerative mechanisms found in plants and the molecular signatures underlying regenerative capacity of certain cell-types, which, in turn, might influence different regenerative capacities between organs and among species. Particularly intriguing is callus formation. As callus is achieved by exogenous hormonal supplementation and it was not thought to be part of endogenous plant regenerative mechanisms, it is unclear why it showed a specific tissue organization and genetic program. More recently, callus has been shown to be formed upon wounding and as part of endogenous regenerative processes. Further studies might shed light on molecular connections and possibly common roles of certain cell-types shared between hormone-induced callus and endogenously-formed callus. Overall, regeneration associates with cells able to change their identity or trans-differentiate, and with apparently low level of differentiation. Thus, it appears that although regeneration uses stem cell regulating factors, it does not require stem cells undergoing asymmetric divisions but rather cells not committed into a specific cell fate or differentiation program.

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Figure legends

Figure 1. Location and structure of the root apical meristem in *Arabidopsis thaliana*. (A) Schematic representation of an adult plant of *Arabidopsis thaliana* showing location of the primary root tip. (B) Schematic representation of hormone flux in the root tip. RAM: Root apical meristem. SCN: Stem cell niche. MZ: Maturation zone. TZ: Transition zone. (C) Stem cell regulators at the SCN of the RAM.

Figure 2. Role of root stem cell regulators and hormone signaling in organ self-regeneration. (A) Schematic representation of changes in expression of stem cell regulators upon quiescent center (QC) ablation. A new root stem cell niche is regenerated after 72 hours (B) Comparison of hormonal interactions between root tip regeneration and embryogenesis.

Figure 3. Role of root stem cell regulators and hormone signaling in whole or *de novo* organ regeneration. (A) Formation of lateral roots in *Arabidopsis thaliana*. Oscillating gene expression leads to specification of founder cells at the xylem pole pericycle. Auxin signaling regulators are shown. (B) Regulation of pluripotency progression from callus formation to organ regeneration during *de novo* organ formation. CIM: Callus inductive medium. SIM: Shoot inductive medium. RIM: Root inductive medium. (C) Regulation of callus and *de novo* root formation upon wounding or excision. Discontinuous lines indicate hypothetical interactions.

Figure 4. Role of the shoot stem cell regulators in regenerative processes. (A) Schematic representation of an adult plant of *Arabidopsis thaliana* showing location of the shoot apical meristem (SAM). (B) Stem cell regulation at the SAM. (C) Progression from pluripotent callus to shoot regeneration.

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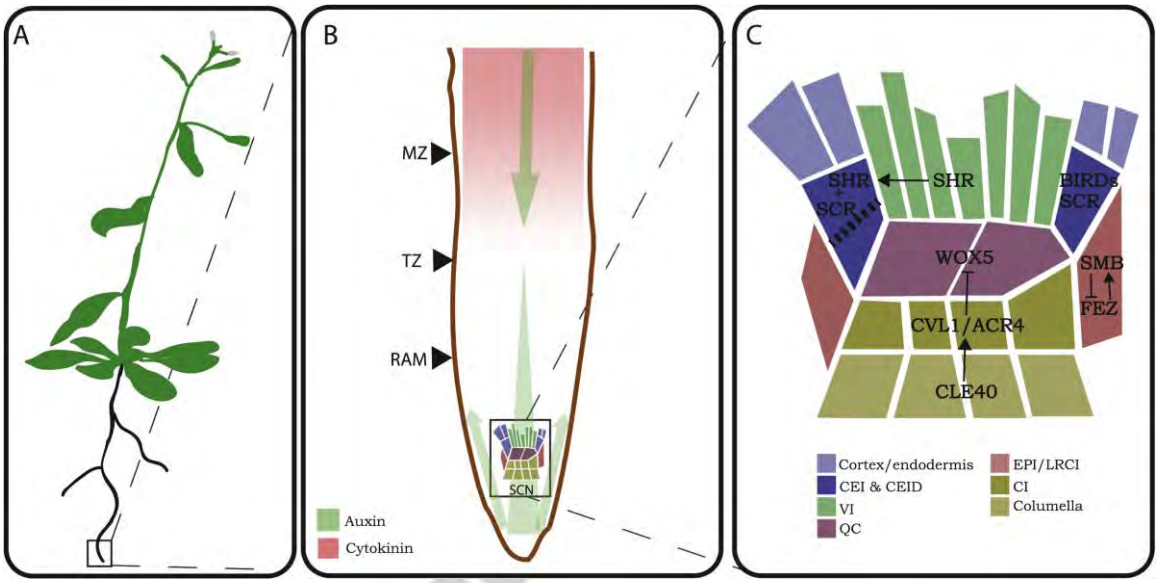

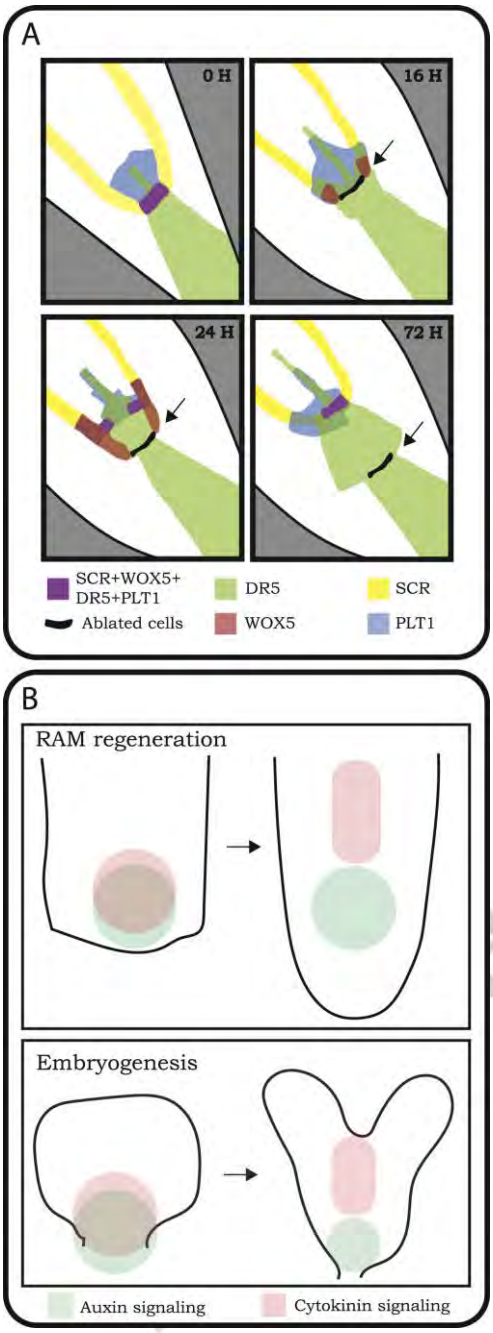
A
0 H
16 H
24 H
72 H
SCR+WOX5+
DR5+PLT1
DR5
SCR
Ablated cells
WOX5
PLT1
B
RAM regeneration
Embryogenesis
Auxin signaling
Cytokinin signaling