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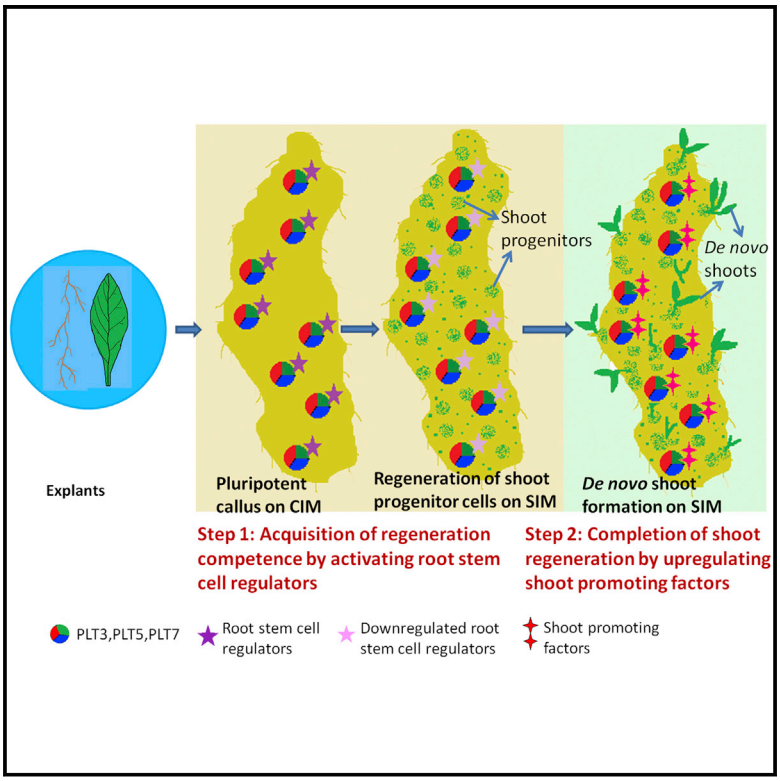
PLETHORA Genes Control Regeneration by a Two-Step Mechanism

Graphical Abstract



Highlights

- *PLT3*, *PLT5*, and *PLT7* genes are essential for de novo shoot regeneration
- PLTs establish regeneration competence by activating root stem cell regulators
- PLTs additionally regulate shoot-promoting *CUC* genes to accomplish the regeneration
- A two-step mechanism of regeneration operates in shoot and root tissues

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In Brief

Kareem et al. uncover intermediate developmental phases of regeneration in plants. Their findings uncouple the acquisition of competence to regenerate shoot progenitors from completion of shoot formation and reveal a two-step mechanism of regeneration that operates in all tissues irrespective of their origin.

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PLETHORA Genes Control Regeneration by a Two-Step Mechanism

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SUMMARY

Regeneration, a remarkable example of developmental plasticity displayed by both plants and animals, involves successive developmental events driven in response to environmental cues. Despite decades of study on the ability of the plant tissues to regenerate a complete fertile shoot system after inductive cues, the mechanisms by which cells acquire pluripotency and subsequently regenerate complete organs remain unknown. Here, we show that three *PLETHORA* (*PLT*) genes, *PLT3*, *PLT5*, and *PLT7*, regulate de novo shoot regeneration in *Arabidopsis* by controlling two distinct developmental events. Cumulative loss of function of these three genes causes the intermediate cell mass, callus, to be incompetent to form shoot progenitors, whereas induction of PLT5 or PLT7 can render shoot regeneration hormone-independent. We further show that PLT3, PLT5, and PLT7 establish pluripotency by activating root stem cell regulators PLT1 and PLT2, as reconstitution of either PLT1 or PLT2 in the *plt3*; *plt5-2*; *plt7* mutant re-established the competence to regenerate shoot progenitor cells but did not lead to the completion of shoot regeneration. PLT3, PLT5, and PLT7 additionally regulate and require the shoot-promoting factor *CUP-SHAPED COTYLEDON2* (*CUC2*) to complete the shoot-formation program. Our findings uncouple the acquisition of competence to regenerate shoot progenitor cells from completion of shoot formation, indicating a two-step mechanism of de novo shoot regeneration that operates in all tested plant tissues irrespective of their origin. Our studies reveal intermediate developmental phases of regeneration and provide a deeper understanding into the mechanistic basis of regeneration.

INTRODUCTION

Regeneration is a common strategy adopted by both plants and animals with functions in tissue repair and propagation [1, 2]. In plants, the regeneration process is widely exploited for in vitro propagation of materials in horticulture. A wide variety of plant tissues (explants) is capable of regenerating an entire organism when supplemented with an appropriate culture medium [1, 2]. In *Arabidopsis,* root and hypocotyl tissues are widely used sources for de novo organogenesis [3, 4]. Modulation of the ratio between the phytohormones auxin and cytokinin in culture media is decisive in specification of de novo shoot or root regeneration [5]. In the commonly used indirect shoot-regeneration system, explants excised from differentiated plant tissues are induced to generate callus, a pluripotent regenerative mass of cells, by incubation on an auxin-rich callus-inducing medium (CIM). Subsequently, de novo shoots can be regenerated from the callus upon incubation on shoot-inducing medium (SIM), which contains high cytokinin-to-auxin ratio [2, 6]. The process of callus formation is thought to be important for the acquisition of competence to form shoot meristems in the succeeding step [7, 8].

A growing body of evidence suggests that activation of the lateral root development program is the common mechanism underlying callus formation from various tissues [4, 9]. Callus formation is abolished in both root and aerial explants of the *aberrant lateral root formation4* (*alf4*) mutant [9], where lateral root formation is impaired due to the failure of initial divisions of pericycle cells [10]. Thus, callus formation involves the activation of genes expressed in lateral root primordia (LRP), and callus shares root-like traits with LRP. However, it is not known whether the root-like trait of callus is required for shoot regeneration and, if so, what molecular components present in the callus are crucial for shoot regeneration.

After induction on SIM, callus develops coordinated polarization of the polar auxin transporter PINFORMED1 (PIN1) and correlated auxin response maxima [3, 11]. An extensive auxin-cytokinin crosstalk is established during shoot meristem initiation, which is critical for induction of the homeodomain transcription factor WUSCHEL (WUS), which specifies de novo stem cells in the center of the regenerating shoot meristem [11].

Regeneration is, therefore, the culmination of developmental events responding to initial exogenous and subsequent endogenous cues. So far, it has proven difficult to dissect different phases of regeneration and therefore to determine the regulatory modules controlling each specific phase. This is a common hurdle to the understanding of the complete regeneration process in plants and in animals. Although many shoot meristem-expressed genes and hormone-related genes have been

implicated in *Arabidopsis* shoot regeneration based on their mutant phenotypes [2, 6, 12], mechanisms underlying the acquisition of regeneration competence and completion of de novo shoot formation remain largely elusive.

Here, we show that plant-specific AP2-family transcription factors, PLETHORA3 (PLT3), PLT5, and PLT7 [13, 14], establish the competence to regenerate shoot progenitor cells by inducing root stem cell regulators PLT1 and PLT2. Independently, PLT3, PLT5, and PLT7 regulate the shoot-promoting factor CUP-SHAPED COTYLEDON2 (CUC2) to permit the de novo shoot regeneration.

RESULTS

PLT3, *PLT5*, and *PLT7* Display Dynamic Expression Patterns during Shoot Regeneration

Recent studies have shown that LRP initiation is required for callus formation, as mutants that fail to initiate LRP are unable to make any callus [9, 15, 16]. To understand the mechanisms controlling the intermediate steps leading to shoot regeneration, mutants that are blocked at different developmental phases of shoot regeneration need to be examined. In a search for genes whose loss of function did not affect callus formation but blocked subsequent steps of de novo shoot regeneration, we considered genes that control lateral organ positioning in *Arabidopsis* [13, 14]. The triple mutant *plt3*; *plt5-2*; *plt7* displays normal shoot outgrowth in planta, but produces aberrant LRP. If a normal lateral root development program is the common mechanism underlying pluripotent callus formation from various plant tissues, *plt3*; *plt5-2*; *plt7* potentially would produce callus abnormal in subsequent regeneration steps.

To probe the role of *PLT3*, *PLT5*, and *PLT7* during de novo shoot regeneration, we first assessed their expression patterns using transgenic lines harboring translational fusion proteins of all three *PLT*s tagged with yellow fluorescent protein (YFP), *PLT3*::*PLT3*:*YFP*, *PLT5*::*PLT5*:*YFP*, and *PLT7*::*PLT7*:*YFP*. These fusion proteins are able to complement the *plt3*; *plt5-2*; *plt7* mutant phenotype and therefore are functional [14]. As reported earlier [14], we observed that all three *PLT*s were expressed at early stages of LRP initiation and in young leaves (Figures 1A, 1K, and 1U). Upon CIM induction, all three *PLT*s were upregulated in proliferating callus cells (Figures 1B–1D, 1L–1N, 1V–1X, 1F', 1G', 1K', 1L', 1P', and 1Q'). At later stages, expression was confined to sub-epidermal layers of young callus (Figures 1E, 1O, and 1Y). Upon transfer to SIM, expression was gradually restricted to the group of cells forming shoot progenitors (Figures 1F–1H, 1P–1R, 1Z–1B', 1H', 1M', and 1R'). Eventually, very high expression of all three *PLT*s was noticed at the surface of regenerated shoot meristems (Figures 1I, 1S, and 1C') and in developing leaf primordia (Figures 1J, 1T, 1D', 1I', 1N', and 1S'). Similar to callus-mediated indirect shoot regeneration, all three *PLT*s were upregulated during direct shoot regeneration from the LRP without the intervening callus phase (Figure S1). All three PLTs were also upregulated during shoot regeneration from LRP on a medium containing cytokinin as a sole hormonal supplement, suggesting that these PLTs are regulated by cytokinin during shoot regeneration (Figures S1E, S1J, and S1O). Our data indicate that *PLT3*, *PLT5*, and *PLT7* display dynamic expression patterns during de novo shoot regeneration.

PLT3, *PLT5*, and *PLT7* Are Necessary for De Novo Shoot Regeneration

We next asked whether the activity of *PLT3*, *PLT5*, and *PLT7* is required for de novo shoot regeneration. Toward this, callus was induced from leaf, cotyledon, hypocotyl, and root from both wild-type and *plt3*; *plt5-2*; *plt7* mutants by incubating these tissues on CIM. A proliferating mass of callus was obtained from both wild-type and *plt3*; *plt5-2*; *plt7* tissues within 10 days of induction on CIM. These calli were incubated on SIM to trigger shoot regeneration. The efficiency of shoot regeneration on SIM was assessed in wild-type and mutant calli at various time points. Green regenerating foci started appearing on wild-type callus after 6 days of induction on SIM (Figure S2A), whereas no regenerating foci were observed in *plt3*; *plt5-2*; *plt7* tissue irrespective of the plant region of its origin (Figure S2D). The first leafy shoots emanated from the wild-type callus after 9 or 10 days of induction (Figure S2B), and more shoots were formed after 14 days (Figures 2A–2D). Shoots were regenerated from all of the tested in wild-type explants as previously reported [4, 17]. Shoot regeneration was completely abolished in *plt3*; *plt5-2*; *plt7* tissue (Figures 2A'–2D'). The triple-mutant tissues did not display any sign of shoot regeneration even after prolonged incubation on SIM, indicating that *plt3*; *plt5-2*; *plt7* callus has lost pluripotency. We further assessed the regeneration potential of double-mutant combinations as well as single *plt* mutants. Though a modest reduction in shoot regeneration was observed in *plt3*;*plt5-2* and *plt5-2*;*plt7* mutants, *plt3*;*plt7* displayed a severe reduction (Figures 2E and S2G). Shoot regeneration was not substantially affected in single mutants (*plt3*, *plt5-2*, and *plt7*; Figures 2E and S2G).

We next examined the conversion of LRP into shoots without an intervening callus phase, upon exposure to cytokinin-rich medium in both wild-type and *plt3*; *plt5-2*; *plt7*. Shoots regenerated from LRP of wild-type root explants within 8–10 days of induction on cytokinin-rich medium (Figure S2C), but not from *plt3*; *plt5-2*; *plt7* LRP (Figure S2F).

Taken together, our data demonstrate that *PLT3*, *PLT5*, and *PLT7* genes are necessary for de novo shoot regeneration, but not for callus formation. The regeneration phenotypes of *plt* mutants remained invariant in different culture conditions reported in the literature [3, 4, 18] (Figures 2E and S2G). Because shoot regeneration was completely abolished in *plt3*; *plt5-2*; *plt7*, we chose the triple mutant for the remaining analyses.

PLT5 or *PLT7* Is Sufficient to Bypass Hormonal Requirements for De Novo Shoot Formation

Next, we investigated whether *PLT* gene expression can replace the requirement for cytokinin application for de novo shoot formation. *PLT5* and *PLT7* were overexpressed in wild-type plants under the control of the *Cauliflower Mosaic Virus* (*CaMV*) *35S* promoter in a dexamethasone (DEX)-inducible fashion (*35S*::*PLT5*:*GR* and *35S*::*PLT7*:*GR*). The callus generated from *35S*::*PLT5*:*GR* or *35S*::*PLT7*:*GR* on CIM was placed on cytokinin-free minimal medium supplemented with 20 μM DEX for induction of PLT activity. De novo shoots regenerated on the hormone-free medium after 2 weeks of DEX induction (Figures 2F and S2H). Nevertheless, unlike cytokinin-induced shoot regeneration, ectopic overexpression of *PLT5* or *PLT7* triggered de novo shoot formation at a low frequency, suggesting that not all of the

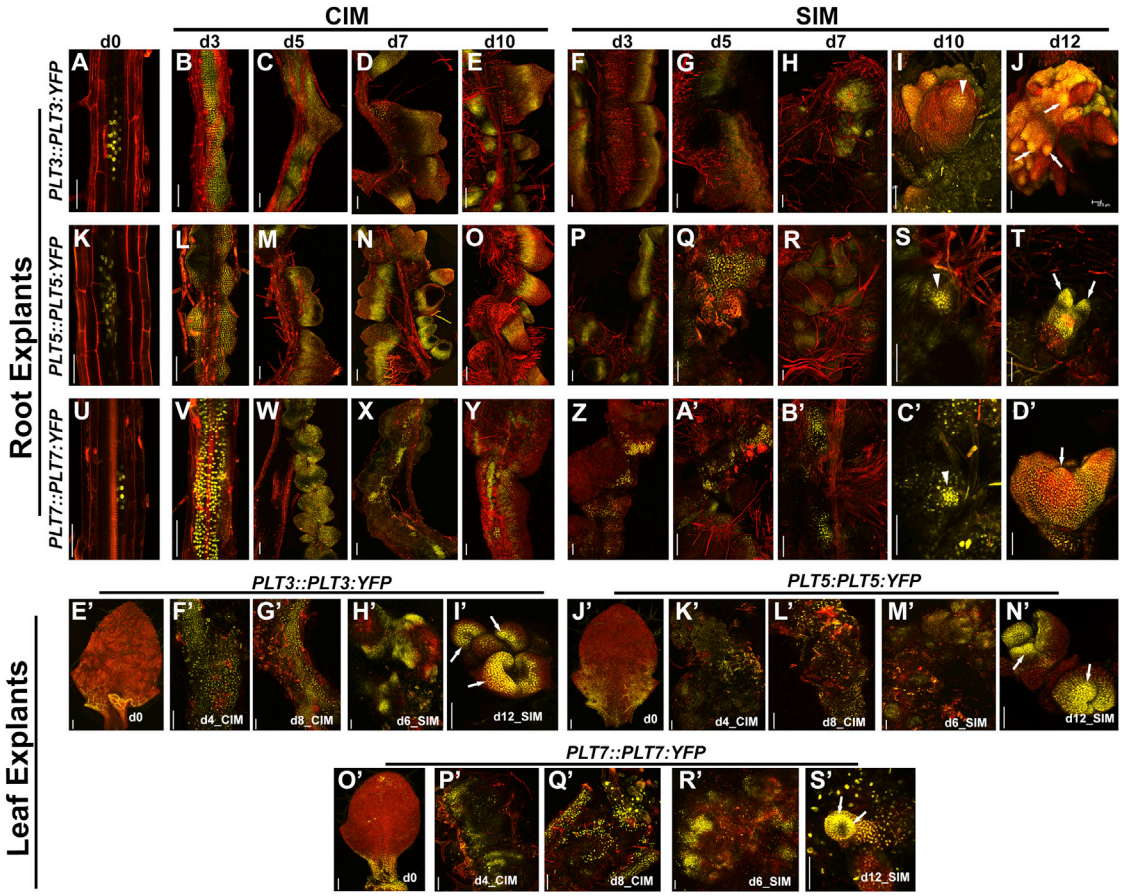

Figure 1. *PLT* Genes Are Upregulated during Shoot Regeneration

(A–D') Expression of *PLT3*::*PLT3*:*vYFP* (A–J), *PLT5*::*PLT5*:*vYFP* (K–T) and *PLT7*::*PLT7*:*vYFP* (U–D') during de novo shoot regeneration from root explants. (A–E, K–O, and U–Y) Expression of all three reporters in both untreated LRP (A, K, and U) and CIM-induced calli (B–E, L–O, and V–Y). Note all three *PLT*s are expressed throughout the callus phase and the expression is confined to the sub-epidermal cells of proliferating callus after 7–10 days (D, E, N, O, X, and Y). Gradual accumulation of expression of all three *PLT*s in shoot-forming cells (F–H, P–R, and Z–B'), nascent shoot meristem (arrowhead; I, S, and C'), and leaf primordia (arrow; J, T, and D') upon SIM treatment.

(E'–S') *PLT3-YFP* (E'–I'), *PLT5-YFP* (J'–N'), and *PLT7-YFP* (O'–S') expression during shoot regeneration from leaf explants. All three *PLT*s are expressed in untreated young rosette leaves (E', J', and O'), callus cells derived from leaf explants on CIM (F', G', K', L', P', and Q'), and callus cells treated on SIM (H', I', M', N', R', and S'). Arrows in (I'), (N'), and (S') mark leaf primordia.

All images are maximum projections of z stacks except (A), (K), and (U), which are single optical sections. Red signal is FM4-64 stain in (I), (J), (S), (T), (C'), (D'), (I'), (N'), and (S'), chlorophyll autofluorescence in (E'), (J'), and (Q'), and propidium iodide stain in the remaining. The scale bar represents 50 μm in (A), (K), and (U) and 100 μm in the rest.

shoot-promoting activities of cytokinin can be mimicked by *PLT5* or *PLT7* overexpression. Our results demonstrate that either PLT5 or PLT7 is sufficient to trigger de novo shoot formation, in addition to its essential role in shoot regeneration.

Shoot Regeneration Stimuli Fail to Establish Correct *PIN1* Expression and Auxin Response Domains in *plt3*; *plt5-2*; *plt7* Mutants

The polar auxin efflux carrier PIN1 is the earliest marker of lateral organ initiation and of regenerating shoot progenitor cells [3, 19, 20]. We therefore compared the pattern of PIN1-GFP (*pPIN1*::*PIN1*:*GFP*) and auxin response sensor DR5-VENUS (*pDR5rev*::*3XVENUS-N7*) expression in wild-type and in *plt3*; *plt5-2*; *plt7* mutants during regeneration. We used calli derived both from root and leaf for this experiment. Both markers were expressed in wild-type and mutant LRP before transfer to CIM (Figures 3A and 3A'). The upregulation of the *DR5* reporter noticed until 4 days after transfer to CIM in both genotypes (Figures 3B, 3B', 3C, and 3C'), whereafter the level of auxin response gradually decreased (Figures 3D–3F and 3D'–3F'). In wild-type, *PIN1-GFP* expression persisted 8 days after induction on CIM (Figures 3C–3F) but diminished 10 days after transfer (data not shown). Conversely, *PIN1* expression was downregulated in *plt3*; *plt5-2*; *plt7* callus by 6 days on CIM and it was undetectable after 8 days (Figures 3D'–3F').

After transfer to SIM, PIN1-GFP was initially detected in the shoot progenitor cells regenerated in wild-type callus, consistent with published data (Figure 3H) [3]. During the emergence of leaf primordia from the wild-type shoot meristem, both *DR5-VENUS* and *PIN1-GFP* signal accumulated in the primordia (Figures 3I

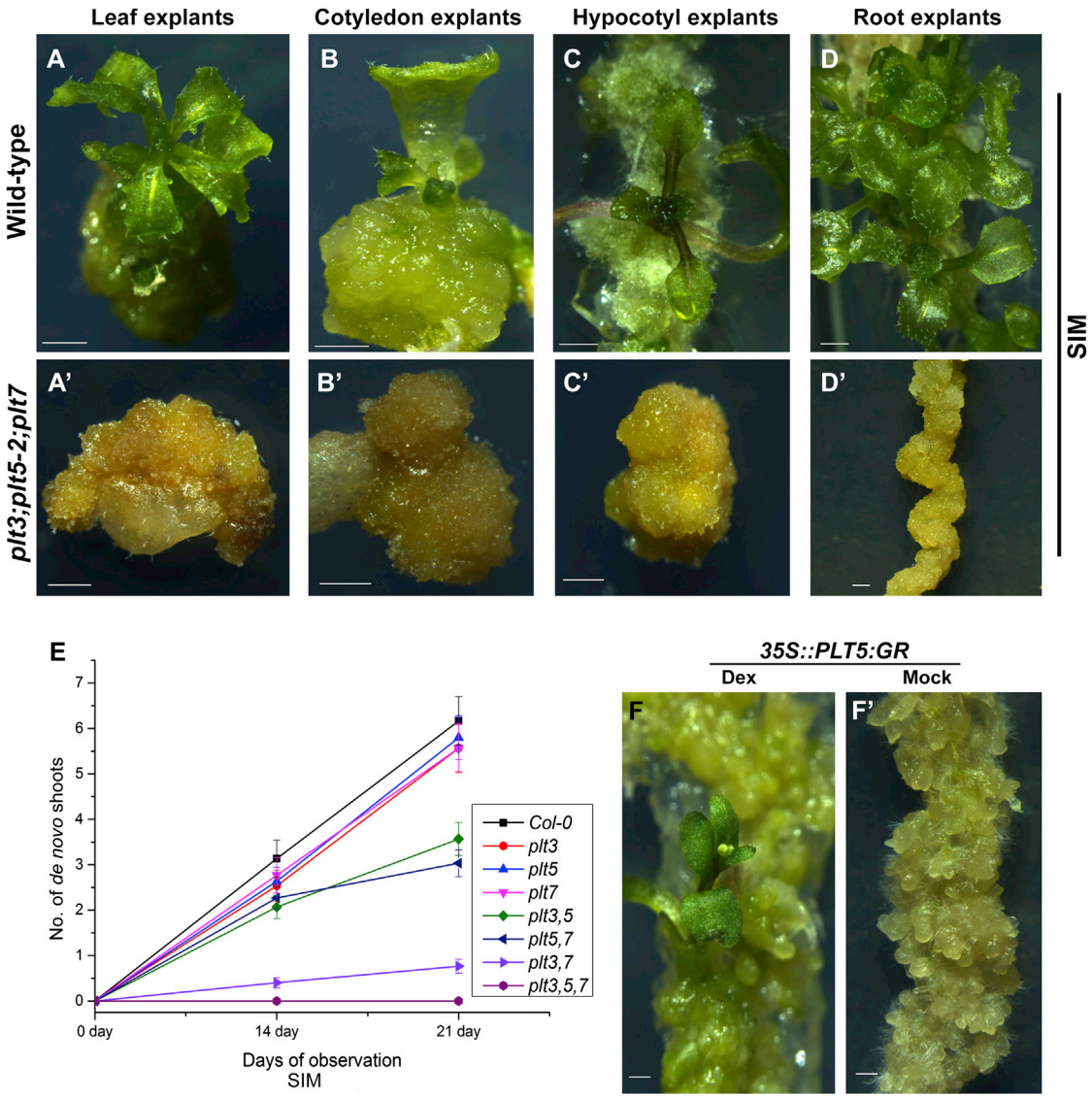

Figure 2. *PLT* Genes Are Necessary and Sufficient for De Novo Shoot Formation

De novo shoot regeneration in wild-type calli derived from (A) leaf, (B) cotyledon, (C) hypocotyl, and (D) root explants after 14 days of SIM treatment.

(A'–D') No shoot regeneration in *plt3*; *plt5-2*; *plt7* calli derived from leaf, cotyledon, hypocotyl, and root explants.

(E) Regeneration efficiency in various combinations of *plt* mutants on SIM. Number of shoots represents shoots formed per explant (~3 cm).

(F) Shoot regeneration in the callus of wild-type;*35S*::*PLT5*:*GR* incubated on hormone-free medium supplemented with DEX.

(F') No shoot regeneration in mock-treated callus of wild-type;*35S*::*PLT5*:*GR*.

The scale bars represent 1 mm. Error bar in (E) represents SEM.

and 3J). On the contrary, *PIN1-GFP* expression was never detected in *plt3*; *plt5-2*; *plt7* callus after transfer to SIM (Figures 3G'–3J'). Moreover, no PIN1-GFP marked shoot progenitor cells developed in the mutant. *DR5* reporter activity was dispersed throughout the *plt3*; *plt5-2*; *plt7* callus, and there was no sign of localized accumulation during incubation on SIM. Furthermore, the VENUS signal intensity was relatively low as compared to wild-type (Figures 3I, 3J, 3I', and 3J'). Therefore, we surmise that polar auxin transport and auxin response gradients are impaired in the triple mutant. The auxin response gradient was also abrogated in mutant LRP when stimulated for direct conversion to shoot (Figures S3A–S3E'). Taken together, our studies demonstrate that PLT3, PLT5, or PLT7 is required during the initial steps of shoot regeneration.

Reconstitution of *PIN1* Expression in *plt3*; *plt5-2*; *plt7* Does Not Restore Shoot Regeneration

Failure to detect *PIN1* expression in *plt3*; *plt5-2*; *plt7* upon SIM treatment led us to ask whether reconstitution of *PIN1* activity could trigger shoot regeneration in the triple mutant. *PIN1-GFP* was introduced into the mutant under the regulation of the artificial auxin-responsive *DR5* promoter (*DR5*::*PIN1*:*GFP*). The experiment was based on the notion that auxin and PIN1 function in a positive regulatory feedback loop and the use of

an auxin-responsive regulatory element to drive PIN1-GFP could maintain this loop in the mutant. Unlike in wild-type transgenic for *DR5*::*PIN1*:*GFP*, neither green foci nor developing shoot meristems were observed in *plt3*; *plt5-2*; *plt7*; *DR5*::*PIN1*:*GFP* callus on SIM, although *PIN1-GFP* was expressed throughout the callus (Figures 3K–3L' and S3F), indicating that forced *PIN1* expression is not able to rescue shoot regeneration in the mutant. Perhaps expression of PIN1 did not rescue the *plt3*; *plt5-2*; *plt7* triple mutant because the correct polarization of PIN1 necessary for shoot regeneration is still not provided. PLT3, PLT5, and PLT7 might regulate factors, which enable a correct polarization of PIN1 and thus shoot outgrowth.

De Novo Shoot-Promoting Activity of Key Regulators Is Impaired in *plt3*; *plt5-2*; *plt7*

We investigated whether the *WUS-CLV3* regulatory feedback loop, which is an integral part of both in planta and de novo shoot meristem development in wild-type [3, 21], was functional in *plt3*; *plt5-2*; *plt7* mutants. *pWUS* activity was dispersed across a wide area of the wild-type callus surface after 4 days on SIM (Figures 4A–4C) but was gradually confined to the center of nascent shoot meristems thereafter (Figures 4D–4F). Unlike in wild-type, *plt3* single mutant, or *plt3*; *plt5-2* double mutant, a locally confined expression pattern of *pWUS*::*CFP* was not established in *plt3*; *plt5-2*; *plt7* triple-mutant tissue (Figures 4A'–4F', S4K, and S4L).

Initially, the *pCLV3*::*CFP* reporter displayed dynamic expression on SIM, and later, the *CLV3* reporter was reinstated exclusively in the center of regenerated shoot meristems in calli of wild-type, *plt3* single mutant, and *plt3*; *plt5-2* double-mutant tissue (Figures 4G–4L, S4M, and S4N). This dynamic pattern of *pCLV3*::*CFP* expression was disrupted in *plt3*; *plt5-2*; *plt7* triple-mutant callus, and it displayed sporadic CLV3 reporter expression in few cells (Figures 4G'–4L'). Consistent with these observations, spatio-temporal expression pattern of *WUS* and *CLV3* failed to be established in *plt3*; *plt5-2*; *plt7* LRP when it was stimulated for direct shoot induction (Figures S4A–S4J'). Taken together, our data suggest that WUS-CLV3 feedback regulatory interaction is lost in the triple mutant, and the mutant explants fail to regenerate cells with functional shoot stem cell identity.

So far, our analysis was based on observations made from regeneration upon external hormone application. Next, we examined whether overexpression of shoot inducers like *WUS* or *ESR2*, which are known to trigger shoot regeneration without external hormone application [18, 22], can instigate de novo shoot formation in *plt3*; *plt5-2*; *plt7* callus. As reported earlier, de novo shoots were formed from wild-type callus after the ectopic overexpression of estradiol-inducible *WUS* (*pG10-90*::*WUS*:*3AT*) or *ESR2* (*pG10-90*::*ESR2*:*3AT*) on hormone-free medium supplemented with β-estradiol (Figures 4M–4O) [18, 22]. On the contrary, there was no sign of direct or callus-mediated shoot regeneration in *plt3*; *plt5-2*; *plt7* following overexpression of *WUS* or *ESR2* (Figures 4M'–4O'). Our data suggest that forced expression of known shoot inducers such as *WUS* or *ESR2* cannot induce shoot regeneration in *plt3*; *plt5-2*; *plt7* and therefore that the mutant has lost the competence to regenerate.

PLT3, PLT5, and PLT7 Activate Root Stem Cell Maintenance Regulators PLT1 and PLT2 to Establish the Competence for De Novo Shoot Regeneration

Callus derived from root as well as shoot tissues expresses root cell fate markers and displays organized structures [9]. However, the functional significance of the activation of root stem cell maintenance regulators in the regenerative mass of cells is not known. Because *plt3*; *plt5-2*; *plt7* mutant callus derived from root or shoot is abnormal in its regenerative capacity, we asked whether root stem cell maintenance regulators are deregulated in *plt3*; *plt5-2*; *plt7* callus. To address this, we first examined the expression of key root stem cell maintenance regulatory genes such as the *SCARECROW* (*SCR*), *PLT1*, and *PLT2*, in both wild-type and mutant calli. These genes are expressed in different cell types of the root meristem (Figures 5A and 5G) [23, 24]. The expression of *PLT1*::*PLT1*:*vYFP*, *PLT2*::*PLT2*:*vYFP* [25], and *pSCR*::*H2B*:*vYFP* was upregulated in proliferating cells of wild-type callus derived from leaf or root explants (Figures 5C–5F, 5I–5L, S5A, and S5B) and sustained throughout the callus phase. In contrast, no expression of these regulators was detected in *plt3*; *plt5-2*; *plt7* callus derived from leaf or root explants at any stage of callus formation (Figures 5C'–5F', 5I'–5L', S5C, and S5D). We did observe some expression of *PLT2* in few callus cells derived from mutant primary root tip (Figures S5E and S5F). We further analyzed the expression of the lateral root cap and epidermis-specific *WEREWOLF* (*WER*) gene [26] in both wild-type and mutant calli derived from leaf and root explants. *pWER*::*H2B*:*vYFP* was detected in the proliferating cells of both wild-type and mutant calli, although the expression pattern and level in the mutant was different from that of wild-type (Figures 5O–5R and 5O'–5R'). Thus, not all the root marker expression is absent in *plt3*; *plt5-2*; *plt7* callus. Further, it is important to note that genes that are not detectably expressed in the mutant LRP also fail to detectably express in the callus derived from shoot or root (Figures 5B'–5F' and 5H'–5L').

To probe the functional significance of the activation of root-expressed genes in the callus, we chose the root stem cell maintenance regulators PLT1 and PLT2 for further analysis, as they are root-specific, unlike SCR and WER, which are also expressed in the shoot [23, 27, 28]. We examined whether PLT1 and PLT2 can be induced by PLT5. Toward this, we performed qRT-PCR and analyzed the expression of *PLT1* and *PLT2* upon the DEX induction of *PLT5* in *35S*::*PLT5*:*GR* callus. Both *PLT1* and *PLT2* were upregulated after 12 hr of induction of *PLT5* (Figure 5S). We next asked whether reconstitution of *PLT1* or *PLT2* expression could re-establish regenerative competence in *plt3*; *plt5-2*; *plt7* and could trigger de novo shoot regeneration. To test this, the coding sequence of *PLT1* tagged with *YFP* was introduced into *plt3*; *plt5-2*; *plt7* under control of a 1.5-kb truncated promoter of *PLT7* (*PLT7*::*cPLT1*:*vYFP*). *PLT7* (1.5 kb) promoter was active only on CIM and not on SIM, similar to the endogenous *PLT1* expression window (Figures S6A, S6C–S6E, S6G, and S6H). Mutant calli derived from both aerial and root explants regained a morphology similar to wild-type after activation of *PLT1* (Figures 6G–6I and S6I–S6K). Upon the induction on SIM, *plt3*; *plt5-2*; *plt7*; *pPLT7*::*cPLT1*:*vYFP* callus turned green, similar to wild-type and unlike *plt3*; *plt5-2*; *plt7* callus (Figures 6A, 6B, and 6D–6F). We also examined the direct regeneration efficiency in *plt3*; *plt5-2*; *plt7*; *pPLT7*::*cPLT1*:*vYFP* and

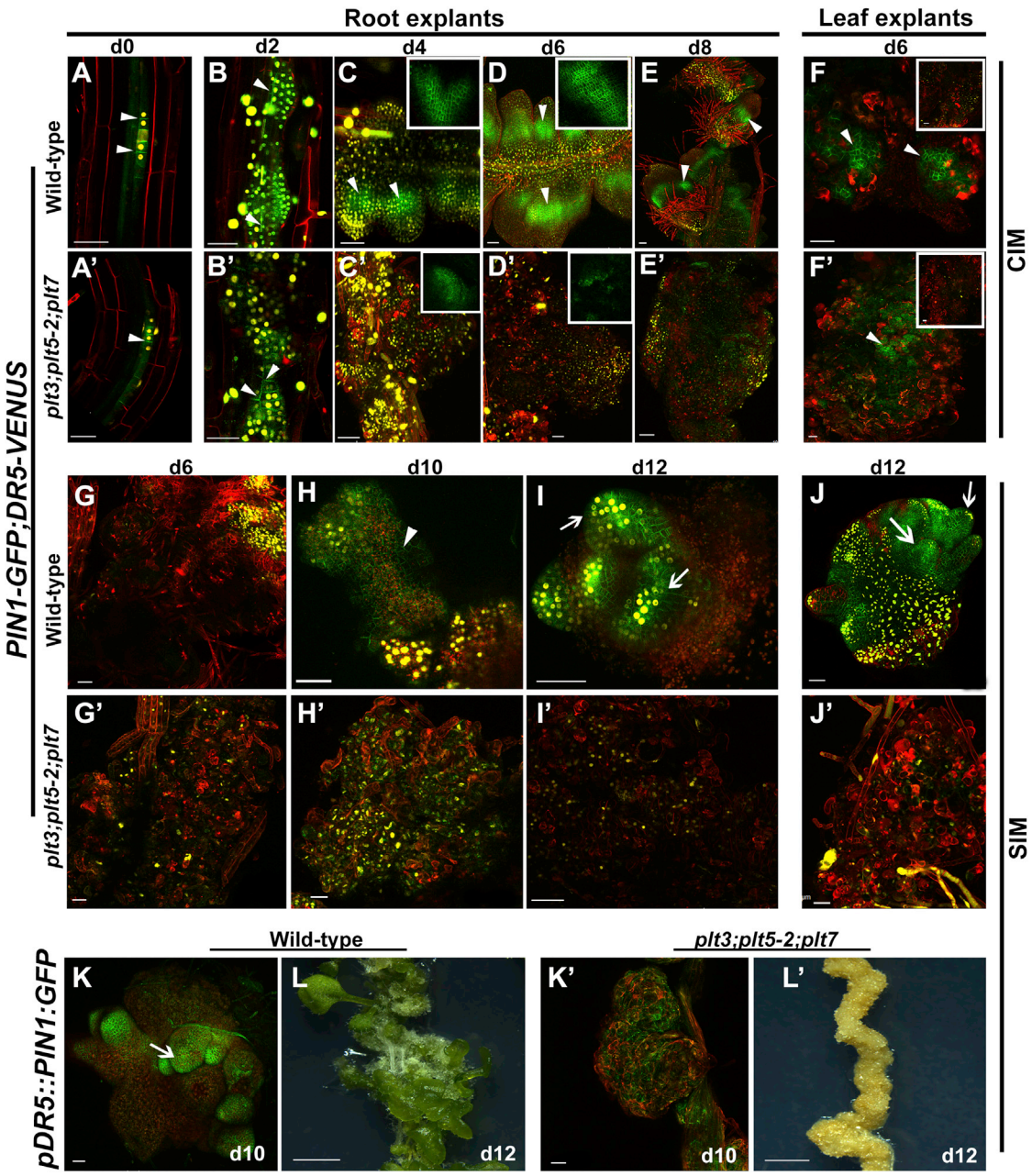

Figure 3. Auxin Responses Are Deregulated in *plt3*; *plt5-2*; *plt7* Mutants after Regeneration Stimulus

(A–J and A'–J') *PIN1*::*PIN1*:*GFP* (green) and *pDR5rev*::*3XVENUS-N7* (yellow) expression in wild-type explants (A–J) and *plt3*; *plt5-2*; *plt7* explants (A'–J'). Expression of both the markers in wild-type (A) and mutant (A') LRP before transfer to CIM. (B–F) Upregulation of *PIN1-GFP* (arrow head) in wild-type calli derived from root explants (B–E) and leaf explants (F) on CIM. (B'–F') *PIN1-GFP* (arrow head) expression is detectable in mutant calli derived from root explants (B'–D') and leaf explants (F') till 6 days on CIM. (E') No detectable *PIN1-GFP* expression in mutant callus by 8 days on CIM. Upregulation of *DR5-VENUS* in both the genotypes till 4 days on CIM (B, C, B', and C') and downregulation of the *VENUS* signal for the following days (D–F and D'–F'). Inset in (C), (D), (C'), and (D') shows *PIN1-GFP* expression and in (F) and (F') shows *DR5-VENUS* expression. (G) Sporadic distribution of *DR5-VENUS* signal and no expression of *PIN1-GFP* in wild-type after 6 days on SIM. (H) Upregulation of *PIN1-GFP* in the developing-shoot meristem (arrowhead) and expression of *DR5-VENUS* in the emerging leaf primordia and in the peripheral callus in wild-type after 10 days on SIM. (I) Accumulation of both *PIN1-GFP* and *DR5-VENUS* signal within leaf primordia (arrow) in root-derived wild-type callus on SIM. (J) Expression of *PIN1-GFP* and *DR5-VENUS* in leaf primordia (arrow) in leaf-derived wild-type callus on SIM. (G') Weak expression of *DR5-VENUS* in *plt3*; *plt5-2*; *plt7* after 6 days on SIM. (H'–J') No *PIN1-GFP* expression but a weak and ubiquitous expression of *DR5-VENUS* in the mutant calli derived from both root and leaf explants after 10–12 days on SIM.

(K) PIN1-GFP localization at the tip of leaf primordia (arrow) in wild-type;*pDR5*::*PIN1*:*GFP* after 10 days on SIM.

(L) Shoot regeneration in wild-type;*pDR5*::*PIN1*:*GFP* after 12 days on SIM.

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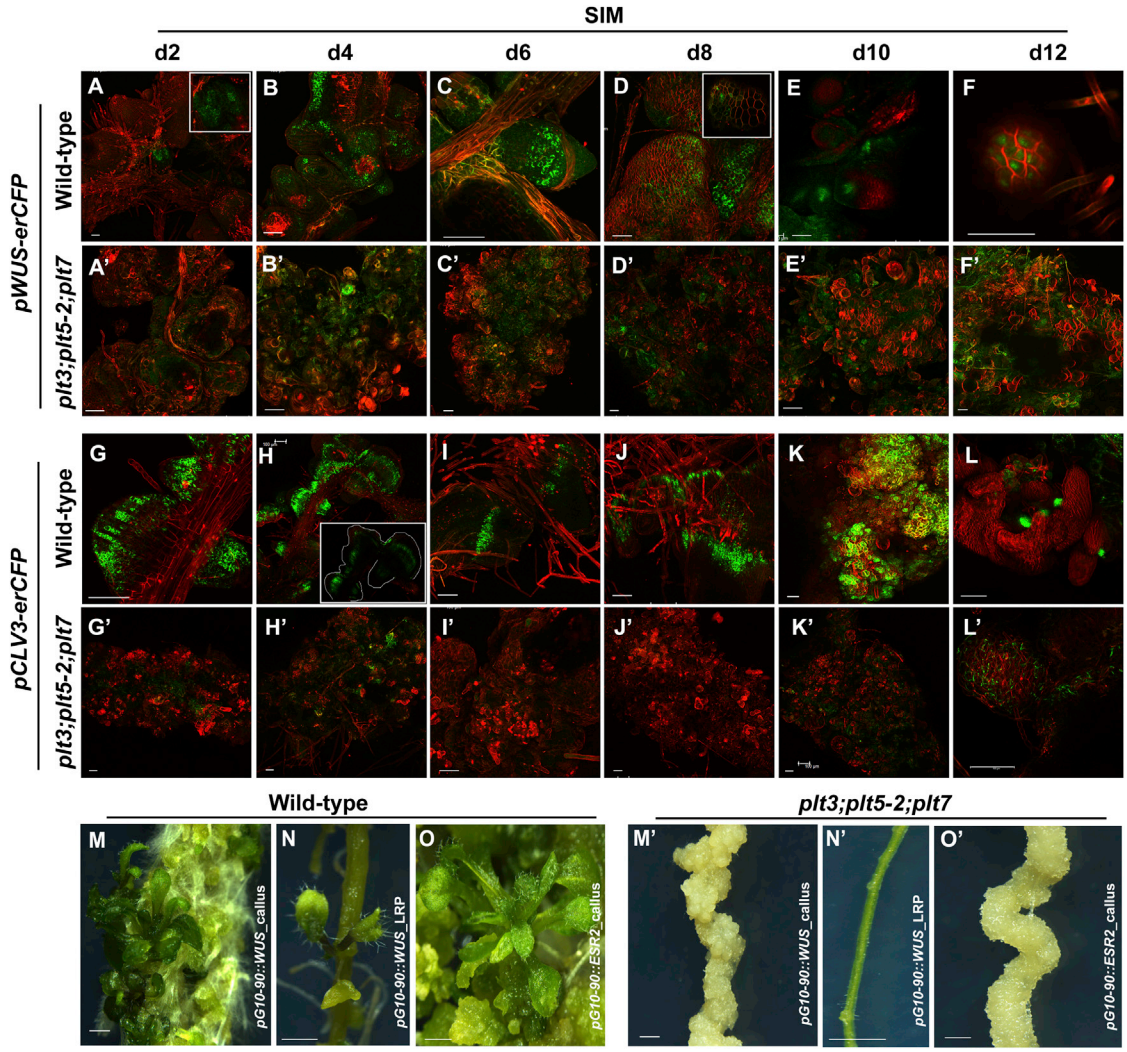

Figure 4. *WUS* and *CLV3* Expression Domains Are Not Properly Established in *plt3*; *plt5-2*; *plt7* Mutants after Regeneration Stimulus

(A–L and A'–L') *pWUS*::*erCFP* and *pCLV3*::*erCFP* expression in calli of wild-type (A–F and G–L) and *plt3; plt5-2; plt7* mutant (A'–F' and G'–L') on SIM. (A) Expression of *WUS*::*erCFP* (green) in the innermost layers of wild-type callus after 2 days SIM treatment. Inset shows *WUS* expression. (B–E) *pWUS-erCFP* expression in a large portion of the wild-type callus after 4–6 days (B and C) and its progressive localization to the center of developing meristems (D and E). Inset in (D) shows *pWUS-CFP* expression in the meristem. (F) The center of shoot meristems marked by WUS-CFP after 12 days in wild-type. (A'–F') Weak expression of *pWUS-CFP* in *plt3*; *plt5-2*; *plt7* callus after 2 days of SIM treatment (A'), which thereafter became scattered within the callus (B'–F'). (G) Expression of *pCLV3*::*erCFP* (green) in wild-type callus after 2 days of SIM treatment. (H–K) *CLV3*::*erCFP* signal encompassing a large part of wild-type callus after 4–10 days of induction. Inset in (H) is single section image showing the CLV3-CFP signal in the inner and middle layers. White lines mark the callus boundary to reveal the callus layers. (L) Upregulation of *CLV3*::*erCFP* only in the meristem center after 12 days of SIM treatment in wild-type. (G') Weak expression of *CLV3*::*erCFP* in *plt3*; *plt5-2*; *plt7* callus after 2 days of induction. (H'–L') Sporadic *CLV3*::*erCFP* expression in *plt3*; *plt5-2*; *plt7* callus after 4–12 days of SIM induction.
(M, M', N, and N') Ectopic overexpression of *WUS* (*G10-90*::*WUS:3AT*) induced de novo shoots from both callus and LRP in wild-type (M and N), but not in *plt3*; *plt5-2*; *plt7* mutant (M' and N'), upon incubation on hormone-free medium supplemented with estradiol.
(O and O') Forced expression of *ESR2* (*G10-90*::*ESR2*:*3AT*) on minimal medium with estradiol induced de novo shoots on wild-type callus (O) whereas mutant callus failed to regenerate shoots (O'). (The brightness and contrast of the image in O have been adjusted).
The scale bar represents 50 μm in (F), 1 mm in (M)–(O'), and 100 μm in the remaining.

found that the LRP turned green on cytokinin-rich medium (Figures S6L–S6N). Though many green foci were observed in *plt3*; *plt5-2*; *plt7*; *pPLT7*::*cPLT1*:*vYFP* callus, none of them displayed shoot outgrowth. PIN1-GFP marked shoot progenitor cells developed on the surface of *plt3*; *plt5-2*; *plt7*; *pPLT7*::*cPLT1*:*vYFP*; *PIN1*:*GFP* callus on SIM (Figures 6J–6L).

(K' and L') Although weak *PIN1-GFP* expression in most parts of the callus (K'), no shoot regeneration in *plt3*; *plt5-2*; *plt7*;*pDR5*::*PIN1*:*GFP* (L').
(A) and (A') are confocal single optical section images, (L) and (L') are bright-field images, and the remaining are confocal images with projections of multiple optical sections. Red color is the propidium iodide stain in (A)–(F') and the FM4-64 stain in (G) and (J)–(J'). Red color in (H) and (I) is autofluorescence. The scale bar represents 50 μm in (A)–(J'), (K), and (K') and 1 mm in (L) and (L').

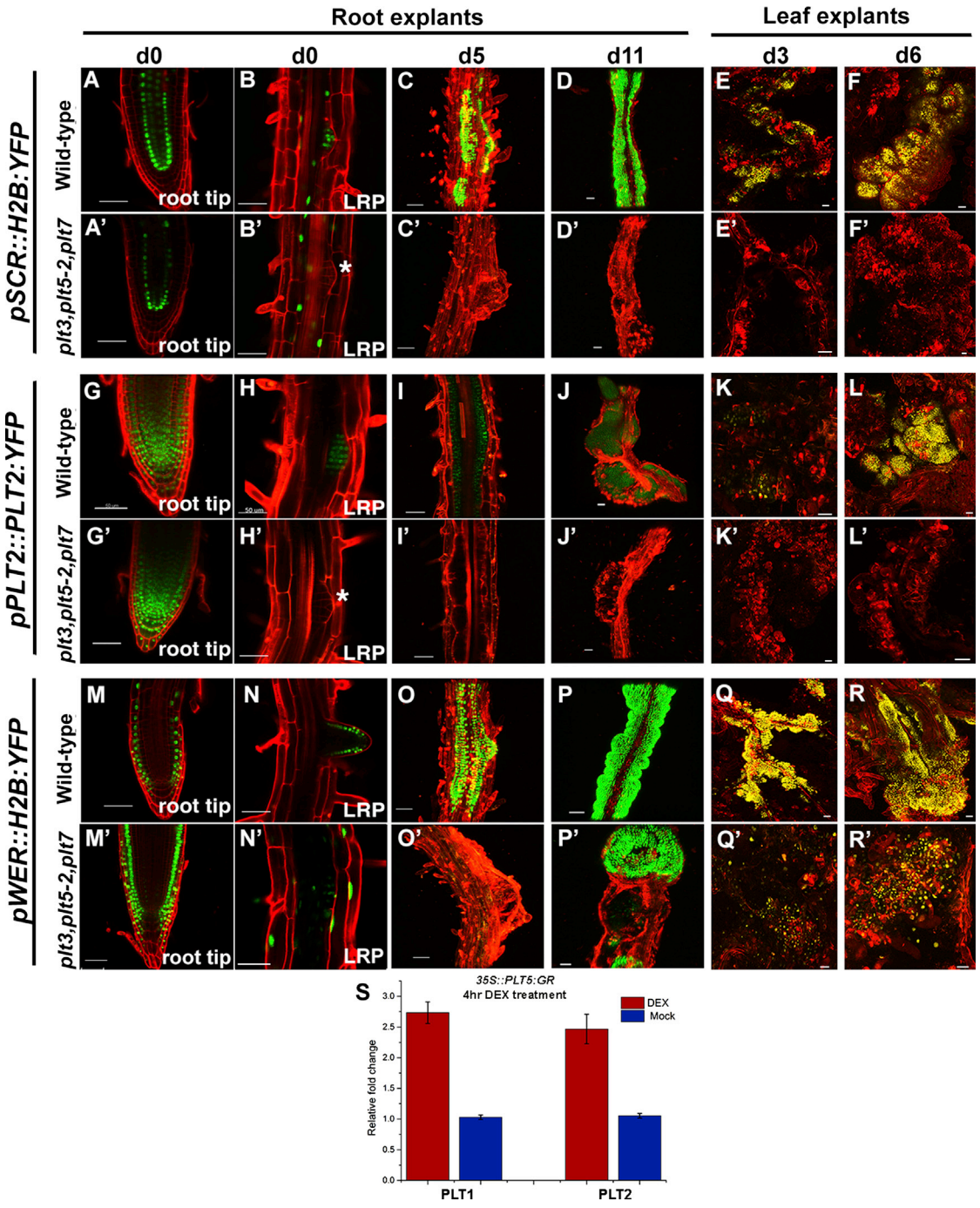

Figure 5. Root Stem Cell Maintenance Regulators Are Not Detectably Expressed in *plt3*; *plt5-2*; *plt7* LRP and Callus

(A–R and A'–R') *pSCR*::*H2B*:*YFP*, *pPLT2*::*PLT2*:*YFP*, and *pWER*::*H2B*:*YFP* expression in wild-type explants (A–F, G–L, and M–R) and *plt3*; *plt5-2*; *plt7* explants (A'–F', G'–L', and M'–R'). The order of the columns from the left is: untreated primary root tip, untreated lateral root primordium, calli derived from root cultured on CIM for 5 days, 11 days, and calli derived from leaf cultured on CIM for 3 days, 6 days. (A–F, G–L, and M–R) Expression of all three reporters in both untreated primary root tip (A, G, and M), LRP (B, H, and N), and CIM-induced calli (C–F, I–L, and O–R) derived from root and leaf explants in wild-type, whereas no expression of *pSCR*::*H2B*:*YFP* and *pPLT2*::*PLT2*:*YFP* in either LRP (B' and H' asterisks) or calli derived from those tissues in the *plt3*; *plt5-2*; *plt7* mutant (C'–F' and I'–L'). (N') Slight expression and (P', Q', and R') partial or weak expression of *pWER*::*H2B*:*YFP* in the LRP and calli of *plt3*; *plt5-2*; *plt7*, respectively. (O') No expression of reporter at all in some calli.

(S) Upregulation of *PLT1* and *PLT2* transcripts upon the induction of *PLT5* measured by qRT-PCR. Expression levels were normalized to *ACTIN2*. Error bar represents SEM from three independent biological replicates.

The scale bars in (A)–(R') represent 50 μm.

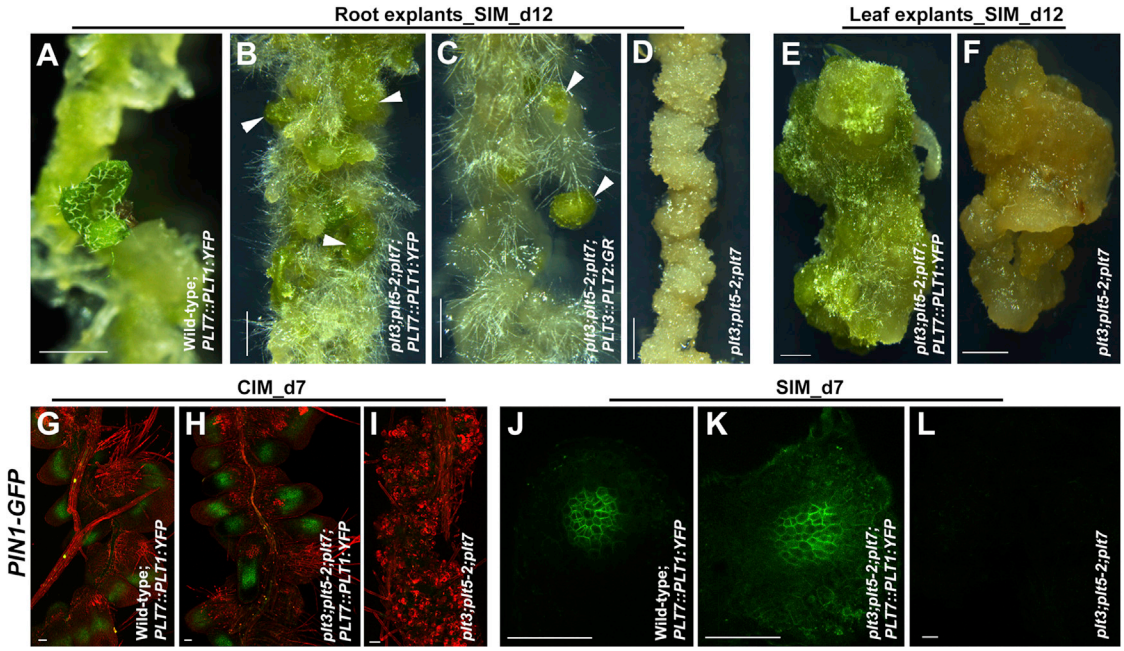

Figure 6. Root Stem Cell Maintenance Regulators Establish Early Competence for Shoot Regeneration

(A) Shoot regeneration in wild-type callus (wild-type;*PLT7*(*1.5kb*)::*cPLT1*:*YFP*) derived from root explant after 12 days on SIM.

(B and C) Turning green of competent calli derived from root explants of *plt3*; *plt5-2*; *plt7*; *PLT7*(*1.5kb*)::*cPLT1*:*vYFP* (B) and *plt3*; *plt5-2*; *plt7*; *PLT3*::*PLT2*:*GR* (C) after 12 days on SIM. Arrow head in (B) and (C) marks green foci.

(D) Incompetent yellowish callus derived from root explant of *plt3*; *plt5-2*; *plt7* on SIM.

(E) Green, competent callus derived from leaf explant of *plt3*; *plt5-2*; *plt7*; *PLT7*(*1.5kb*)::*cPLT1*:*vYFP*

(F) *plt3*; *plt5-2*; *plt7* callus derived from leaf explant remained yellowish on SIM.

(G and H) *PIN1-GFP* expression in the callus of wild-type; *PLT7*(*1.5kb*)::*cPLT1*:*YFP*, *PIN1*::*PIN1*:*GFP* (G) and *plt3*; *plt5-2*; *plt7*; *PLT7*(*1.5kb*)::*cPLT1*:*vYFP*; *PIN1*::*PIN1*:*GFP* (H) after 7 days on CIM.

(I) Disorganized callus cells with undetectable *PIN1-GFP* expression in *plt3*; *plt5-2*; *plt7*; *PIN1*::*PIN1*:*GFP* on CIM.

(J and K) Shoot progenitor cells labeled with PIN1-GFP in the callus of wild-type; *PLT7*(*1.5kb*)::*cPLT1*:*vYFP*, *PIN1*::*PIN1*:*GFP* (J) and *plt3*; *plt5-2*; *plt7*; *PLT7*(*1.5kb*)::*cPLT1*:*vYFP*, *PIN1*::*PIN1*:*GFP* (K) after 7 days on SIM.

(L) No *PIN1-GFP* expression or shoot progenitor cell formation in *plt3*; *plt5-2*; *plt7*;*PIN1*::*PIN1*:*GFP* on SIM.

Brightness and contrast in (A) have been adjusted. The scale bars represent 1 mm in (A)–(F) and 50 μm in (G)–(L). Red color in (G)–(I) is propidium iodide. No stain was used for cell boundaries in (J)–(L).

Thus, activation of *PLT1* in *plt3*; *plt5-2*; *plt7* callus can reinstate the competence for regeneration of shoot progenitor cells, though not of shoots. We also introduced a steroid-inducible version of *PLT2* under control of a *PLT3* promoter (*PLT3*::*PLT2*:*GR*) in *plt3*; *plt5-2*; *plt7* mutants. Nuclear entry of PLT2 was facilitated upon transient steroid induction in *plt3*; *plt5-2*; *plt7*; *PLT3*::*PLT2*:*GR* callus, and the induction was withdrawn prior to the transfer to SIM to recapitulate the expression timing of endogenous *PLT2* (endogenous *PLT2* is upregulated upon callus formation and downregulated upon transfer of callus onto SIM in wild-type; Figures S6B and S6F). Here too, calli derived from aerial as well as root explants regained pluripotency and shoot progenitor cells were regenerated on cytokinin-rich medium (Figures 6C and S6P). Persistent induction with the steroid and thus constitutive PLT2 activity even on SIM abolished any sign of shoot regeneration (Figure S6Q). Unlike *PLT2*, expression of *PLT3* under control of a *PLT3* promoter accomplished de novo shoot formation in *plt3*; *plt5-2*; *plt7* and displayed regeneration ability as do *plt5-2*; *plt7* double mutants, demonstrating that the *PLT3* promoter used is functional during acquisition of pluripotency and shoot regeneration like the endogenous one (Figure S6R). The calli derived from both aerial and root explants displayed similar response upon activation of PLT1 or PLT2. This further reinforces the notion that both aerial and root explants pass through a phase of competence where cells require root stem cell maintenance regulators to establish pluripotency. Whereas activation of PLT1 or PLT2 in *plt3*; *plt5-2*; *plt7* can restore pluripotency and shoot progenitor cells can be regenerated on cytokinin-rich medium, a complete shoot formation program is not achieved.

PLT3, PLT5, and PLT7 Regulate and Require the Activity of Lateral Organ Boundary Regulators *CUC* to Accomplish De Novo Shoot Formation

We searched for factors that (1) can promote shoot regeneration in *plt3*; *plt5-2*; *plt7*; *pPLT7*::*cPLT1*:*vYFP* and (2) are regulated by PLT3, PLT5, and PLT7. It has been described earlier that lateral organ boundary regulator gene *CUC2* plays a major role in de novo shoot regeneration [3]. Ectopic overexpression of either of the functionally redundant genes *CUC1* or *CUC2* can enhance de novo shoot formation, and the corresponding double mutant *cuc1*;*cuc2* displays reduced shoot regeneration [29]. Moreover,

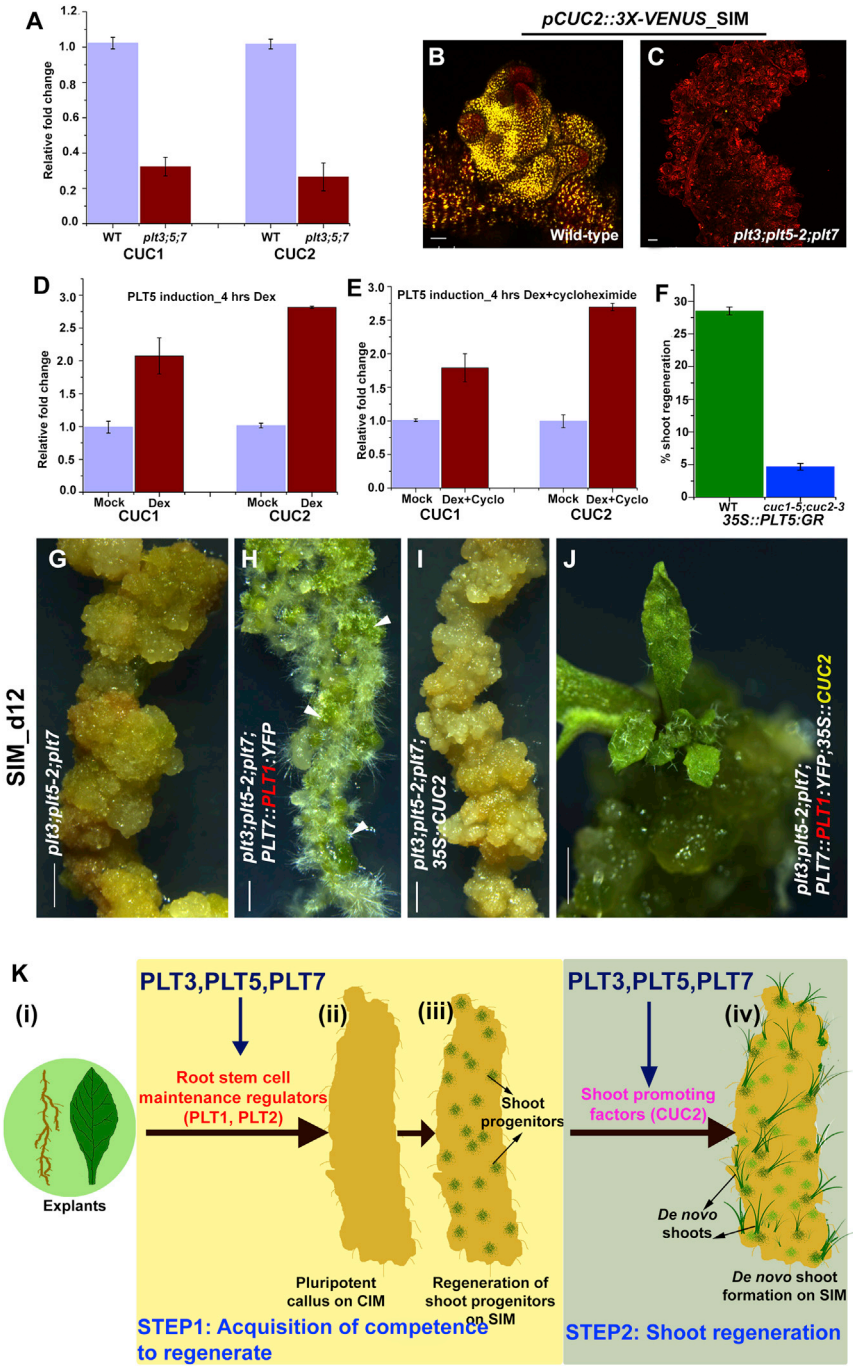

Figure 7. PLT3, PLT5, and PLT7 Control De Novo Shoot Regeneration by a Two-Step Mechanism

(A) *CUC1* and *CUC2* transcript levels in wild-type and *plt3*; *plt5-2*; *plt7* mutant calli after 10 days of induction on SIM, measured by qRT-PCR.

(B and C) Upregulation of *pCUC2::3X-VENUS* in wild-type callus on SIM (B) and its downregulation in *plt3*; *plt5-2*; *plt7* callus (C).

(D and E) *CUC1* and *CUC2* transcript levels after 4 hr of *PLT5* induction by DEX (D) and DEX with cycloheximide treatment (E), measured by qRT-PCR. Expression levels were normalized to *ACTIN2*.

(F) Percentage of shoots formed in wild-type; *35S::PLT5:GR* and *cuc1-5,cuc2-3;35S::PLT5:GR* after 4 weeks of culture on hormone-free medium supplemented with DEX.

(G) Complete abolishment of de novo shoot regeneration in *plt3*; *plt5-2*; *plt7* upon SIM induction.

(H) Regain of pluripotency and regeneration of shoot progenitors in the PLT1-reconstituted *plt3*; *plt5-2*; *plt7* callus cells. Arrowheads represent green foci.

(I) No de novo shoot formation in *plt3*; *plt5-2*; *plt7*; *35S::CUC2* upon SIM treatment

(J) Complete shoot regeneration on ectopic overexpression of *CUC2* in *plt3*; *plt5-2*; *plt7*;*PLT7::PLT1-YFP* on SIM.

(K) Schematic representation of a two-step mechanism of shoot regeneration. First, PLT3, PLT5, and PLT7 control the expression of root stem cell maintenance regulators conferring regenerative competence, and second, they regulate shoot-promoting factors leading to the initiation of shoot regeneration. (K-i) Explants derived from aerial or root tissues. (K-ii) PLT3, PLT5, and PLT7 determine pluripotency by regulating the root stem cell maintenance regulators, PLT1 and PLT2. (K-iii) Pluripotent callus can regenerate shoot progenitor cells on SIM. Root stem cell maintenance regulators are downregulated on SIM. (K-iv) Shoot progenitor cells further require shoot-promoting factors (CUC2) regulated by PLT3, PLT5, and PLT7 to complete the process of shoot regeneration.

Error bars in (A), (D), and (E) represent SEM from three independent biological replicates. The scale bars in (B) and (C) represent 50 μm and in (G)–(I) represent 1 mm.

PLT3, *PLT5*, and *PLT7* display overlapping expression with *CUC2* during regeneration (present study) [3]. We therefore asked whether PLTs regulate *CUC* expression to promote shoot regeneration. We first determined the expression status of *CUC1* and *CUC2* at the transcript level in *plt3*; *plt5-2*; *plt7* callus after 10 days of induction on SIM by qRT-PCR. Both *CUC1* and *CUC2* were downregulated in the *plt3*; *plt5-2*; *plt7* mutant relative to wild-type (Figure 7A). We further examined the expression pattern of *pCUC2::3X-VENUS* by live imaging and consistently observed lower levels of *CUC2* expression in the mutant on SIM, as compared to wild-type (Figures 7B and 7C). We next investigated whether the *CUC* genes can be induced by *PLT*s. For this, we carried out qRT-PCR and analyzed the expression levels of *CUC1* and *CUC2* upon the induction of *PLT5* in wild-type callus harboring *35S::PLT5:GR*. We observed increased transcript levels of both *CUC1* and *CUC2* after 4 and 8 hr of PLT5 induction even when the translational machinery was inhibited by cycloheximide (Figures 7D, 7E, and S7A). These results demonstrate that PLTs promote the expression of *CUC* genes during de novo shoot formation.

To test whether PLTs require activity of *CUC* genes for de novo shoot regeneration, we induced *PLT5* overexpression in the *cuc1-5*; *cuc2-3* mutant (*cuc1-5*;*cuc2-3*; *35S*::*PLT5:GR*). Calli of both wild-type; *35S*::*PLT5*:*GR* and *cuc1-5*; *cuc2-3*; *35S*::*PLT5*:*GR* were incubated on hormone-free medium supplemented with DEX. Shoot regeneration was highly compromised in *cuc1-5*; *cuc2-3*; *35S*::*PLT5*:*GR* in comparison to wild-type; *35S*::*PLT5*:*GR* (Figure 7F). Shoot regeneration efficiency was reduced by 90% in *cuc1-5*; *cuc2-3*; *35S*::*PLT5*:*GR*, suggesting that *PLT5* requires CUC function for de novo shoot formation. Taken together, our results indicate that PLTs regulate *CUC* genes to promote a second stage in shoot regeneration.

Finally, we asked whether *CUC* genes can promote complete shoot regeneration in *plt3*; *plt5-2*; *plt7*; *pPLT7*::*cPLT1*:*vYFP. CUC1* and *CUC2* redundantly control various developmental processes [3, 23, 29, 30]. Among these two, we chose CUC2 as its role is more elaborately analyzed in leaf development [30]. We therefore overexpressed *CUC2* in *plt3*; *plt5-2*; *plt7* mutants (*plt3*; *plt5-2*; *plt7*; *pPLT7*::*cPLT1*:*vYFP*; *35S*::*CUC2*), and shoot regeneration was evaluated on SIM. *plt3*; *plt5-2*; *plt7*; *pPLT7*::*cPLT1*:*vYFP*; *35S*::*CUC2* callus displayed de novo shoot regeneration on SIM (Figures 7G–7J). De novo shoots were regenerated after 10 days of induction on SIM although the proficiency of shoot regeneration was low in comparison to wild-type. On contrary, in the absence of root stem cell maintenance regulators, overexpression of *CUC2* in *plt3*; *plt5-2*; *plt7* (*plt3*; *plt5-2*; *plt7*; *35S*::*CUC2*) did not lead to any sign of shoot regeneration (Figures 7I, S7B, and S7C). *plt3*; *plt5-2*; *plt7*; *35S*::*CUC2* callus was similar to that of *plt3*; *plt5-2*; *plt7*, and it remained yellowish upon SIM induction, suggesting that CUC2 requires the pluripotent state established by root stem cell maintenance regulators PLT1 or PLT2 to accomplish shoot regeneration. Consistent with these results, whereas regeneration proficiency of shoot progenitors was reduced in *plt1*; *plt2* double mutants, the formation of shoot progenitors was not significantly altered in *cuc1-5*; *cuc2-3* mutants (Figures S7D and S7E). *cuc1-5*; *cuc2-3* mutants were mainly compromised in complete shoot formation (Figure S7E) [29].

Taken together, our data suggest a two-step mechanism of de novo shoot regeneration, wherein PLT3, PLT5, and PLT7 initially promote pluripotency by inducing root stem cell maintenance regulators and later activate shoot-specific *CUC* genes to accomplish the formation of de novo shoots (Figure 7K).

DISCUSSION

Ability to regenerate root or shoot from plant tissue has been widely exploited over decades. But the mechanisms by which the external hormone application establishes pluripotency and ensures the completion of organ formation remain largely unknown. Several regulators of de novo shoot regeneration such as WUS, STM, and MP have been identified. Loss-of-function mutants of these regulators do regenerate shoots, though the regeneration efficiency is significantly reduced [3, 31, 32]. Our studies discover previously unrecognized critical roles of *PLT* genes in establishing pluripotency and their absolute necessity for shoot regeneration.

Capacity for lateral root initiation is essential for callus formation from root as well as shoot [7, 9]. Callus displays root-like organization and expresses root-specific genes [9]. The functional significance of this in the callus remained elusive. Our studies uncover the importance of root-like traits of callus and determine the function of root stem cell regulators during de novo shoot regeneration. A *plt3*; *plt5-2*; *plt7* mutant does make LRP, and it consistently makes callus as well. But the mutant callus derived from shoot or root tissues lacks root stem cell regulators and is not pluripotent as it fails to regenerate shoots. Therefore, callus formation on its own is not sufficient for shoot regeneration. PLT3, PLT5, and PLT7 activate the root stem cell regulators PLT1 and PLT2 to establish pluripotency. Once cells acquire pluripotency and thus regeneration competence, subsequent steps of regeneration are triggered that can either lead to regeneration of intermediate structures or of complete organs. Reconstitution of either PLT1 or PLT2 activity in *plt3*; *plt5-2*; *plt7* re-establishes the competence to regenerate shoot progenitors, but complete shoot regeneration is never achieved despite restoration of the wild-type callus traits (Figure 7H). A subsequent step is required to accomplish shoot formation. This study demonstrates the functional significance of expression of root-specific genes in the callus, i.e., to establish competence for shoot regeneration. PLT3, PLT5, and PLT7 additionally regulate the shoot-promoting factor CUC2 and require its activity to accomplish shoot formation. *CUC* genes become induced in elevated-hormone media [33]. However, PLT-mediated activation of *CUC2* during regeneration is not an indirect output of PLT-mediated upregulation of the auxin biosynthesis genes *YUC1* and *YUC4* [34] as (1) reconstitution of *YUC4*, and thus auxin biosynthesis in *plt3*; *plt5-2*; *plt7* mutant, did not restore shoot regeneration (data not shown) and (2) *CUC2* is likely to be a direct target of PLT. Previous work shows that *CUC1* and *CUC2* enhance shoot regeneration upon external hormonal application [29]. However, several questions pertaining to role of *CUC* genes during regeneration remain unanswered. For example, how do *CUC* genes promote shoot regeneration? When is CUC activity required, and how are *CUC* genes regulated during regeneration? Our study reveals the temporal regulatory action of *CUC2* during shoot regeneration and demonstrates that PLTs regulate *CUC* expression. In the absence of root stem cell regulators, *CUC2* overexpression is unable to restore shoot regeneration in *plt3*; *plt5-2*; *plt7*, suggesting CUC2 activity in shoot regeneration is dependent on the prior function of root stem cell regulators. CUC2 activity is required once shoot progenitors are regenerated, and it is essential to initiate the regeneration of lateral organs at the periphery of shoot progenitors. How does CUC2 complete the shoot formation program from shoot progenitor cells? A possible mode of action is to promote PIN polarity at the periphery of shoot progenitors and thereby lateral organ outgrowth [20, 30]. Regeneration of complete shoot is compromised, but not abolished, in *cuc1-5*;*cuc2-3* mutant, suggesting the necessity of additional shoot-promoting factors to facilitate the shoot outgrowth.

During regeneration, prior to shoot outgrowth, there are several developmental phases from acquisition of the competence for regeneration to promotion of shoot growth, which are dynamically regulated and are critical for completing the process [3, 11]. One of the reasons why molecular mechanisms of de novo shoot regeneration have remained unknown so far is the difficulty in linking or uncoupling different developmental phases of shoot regeneration. It is only very recently that the complex shoot-regeneration process has been dissected into phases

and the links between them examined. A recent report by Motte et al. [35] observed wide natural variation in different parameters such as callus development, callus greening, formation of primordia, and shoots during shoot regeneration across 88 *Arabidopsis* accessions. They performed correlation analysis between the traits. It is important to note that shoot primordium initiation and complete shoot formation are separable processes. Consistent with their findings, our results suggest that acquisition of competence to regenerate shoot progenitor cells (callus greening) can be uncoupled from completion of shoot formation and reinforce the notion that ability to generate green callus does not necessarily ensure shoot regeneration. Our studies further provide the molecular basis of such an uncoupling. PLT3, PLT5, and PLT7 redundantly control the intermediate steps leading to de novo shoot regeneration by regulating two distinct sets of regulators: the root stem cell regulators PLT1 and PLT2 to establish pluripotency and thus the competence to regenerate shoot progenitor cells and shoot-promoting factors like CUC2 to allow shoot regeneration (Figure 7K). These two distinct regulatory modules function downstream of external regeneration stimuli (auxin and cytokinin). It will be revealing to probe the PLT-regulated modules in *Arabidopsis* accessions that display natural variation in regeneration responses. Regulatory modules controlling intermediate steps of organ regeneration remain to be elucidated across the plant kingdom.

In summary, our findings demonstrate a two-step mechanism of shoot regeneration that operates in all tested plant tissues. PLT3, PLT5, and PLT7 initially determine a competent state for regeneration by regulating root stem cell regulators and trigger regeneration (Figure 7K, i–iii). They additionally regulate and require the shoot-promoting factors to complete the process (Figure 7K, iii and iv). *PLT*-like genes are present in multiple plant species [13, 36]. It is tempting to speculate that a PLT-mediated mechanistic module might be utilized as a common strategy to regenerate desired organs in plant species where de novo shoot regeneration is naturally blocked at intermediate developmental phases.

EXPERIMENTAL PROCEDURES

Detailed experimental procedures are described in Supplemental Experimental Procedures.

Plant Materials and Molecular Cloning

Arabidopsis thaliana ecotype Columbia (Col-0) was used as wild-type. The origins of *plt3-1*, *plt5-2*, and *plt7-1* double and triple combinations of *plt* mutants have been described previously [13]. Details of plant lines and constructs are described in Supplemental Information. All the constructs were cloned into pCAMBIA 1300 binary vector using the Multisite Gateway recombination cloning system (Invitrogen) and thereafter introduced into *Agrobacterium tumefaciens* strain C58 [37] by electroporation. Stable transgenic plants were generated by the floral-dip method [38].

Regeneration Assays

Root and hypocotyl explants were collected from 10 dpg (days post germination) seedlings grown on MS basal salt medium (Sigma). Cotyledon explants were collected at 4 dpg, and leaf explants were taken 5 days post leaf formation. Explants were induced for callus formation followed by shoot regeneration on suitable culture media as described in Supplemental Information. The cultures were incubated at 22°C and 70% relative humidity under continuous white light.

Microscopic Imaging

Bright-field images of regenerating callus and de novo shoots were captured using a Leica M205FA stereo microscope. For confocal imaging, root and callus samples were treated with 10 μg/ml propidium iodide (Sigma) to stain the cell boundaries. 10 μg/ml FM4-64 dye (Invitrogen) was used to stain the cell membrane of regenerating shoot tissue arising from the callus on SIM. Confocal imaging was done by using a Leica TCS SP5 II laser scanning microscope with a 10× air objective, 20× oil immersion objective, or a 40× water-dipping lens. Settings for confocal imaging are given in Supplemental Information. The projection view of the images was reconstructed from the z stacks with Leica LAS-AF software. Images were compiled using Adobe Photoshop CS6.

DEX Induction for De Novo Shoot Formation

Callus was derived from wild-type;*35S*::*PLT5*:*GR*, wild-type;*35S*::*PLT7*:*GR*, and *cuc1-5*; *cuc2-3*; *35S*::*PLT5*:*GR* on CIM. These pluripotent calli were induced on MS agar plate (without any hormone) supplemented with 20 μM DEX (Sigma) for shoot regeneration. The cultures were incubated for 3 or 4 weeks under the regeneration conditions mentioned above, and the de novo shoots formed were quantified per explant. Root explants of *plt3*; *plt5-2*; *plt7*; *PLT3*::*PLT2*:*GR* were induced on CIM with 20 μM DEX for pluripotent callus formation. After 3 days of induction on CIM with DEX, the tissues were washed several times in sterile water to remove the residual DEX and placed on fresh CIM without DEX content. After 10 days of total CIM treatment, the tissues were transferred to SIM for shoot regeneration.

qRT-PCR

For qRT-PCR, PLT5 was induced in wild-type;*35S*::*PLT5*:*GR* callus by treating with 20 μM DEX or 20 μM DEX with 10 μM cycloheximide (Sigma) in liquid MS medium for 4 hr and 8 hr and the callus harvested for RNA extraction. In case of cycloheximide treatment, samples were pre-treated with 10 μM cycloheximide for 20 min before DEX addition. Mock treatment was performed using MS liquid medium supplemented only with DMSO or 10 μM cycloheximide. To assess the differential gene expression level between wild-type and *plt3*; *plt5-2*; *plt7* mutant, calli of both the genotypes were collected for RNA extraction after 10 days of treatment on SIM. Total RNA was extracted from callus samples using a Spectrum Plant Total RNA kit (Sigma) and subjected to on-column DNase treatment according to the manufacturer's guidelines. cDNAs were synthesized from 1 μg total RNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). qRT-PCR was performed in 25 μl reaction volume containing 12.5 μl SYBR Green PCR master mix (Takyon- Eurogentec), 100 nM gene-specific primers (Table S1) and 100 ng cDNA in a QIAGEN Rotor Gene thermocycler. All reactions were performed with RNA derived from three independent biological replicates. Each biological sample was tested in technical triplicate. *ACTIN2* (*ACT2*) was used to normalize the result. The relative gene expression was represented as fold-change value by calculating $-\Delta\Delta C^T$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.02.022.

AUTHOR CONTRIBUTIONS

A.K. and K.P. conceived and designed the research. A.K., K.D., A.J.P., Z.B.T., K.S., and P.V.A. performed research. A.K., K.D., K.S., and K.P. analyzed data. A.K., K.D., and K.P. wrote and revised the paper. K.S., E.M.M., and B.S. discussed the data and edited and revised the manuscript. Y.D., V.P., and B.S. contributed *pPLT7-cPLT1-VENUS*, *pWUS-CFP*, and *pCLV3-CFP* lines.

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