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PLETHORA Genes Control Regeneration by a Two-step Mechanism

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Summary

Background—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 complete fertile shoot system after inductive cues, the mechanisms by which cells acquire pluripotency and subsequently regenerate complete organs remain unknown.

Results—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* to complete the shoot formation program.

Conclusions—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 tissues irrespective of their origin. Our studies reveal intermediate developmental phases of regeneration and provide a deeper understanding into the mechanistic basis of regeneration.

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Author contributions:
AK and KP conceived and designed the research. AK, KD, AJP, ZBT, KS and PVA performed research. AK, KD, KS and KP analyzed data. AK, KD and KP wrote and revised the paper. KS, EMM, BS discussed the data and edited and revised the manuscript. YD, VP and BS contributed *pPLT7-cPLT1-VENUS*, *pWUS-CFP* and *pCLV3-CFP* lines.

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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]. Similarly, when *LATERAL ORGAN BOUNDARIES DOMAIN (LBD)* genes are suppressed in *Arabidopsis*, lateral root formation is highly compromised and moreover, no callus is formed even after high auxin treatment [11]. Thus, callus formation involves the activation of various genes expressed in lateral root primordia (LRP), and callus shares root-like traits with LRP. However it is not known whether the root4 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, 12]. The resulting auxin distribution requires local auxin biosynthesis and polar auxin transport mediated by *YUCCA* (*YUC*) genes and PIN1 respectively [12]. Auxin response factor MONOPTEROS (MP/ARF5) is upregulated during shoot regeneration. A non-repressible MP variant promotes *de novo* shoot formation in the presence of cytokinin-rich medium [13]. During shoot regeneration, callus cells display dynamic changes in gene expression pattern and several regulatory interactions promoting shoot formation are established on or below the rough surface of the callus [3, 4, 12]. 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 [12]. At this point, the shoot patterning gene *CUP SHAPED COTYLEDON2* (*CUC2*) is expressed in a region surrounding the shoot progenitor cells [3]. Subsequently a fully developed shoot meristem is formed and key shoot developmental genes such as *SHOOT MERISTEMLESS (STM)* and *CLAVATA3 (CLV3)* are upregulated in the new meristem [3, 4].

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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 the determine 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, 14], 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 [15, 16], 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 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 lateral root primordium initiation is required for callus formation, as mutants which fail to initiate lateral root primordia are unable to make any callus [9, 11, 17]. 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* [15, 16]. The triple mutant *plt3; plt5-2; plt7* displays normal shoot outgrowth *in planta*, but produces aberrant lateral root primordia. 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 *PLTs* 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 [16]. We employed laser scanning confocal microscope live imaging to monitor the expression pattern of all three *PLT*s during callus formation and shoot regeneration in wild-type. As reported earlier [16], we observed that all three *PLTs* were expressed at early stages of LRP initiation and in young leaves (Fig. 1 A,K,U). Upon CIM induction, all three *PLTs* were upregulated in proliferating callus cells (Fig. 1 B–D, L–N, V–W, F', G', K', L', P', Q'). Upregulation occurred as early as 5 hrs after CIM induction (data not shown) and was sustained in all the proliferating cells of callus throughout growth on CIM. However at later stages, expression was confined to subepidermal layers of young callus (Fig. 1 E, O, Y). Upon transfer to SIM, expression was gradually restricted to the group of cells forming shoot progenitors (Fig. 1 F–H, P–R, Z–B', H', M', R'). Eventually very high expression of all three *PLTs* was noticed at the surface of shoot meristems formed *de novo* (Fig. 1 I, S, C') and in developing leaf primordia (Fig. 1 J, T, D', I' N', S'). Similar to callus-mediated indirect shoot regeneration,

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all three *PLT*s were upregulated during direct shoot regeneration from the LRP without the intervening callus phase (Fig. 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 (Fig. S1 E, J, O). 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. Towards 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 (Fig. S2 A) whereas no regenerating foci were observed in *plt3; plt5-2; plt7* tissue irrespective of the plant region of its origin (Fig. S2 D). The first leafy shoots emanated from the wild-type callus after 9–10 days of induction (Fig. S2 B) and more shoots were formed after 14 days (Fig. 2 A–D). Shoots were regenerated from all of the tested in wild type explants as previously reported [4, 18]. Shoot regeneration was completely abolished in *plt3; plt5-2; plt7* tissue (Fig. 2 A'–D'). 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 (Fig. 2 E, S2 G). Shoot regeneration was not substantially affected in single mutants (*plt3, plt5-2* and *plt7*) (Fig. 2 E, S2 G).

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 cytokininrich medium (Fig. S2 C) but not from *plt3; plt5-2; plt7* LRP (Fig. S2 F). These results indicate an essential regulatory role of *PLT* genes in controlling shoot regeneration. 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, 19] (Fig. 2 E, S2 G). Since 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

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minimal medium supplemented with 20μM DEX for induction of PLT activity. *De novo* shoots regenerated on the hormone-free medium after two weeks of DEX induction (Fig. 2 F, S2 H). 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 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, 20, 21]. We therefore compared the pattern of PIN1-GFP (*pPIN1*::*PIN1:GFP*) and auxin response sensor DR5-VENUS (*pDR5rev:: 3XVENUSN7*) 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 (Fig. 3 A,A'). Two days after transfer to CIM, proliferating cells were marked by DR5-VENUS expression (Fig. 3 B,B'). The upregulation of the *DR5* reporter continued until 4 days after transfer in both genotypes (Fig. 3 C, C'), whereafter the level of auxin response gradually decreased (Fig. 3 D–F, D'–F'). *PIN1-GFP* was observed in 2-day old proliferating cells on CIM both in wild type and mutant (Fig. 3 B, B'). In wild-type, *PIN1-GFP* expression persisted 8 days after induction on CIM (Fig. 3 C–F) 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 (Fig. 3 D'–F').

After transfer to SIM, PIN1-GFP was initially detected in the shoot progenitor cells regenerated in wild-type callus, consistent with published data (Fig. 3 H) [3]. *PIN1-GFP* expression was uniform throughout the superficial layer of the developing shoot meristem while *DR5* reporter was low or undetectable in the shoot meristem region marked by *PIN1* upregulation (Fig. 3 H). *DR5* reporter activity was however observed in the callus cells that were not forming shoot meristem. During the emergence of leaf primordia from the wild-type shoot meristem, both *DR5-VENUS* and *PIN1-GFP* signal accumulated in the primordia (Fig. 3 I,J). On the contrary, *PIN1-GFP* expression was never detected in *plt3; plt5-2; plt7* callus after transfer to SIM (Fig. 3 G'–J'). 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 (Fig. 3 I,J,I',J'). 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 (Fig. S3). Taken together our studies demonstrate that PLT3, PLT5 or PLT7 is required during the initial steps of shoot regeneration.

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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 if 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 (Fig. 3 K–L', S4), indicating that forced *PIN1* expression is not able to rescue shoot regeneration in the mutant.

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, 22], was functional in *plt3; plt5-2; plt7* mutants. After 2 days of induction on SIM, expression of *pWUS::CFP* was noticed in the inner cell layers of the wild-type callus (Fig. 4A). *pWUS* activity was dispersed across a wide area of the wild-type callus surface after 4 days (Fig. 4B,C) but was gradually confined to the center of nascent shoot meristems thereafter (Fig. 4 D–F). 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 (Fig. 4 A'–F', S6 A, B).

The *pCLV3::CFP* reporter was upregulated in the inner cell layers of wild-type callus after 2 days of induction on SIM (Fig. 4 G). After 4 days, a strong signal was detected in the inner layers as well as in the middle layers of callus cells (Fig. 4 H). Later, expression diminished gradually from the areas of shoot-forming cells, consistent with earlier experiments (Fig. 4 I–K) [3]. After 12 days on SIM, 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 (Fig. 4 L, S6 C, D). This dynamic pattern of *pCLV3:: CFP* expression was disrupted in *plt3; plt5-2; plt7* triple mutant callus (Fig. 4 G'–L'). After 2 days of induction on SIM, weak CLV3 reporter expression was observed in the mutant callus (Fig. 4 G'). At 12 days on SIM, when wild-type callus displayed confined localization of the CLV3 reporter at the centre of new shoot meristems, the mutant callus displayed sporadic CLV3 reporter expression in few cells (Fig. 4 L'). 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 (Fig. S5). *WUS* has been shown to be required for the conversion of LRP into shoots [19]. Though the mutant LRP expresses WUS upon exposure to high cytokinin, it was unable to produce shoots. 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 if overexpression of shoot-inducers like *WUS* or *ESR2*, which are known to trigger shoot regeneration without external hormone application,

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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 estradiolinducible *WUS* (*pG10-90::WUS:3AT)* or *ESR2* (*pG10-90::ESR2:3AT*) on hormone-free medium supplemented with β-estradiol (Fig. 4 M–O) [19, 23]. 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* (Fig. 4 M'–O'). 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. Since *plt3; plt5-2; plt7* mutant callus derived from root or shoot is abnormal in its regenerative capacity, we asked if 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 (Fig. 5A,G) [24, 25]. The expression of *PLT1::PLT1:vYFP, PLT2::PLT2:vYFP*, and *pSCR::H2B:vYFP* was upregulated in proliferating cells of wild-type callus derived from leaf or root explants (Fig. 5 C–F, I–L, S7A, B) 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 (Fig. 5 C'–F', I'–L', S7C, D). We did observe some expression of *PLT2* in few callus cells derived from mutant primary root tip (Fig. S8). 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 (Fig. 5 O–R, O'–R'). 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 (Fig. 5 B'–F', H'–L').

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 [24, 27, 28]. We examined if PLT1 and PLT2 can be induced by PLT5. Towards this we performed quantitative RT-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 hours of induction of *PLT5* (Fig. 5S). We next asked if 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*

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(*PLT7::cPLT1:vYFP*). *PLT7* (1.5 kb) promoter was active only on CIM and not on SIM (Fig. S9). Mutant calli derived from both aerial and root explants regained a morphology similar to wild-type after activation of *PLT1* (Fig. 6 G–I, S10). Upon the induction on SIM, *plt3; plt5-2; plt7*; *pPLT7::cPLT1:YFP* callus turned green, similar to wild-type and unlike *plt3; plt5-2; plt7* callus (Fig. 6 A,B,D–F). We also examined the direct regeneration efficiency in *plt3; plt5-2; plt7*; *pPLT7::cPLT1:vYFP* and found that the LRP turned green on cytokinin-rich medium (Fig S11 A–C). 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:YFP; PIN1:GFP* callus on SIM (Fig. 6 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 *PLT1* and *PLT2* are upregulated upon callus formation and downregulated upon transfer of callus onto SIM in wild-type (Fig. S12)). Here too, calli derived from aerial as well as root explants regained pluripotency and shoot progenitor cells were regenerated on cytokinin rich medium (Fig. 6 C, S13 B). Persistent induction with the steroid and thus constitutive PLT2 activity even on SIM abolished any sign of shoot regeneration (Fig. S13 C). 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 (Fig. S13 D). 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. While 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 (i) can promote shoot regeneration in *plt3; plt5-2; plt7*; *pPLT7::cPLT1:YFP* and (ii) are regulated by PLT3, PLT5 and PLT7. It has been described earlier that lateral organ boundary regulator gene *CUC*2 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, *PLT3*, *PLT5* and *PLT7* display overlapping expression with *CUC2* during regeneration (present study; [3]). We therefore asked if 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 quantitative RT-PCR. Both *CUC1* and *CUC2* were downregulated in the *plt3; plt5-2; plt7* mutant relative to wild type (Fig 7A). We further examined the expression pattern of *pCUC2::3X-VENUS* by live imaging and

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consistently observed lower levels of *CUC2* expression in the mutant on SIM, as compared to wild type (Fig. 7 B,C). We next investigated whether the *CUC* genes can be induced by *PLTs*. For this we carried out quantitative RT-PCR and analyzed the expression levels of *CUC1* and *CUC2* upon the induction of *PLT5* in wild-type callus harbouring *35S::PLT5:GR*. We observed increased transcript levels of both *CUC1* and *CUC2* after 4 and 8 h of PLT5 induction even when the translational machinery was inhibited by cycloheximide (Fig. 7 D,E, S14). 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* (Fig. 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 if *CUC* genes can promote complete shoot regeneration in *plt3; plt5-2; plt7*; *pPLT7::cPLT1:YFP. CUC1* and *CUC2* redundantly control various developmental processes [3, 24, 29, 30]. Among these two, we chose CUC2 as its role is more elaborately analysed 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 (Fig. 7 G–J). *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 (Fig. 7I, S15). *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, while 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 (Fig. S16, S17). *cuc1-5; cuc2-3* mutants were mainly compromised in complete shoot formation (Fig. S17) [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 (Fig. 7 K).

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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, 13, 31]. Our studies discover previously unrecognized critical roles of *PLETHORA* genes in establishing pluripotency and their absolute necessity for shoot regeneration. While PLT5 can induce *de novo* shoot assembly in a hormone-independent manner on the surface of pluripotent callus, it failed to induce shoots from lateral root primordia, as the function of PLT5 is to promote the outgrowth of LRP during normal development [16]. However, one cannot rule out the possibility that conversion of LRP to a shoot requires forced expression of other shoot-promoting factors.

Capacity for lateral root initiation is essential for callus formation from root as well as shoot [7, 9]. Callus displays root-like organisation 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 (Fig. 7 H). 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 [32]. 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* [33] as (i) reconstitution of *YUC4*, and thus auxin biosynthesis in *plt3; plt5-2; plt7* mutant, did not restore shoot regeneration (data not shown), (ii) *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

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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 [21, 30].

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, 12]. 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. [34] 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 (Fig. 7 K). 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 (Fig. 7 K i–iii). They additionally regulate and require the shoot promoting factors CUC1 and CUC2 to complete the process (Fig.7 K iii, iv). *PLT*-like genes are present in multiple plant species [15, 35]. 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.

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EXPERIMENTAL PROCEDURE

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used as wildtype and all plants used in this study were in the Col-0 background. The origins of *plt3-1, plt5-2* and *plt7-1*, double and triple combinations of *plt* mutants [15] and the *cuc1-5;cuc2-3* double mutant [36] have been described previously. The *cuc1-5; cuc2-3*; *35S::PLT5:GR* transgenic line was generated by crossing *cuc1-5; cuc2-3/+* and wild-type; *35S::PLT5:GR*. To generate *plt3; plt5-2; plt7* mutants expressing *PLT1* along with *CUC2* overexpression, a genetic cross was made between *plt3; plt5-2; plt7; PLT7(1.5kb)::cPLT1:YFP* and *plt3; plt5-2; plt7; 35S::CUC2* (T1 line). Two independent transgenic lines of *plt3; plt5-2; plt7; 35S::CUC2* displaying a strong phenotype of *CUC2* overexpression as previously reported [36] were crossed with *plt3; plt5-2; plt7; PLT7(1.5kb)::cPLT1:YFP.* Seeds obtained from these crosses were germinated and the regeneration experiment was performed using seedlings of genotype *plt3; plt5-2; plt7; PLT7(1.5kb)::cPLT1:YFP; 35S::CUC2.* Plants were grown on Murashige and Skoog (MS) basal salt medium (Sigma) at 22°C and 70% relative humidity under a 16 h light/ 8 h dark cycle.

Constructs, molecular cloning and plant transformation

Translational fusions, *PLT1::PLT1:vYFP, PLT2::PLT2:vYFP* [37], *PLT3::PLT3:vYFP, PLT5::PLT5:vYFP* and *PLT7::PLT7:vYFP* [15] were described previously. Transgenic lines harbouring the inducible *35S::PLT5:GR* construct [15], the double marker construct *PIN1::PIN1:GFP; DR5::3XVENUS-N7* [33] and the transcriptional fusion constructs, *pWUS::erCFP, pCLV3::erCFP* [33] were described previously.

The *pDR5::PIN1:GFP* was constructed by placing *pDR5rev* promoter [21] upstream to the *PIN1-GFP* coding region. To generate *pG10-90::WUS* and *pG10-90::ESR2* constructs, the coding region of *WUS* or *ESR2* including introns amplified from Col-0 genomic DNA was placed under the control of estradiol-inducible *G10-90* promoter [37, 38] The transcriptional fusion constructs of *pSCR::H2B:vYFP* and *pWER::H2B:vYFP* were generated by cloning the upstream regulatory sequences of *SCR* [39] or *WER* [37, 38]with the coding region of *vYFP* with nuclear localization signal (Histone2B-H2B). *PLT7(1.5kb)::cPLT1:vYFP* (Du and Scheres, unpublished) contains a 1.5 kb truncated upstream regulatory sequences of *PLT7*, cDNA sequence of *PLT1* gene fused in translational frame with the *vYFP*. To generate a *PLT3::PLT2:GR* construct, the genomic sequence of *PLT2* was fused to the 5' end of the gene encoding rat glucocorticoid receptor (GR) [40] and cloned under the control of a heterologous *PLT3* (7.7 kb) promoter [15]. Coding sequences of *CUC1* or *CUC2* including introns were amplified from Col-0 genomic DNA and incorporated between a 35S promoter of *CaMV* and the nopaline synthase terminator to generate the constructs of *35S::CUC1* and *35S::CUC2.* To generate *pCUC2::3X-VENUS*, a 3.2kb upstream regulatory sequence of *CUC2* was amplified from Col-0 genomic DNA and fused with *3X-VENUS* [41]. The primers used for PCR amplification are listed in Table S1 in the supplemental information. All the constructs were cloned into pCAMBIA 1300 binary vector using the Multisite Gateway recombination cloning system (Invitrogen) and thereafter introduced into

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Agrobacterium tumefaciens strain C58 [42] by electroporation. Stable transgenic plants were generated by the floral-dip method [43].

Regeneration assays

Root and hypocotyl explants were collected from 10 dpg (day 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. For indirect regeneration, explants were first cultured on callus inducing medium (CIM) consisting of Gamborg's B-5 basal salt (Sigma), 20 g/l glucose (Sigma), 0.5 g/l MES (Sigma), 1× Gamborg's vitamin solution (Sigma) and 0.8% agar. The plant hormones used were 500 μg/l (for root and hypocotyl explants) or 2 mg/l (for cotyledon and leaf explants) of 2,4-D (Sigma) and 50 μg/l of kinetin (Sigma). The pH was adjusted to 5.7 with 1M KOH. After 10 days of culture on CIM, the calli were transferred onto shoot inducing medium (SIM) consisting of 1× MS basal salt mixture, 10 g/l sucrose (Sigma), 0.5 g/l MES (Sigma), 1× Gamborg's vitamin solution (Sigma), 0.8% agar, 2 μg/ml trans-zeatin (Sigma), 0.4 μg/ml indole-3-butyric acid (IBA; Sigma) and 1 μg/ml d-biotin (Sigma). Explants were also treated with other culture conditions reported previously [4], in which root explants from 7 dpg seedlings were collected and transferred onto CIM for callus induction. After 5 days of CIM treatment, callus cultures were transferred onto 'SIM-2', for shoot regeneration, which was prepared with CIM basal composition but was supplemented with 24.6 μM 6-(γ,γ-Dimethylallylamino)purine (2-ip) (Sigma) and 0.9 μM Indole-3-acetic acid (IAA) (Sigma) as hormonal sources. For direct regeneration experiments, the root explants from 7dpg plant were shifted onto SIM or SIM-2. The cultures for direct or indirect shoot regeneration were incubated on SIM or SIM-2 for 2-3 weeks at 22°C and 70% relative humidity under continuous white light (45 μmol m^{-2} s^{-1} photon flux intensity from cool white fluorescent tungsten tubes). Regenerated shoots were defined as two or more leaves initiated in a radial pattern around a supposed shoot meristem.

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. For the detection of CFP, we used a 458 nm laser line and 465–515 nm detection band. GFP was excited with the 488 nm laser line and collected using 495–530 nm detection band and for YFP/VENUS we used 514 nm laser line in conjunction with a 520–545 nm detection band. Propidium iodide signal was detected by using a 514 nm or 561 nm laser line for excitation and 585–650 nm detection band for collection of the signal. The same lasers were used for the excitation of FM4-64, but the emission signal was collected through a 620–750 nm band-pass filter. Autofluorescence of chlorophyll was excited by any of these wavelengths and we chose a 650–750 nm band-pass filter for collection. The projection view of the images was reconstructed from the Z-stacks with Leica LAS-AF software.

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Imaging was done for Figure 5 using a Zeiss LSM 710 Meta confocal microscope with a 10×, ×20× or 40× water-dipping lens. To detect the signal of propidium iodide staining, a 488 nm laser line was used for excitation and a 585–615nm band-pass filter in conjunction with a 545nm secondary dichroic was used for collection of the signal. For the detection of other fluorescent markers, similar sets of laser and filters were used to those already described [21, 44]. The Z-stacks were reconstructed into a projection view using IMARIS software. 30 samples were imaged for each marker line to confirm that observed patterns were representative of the respective markers. Adobe Photoshop CS6 was used to arrange the images and occasionally to process images by adjusting the background using the brush tool under quick menu.

Dexamethasone 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 20μM dexamethasone (DEX) (Sigma) for shoot regeneration. The cultures were incubated for 3–4 weeks under the regeneration conditions mentioned above and the *de novo* shoots formed were quantified per explant.

Quantitative Reverse Transcription PCR

For quantitative RT-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 hrs and 8 hrs 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 1μg total RNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative RT-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 100ng 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 – C^{T}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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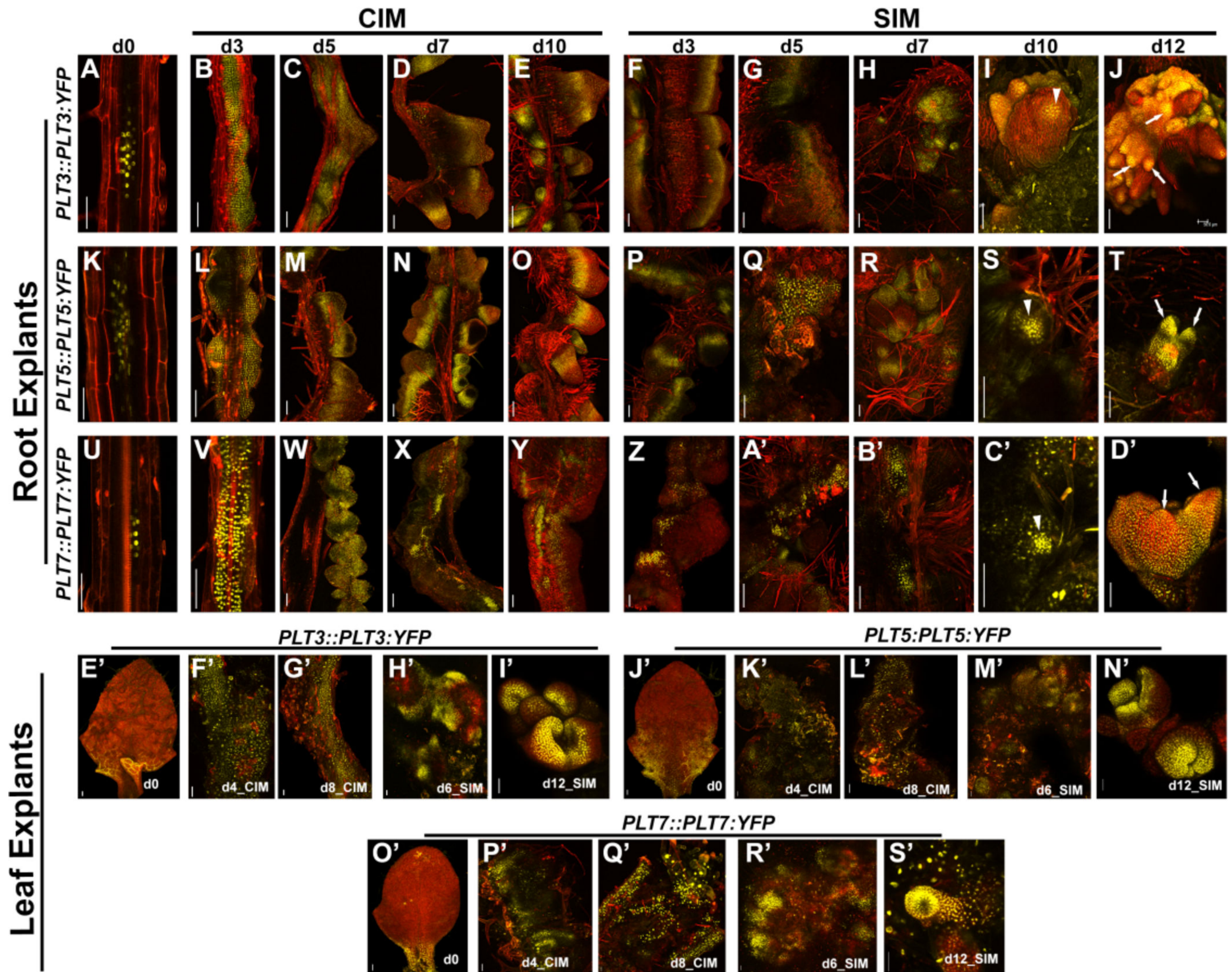

Figure 1. 1. *PLT* genes are upregulated during shoot regeneration

(A, E') *PLT3::PLT3:vYFP* (K, J') *PLT5::PLT5:vYFP* and (U, O') *PLT7::PLT7:vYFP* expression in untreated LRP and young leaves. (B) Proliferating cells after 3 days of CIM induction are marked with, *PLT3-YFP* (L) *PLT5-YFP* and (V) *PLT7-YFP*. (C–E, M–O, W–Y, F', G', K', L', P',Q') Upregulation of all three *PLT*s is maintained throughout the callus phase and the expression becomes mostly confined to the sub-epidermal cells of proliferating callus after 7–10 days. (F–H, P–R, Z–B', H', M', R') Upon SIM treatment the expression of all three *PLTs* gradually get accumulated in the shoot forming cells in callus. (I) A high expression of *PLT3-YFP* (S) *PLT5-YFP* and (C') *PLT7-YFP* expression in nascent shoot meristem (arrowhead) after 10 days on SIM. (J, T, D', I', N', S') All three PLT signals accumulated in leaf primordia (arrow) that emerged from the meristem periphery after 12 days of induction. All images are maximum projections of z-stacks except A, K and U, which are single optical sections. Red signal reflects propidium iodide stain in (A–H, K–R, U–B'), and FM4-64 stain in the remaining. Scale bar: 50 μm in (A,K,U) and 100 μm in the remaining.

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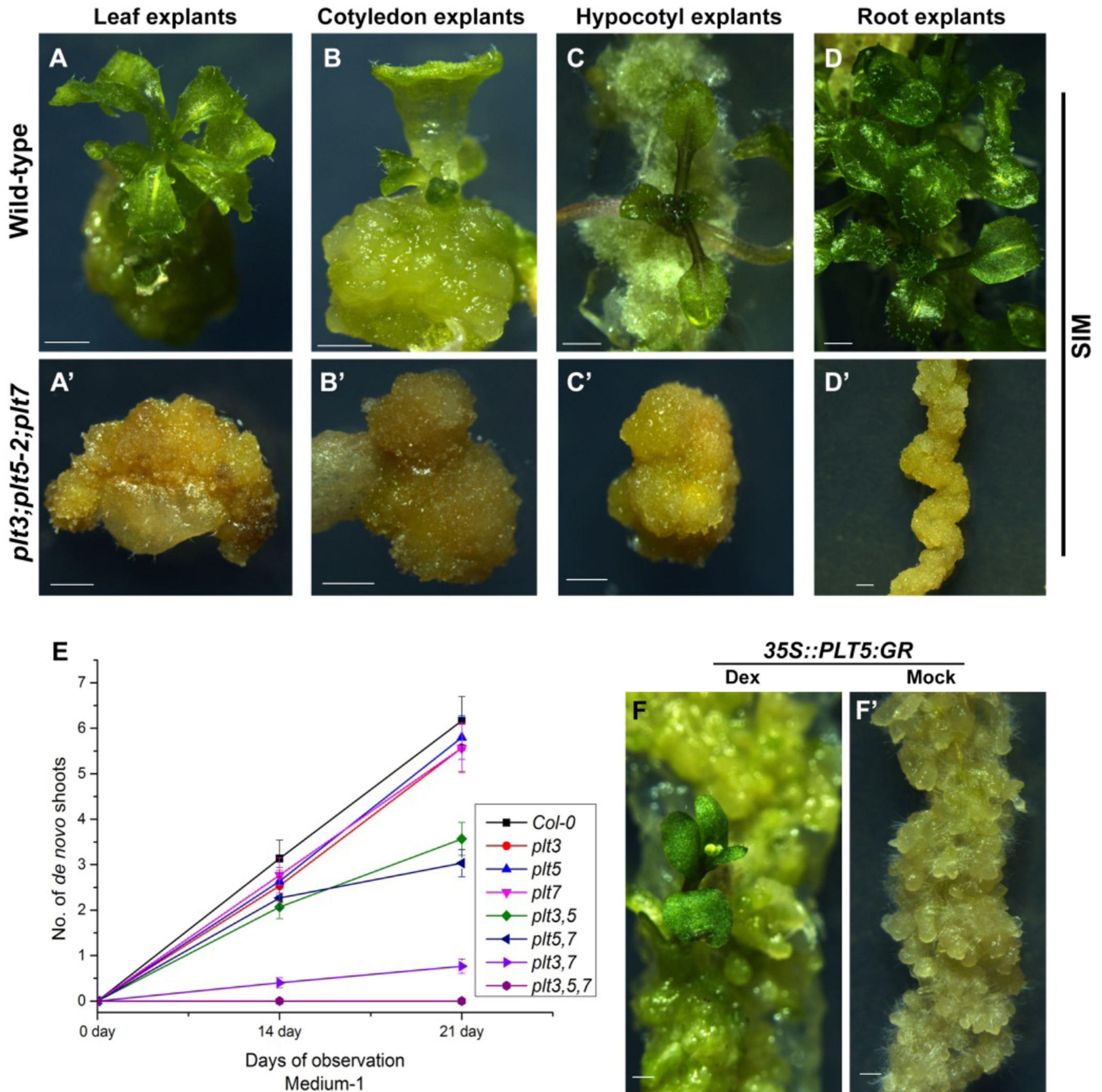

Figure 2. *PLT* genes are necessary and sufficient for *de novo* shoot formation

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') Shoot regeneration is abolished in *plt3; plt5-2; plt7* calli derived from leaf, cotyledon, hypocotyl and root explants. (E) Regeneration efficiency in various combinations of *plt* mutants. Number of shoots were scored per explant and the average length of explant was 3 cm. (F) Shoot regeneration in the callus of wildtype; *35S::PLT5:GR* incubated on hormone-free medium supplemented with dexamethasone. (F') No shoot regeneration in mock-treated callus of

wild-type;*35S::PLT5:GR*. Scale bar: 1mm. Error bar in (E) represents standard error of the mean.

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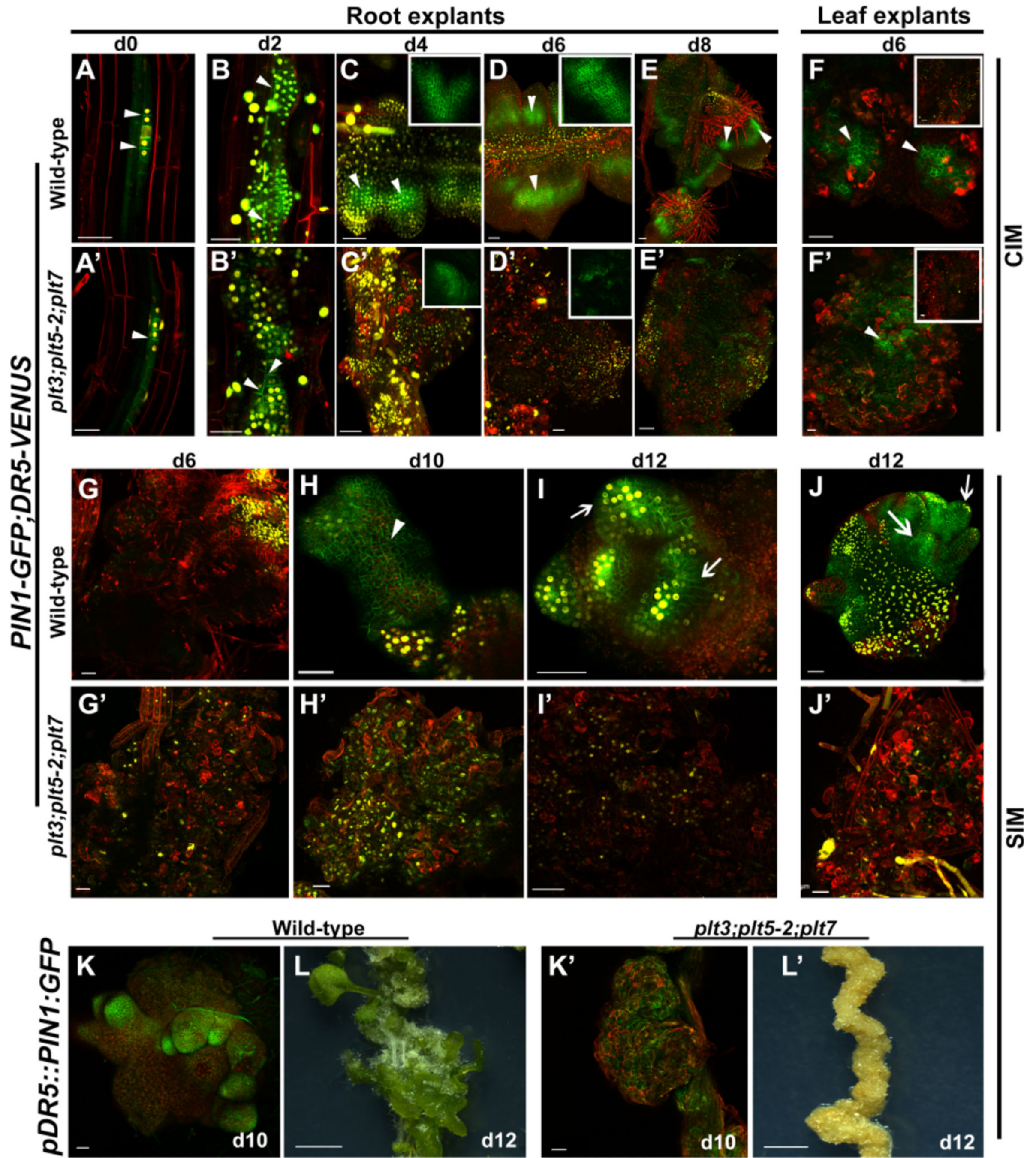

Figure 3. Auxin responses are deregulated in *plt3; plt5-2; plt7* mutants after regeneration stimulus

(A,A') *PIN1*::*PIN1:GFP* (green with arrowhead) and *pDR5rev::3XVENUS-N7* (yellow) expression in LRP of both wild-type and *plt3; plt5-2; plt7* on pre-CIM medium. (B,B') Upregulation of both *DR5-VENUS* and *PIN1-GFP* in proliferating cells of both genotypes after 2 days on CIM.. (C,C') *DR5-VENUS* signal is accumulated throughout the proliferating cells after 4 days in both the genotypes while *PIN1-GFP* (arrowhead) is expressed in the sub-epidermal cells. Inset shows *PIN1-GFP* expression. (D,E) Downregulation of *DR5–VENUS* and *PIN1-GFP* in wild-type callus on CIM after 6 and 8

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days respectively. Inset shows *PIN1-GFP* expression. (D',E') Downregulation of both *DR5–VENUS* and *PIN1-GFP* in the mutant after 6 days on CIM and no *PIN1-GFP* expression is detected after 8 days. (F,F') Expression of *DR5–VENUS* and *PIN1-GFP* (arrowhead) in leaf-derived calli of both wild-type and mutant after 6 days on CIM. Inset shows *DR5–VENUS* signal (G) After 6 days on SIM, sporadic distribution of *DR5–VENUS* signal and no *PIN1-GFP* expression in wild-type. (H) Upregulation of *PIN1-GFP* in developing-shoot meristem (arrowhead) in wild-type after 10 days on SIM. *DR5–VENUS* is expressed within the initiating leaf primordia and in the peripheral callus. (I) Both *PIN1-GFP* and *DR5–VENUS* signal accumulated within leaf primordia (arrow) after 12 days. (J) Upregulation of *PIN1-GFP* and *DR5–VENUS* fluorescence in leaf primordia (arrow) occurred in the callus derived from leaf explants on SIM. (G') Weak expression of *DR5-VENUS* in *plt3; plt5-2; plt7* after 6 days on SIM. (H'-J') No *PIN1-GFP* expression and a weak and ubiquitous expression of *DR5– VENUS* without any localized signal accumulation in the mutant calli derived from both root and leaf explants after 10–12 days on SIM. (K) PIN1-GFP is localized the tip of leaf primordia (arrow) in wild-type*;pDR5::PIN1:GFP* after 10 days on SIM. (K') No shoot meristem formation in *plt3; plt5-2; plt7;pDR5::PIN1:GFP* although a weak *PIN1-GFP* expression is observable in most parts of the callus. (L) Shoot regeneration in wild-type*;pDR5::PIN1:GFP* after 12 days on SIM. (L') No shoot regeneration in *plt3; plt5-2; plt7;pDR5::PIN1:GFP* on SIM. (S) The panels (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 colour is the propidium iodide stain in (A–F') and the FM4-64 stain in (G,G'-J'),. Red colour in (H,I) is autofluorescence. Scale bar= 50μm in (A–J', K,K') and 1 mm in (L,L')

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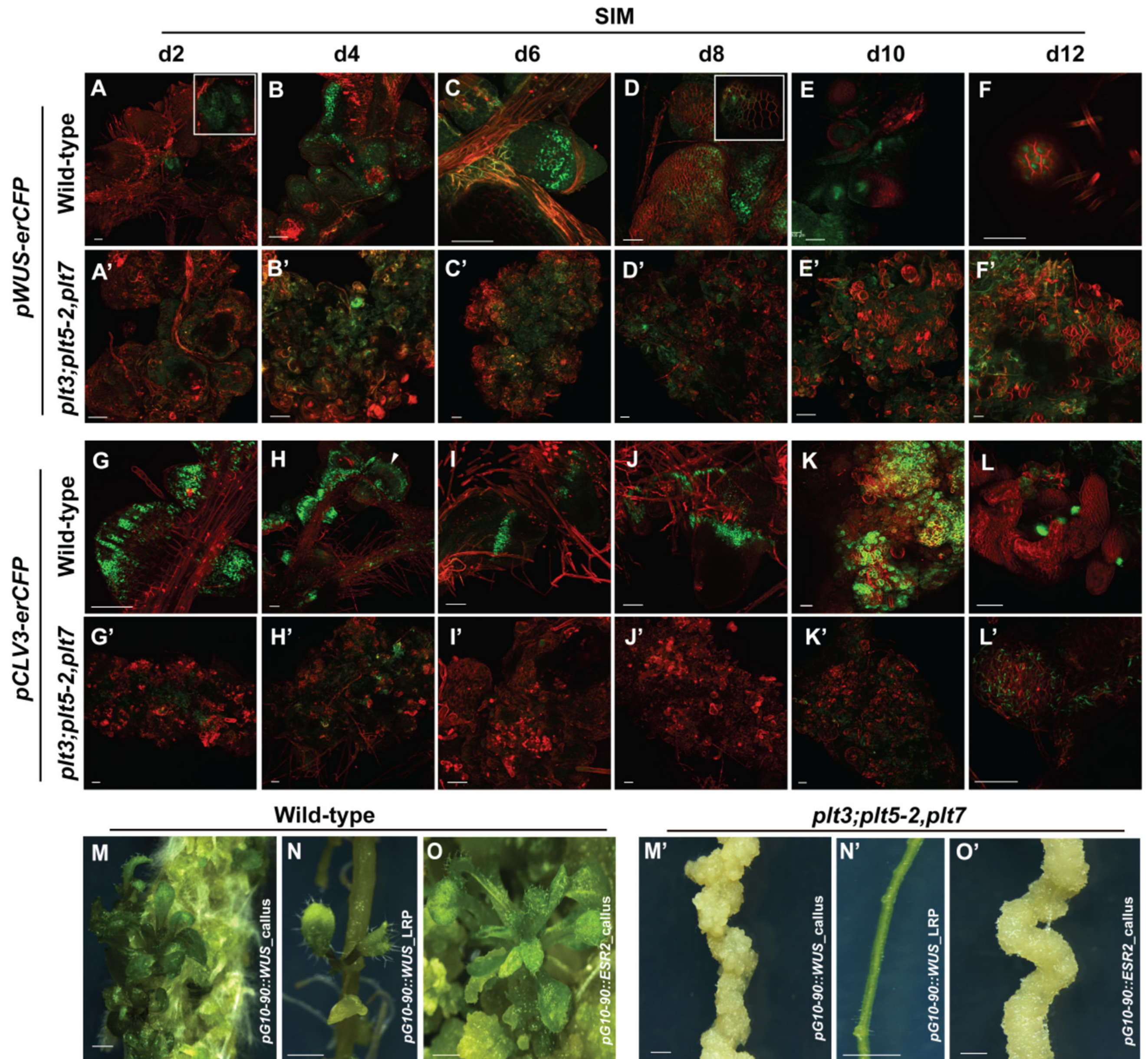

Figure 4. *WUS* and *CLV3* expression domains are not properly established in *plt3; plt5-2; plt7* mutants after regeneration stimulus

(A) 2 days after transfer to SIM, *WUS::erCFP* was expressed in the innermost layers of proliferating cells in wild-type. (B,C) *WUS* expression was distributed in a large portion of the wild-type callus after 4–6 days and (D,E) it became progressively restricted to the centre of developing meristems. Inset in (D) shows *WUS* expression in the meristem centre. (F) After 12 days, WUS-CFP marked the centre of shoot meristems in wild-type. (A') In *plt3; plt5-2; plt7* callus, *WUS* was weakly expressed after 2 days of SIM induction and (B'-D') became scattered within the callus. (E',F') Scattered distribution continued after 10–12 days of induction, without any confined accumulation. (G) *CLV3::erCFP* expression initially arose in wild-type callus after 2 days of SIM induction. (H–K) After 4–10 days of induction, *CLV3::erCFP* signal expanded to encompass a large part of wild-type callus. (L)

CLV3::erCFP was upregulated only in the meristem centre after 12 days of SIM induction in wild-type. (G') Weak expression of *CLV3::erCFP* in *plt3; plt5-2; plt7* callus after 2 days of induction. (H'-K') Sporadic *CLV3::erCFP* expression in *plt3; plt5-2; plt7* callus after 4–10 days of SIM induction. (L') *CLV3::erCFP* signal remained sporadic without any localized upregulation after 12 days of induction. (M,N) Ectopic overexpression of *WUS* (*G10-90::WUS:3AT*) in wild-type induced *de novo* shoots from both callus and LRP upon incubation on hormone free medium supplemented with estradiol. (M',N') Overexpression of *WUS* in *plt3; plt5-2; plt7* mutant tissue did not induce *de novo* shoots on callus or LRP. (O) Forced induction of *ESR2* (*G10-90::ESR2:3AT*) on minimal medium with estradiol induced *de novo* shoots on wild-type callus while (O') mutant callus failed to regenerate shoots. Scale bar: 50μm in (A–L'); 1mm in (M–O').

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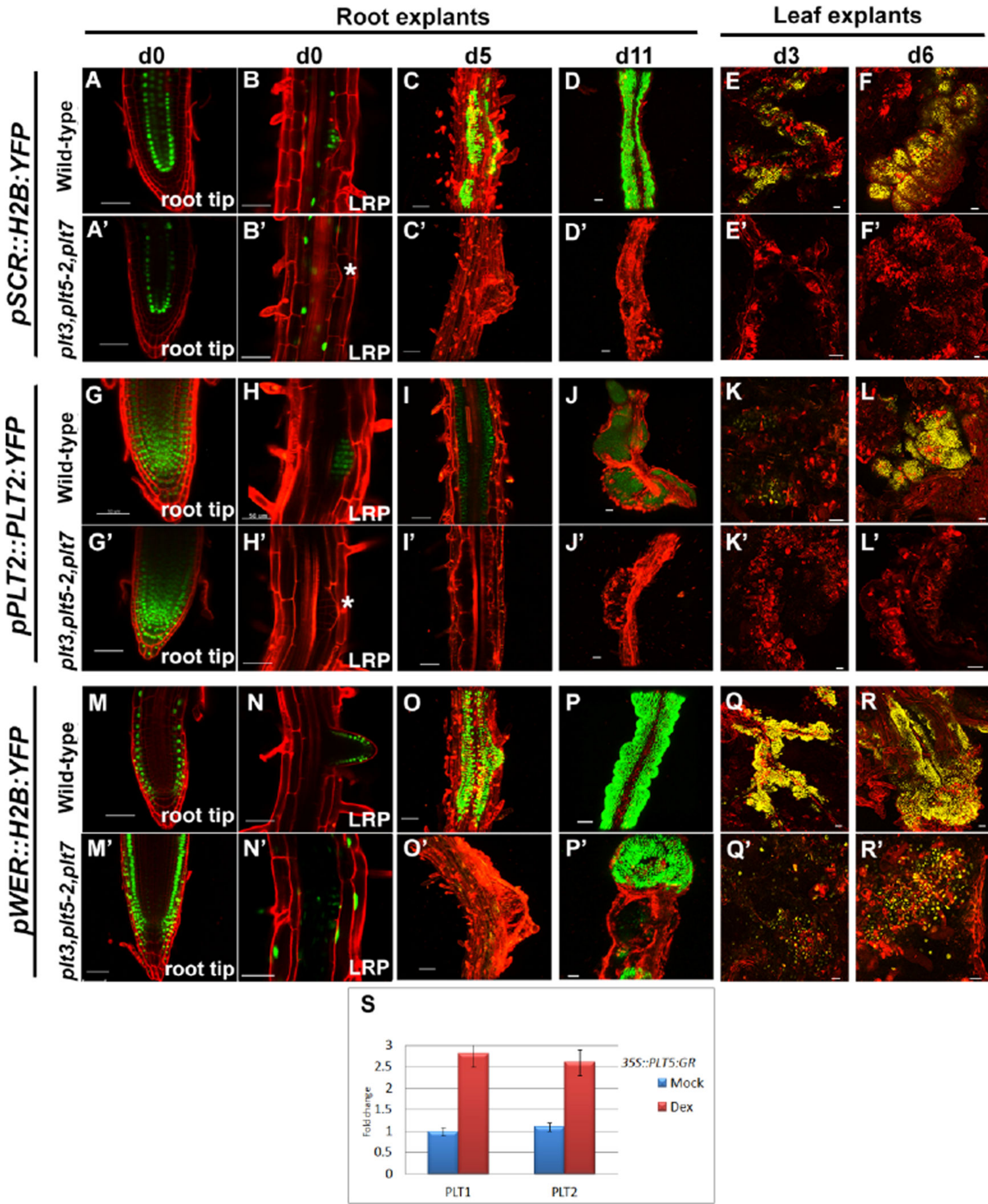

Figure 5. Root stem cell maintenance regulators are not detectably expressed in *plt3; plt5-2; plt7* LRP and callus

(A–F, G–L, M–R) *pSCR::H2B:YFP*, *pPLT2::PLT2:YFP*, and *pWER::H2B:YFP* expression in wild-type explants and (A'–F', G'–L', M'–R') *plt3; plt5-2; plt7* explants. 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, 11days, and calli derived from leaf cultured on CIM for 3 days, 6 days. While in wild-type explants, all three reporters are expressed in both untreated primary root tip, LRP, and CIM-induced calli derived from root and leaf (A–F, G–L, M–R), in the *plt3; plt5-2; plt7* mutant explants, *pSCR::H2B:YFP* and

pPLT2::PLT2:YFP are expressed neither in LRP (B', H' asterisks) nor calli derived from those tissues (C'–F', I'–L'). (N') The LRP of *plt3; plt5-2; plt7* displays slight *pWER::H2B:YFP* expression. (P', Q', R') In calli of *plt3; plt5-2; plt7*, partial or weak expression is observed. (O') Some callus does not express the reporter at all. (S) Upregulation of *PLT1* and *PLT2* transcripts upon the induction of *PLT5* measured by quantitative RT-PCR. Expression levels were normalized to *ACTIN2*. Error bar represents standard error of the mean from three independent biological replicates. Scale bar in (A–R') = 50μm.

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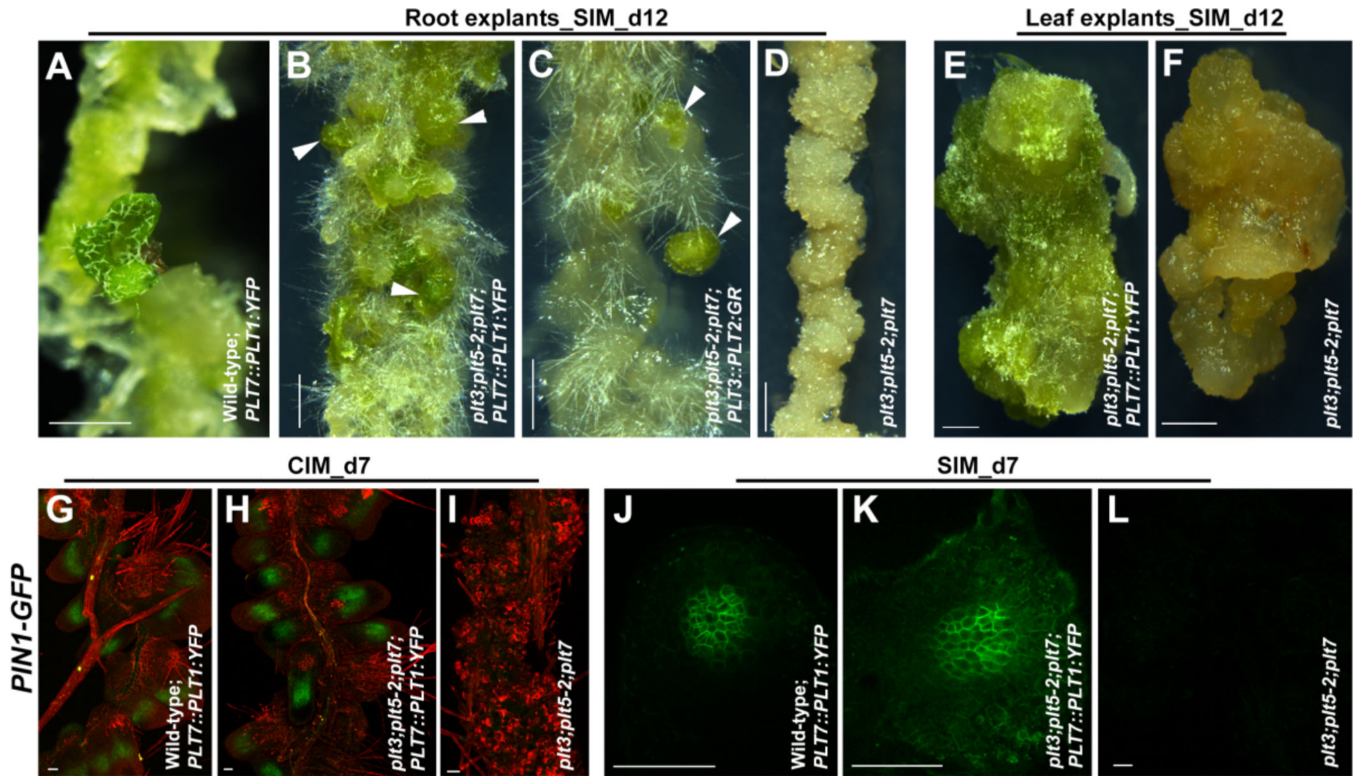

Figure 6. Root stem cell maintenance regulators establish early competence for shoot regeneration

(A) Shoot regeneration in wild-type callus (Wild-type;*PLT7::PLT1:YFP*) derived from root explant after 12 days on SIM. (B,E) Competent calli turned green in *plt3; plt5-2; plt7;PLT7::PLT1:vYFP* root and leaf explants and in (C) *plt3; plt5-2; plt7;PLT3::PLT2:GR* root explants after 12 days on SIM. (D,F) Callus derived from root and leaf explants of *plt3; plt5-2; plt7* remained yellowish on SIM. (G) Expression of *PIN1-GFP* in Wild-type*; PIN1::PIN1:GFP* after 7 days on CIM. (H) *plt3; plt5-2; plt7;PLT7::PLT1:vYFP*, *PIN1::PIN1:GFP* callus regained cellular morphology typical of wild-type and expressed *PIN1-GFP* after 7 days on CIM (I) Disorganized callus cells without *PIN1-GFP* expression in *plt3; plt5-2; plt7;PIN1::PIN1:GFP* on CIM. (J) Shoot progenitor cells labeled with PIN1-GFP in wild-type callus and (K) in *plt3; plt5-2; plt7;PLT7::PLT1:vYFP*, *PIN1::PIN1:GFP* 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. Scale bar: 1mm in (A–F) and 50μm in (G–L). Red color in (G–I) is propidium iodide. No stain was used for cell boundaries in (J–L).

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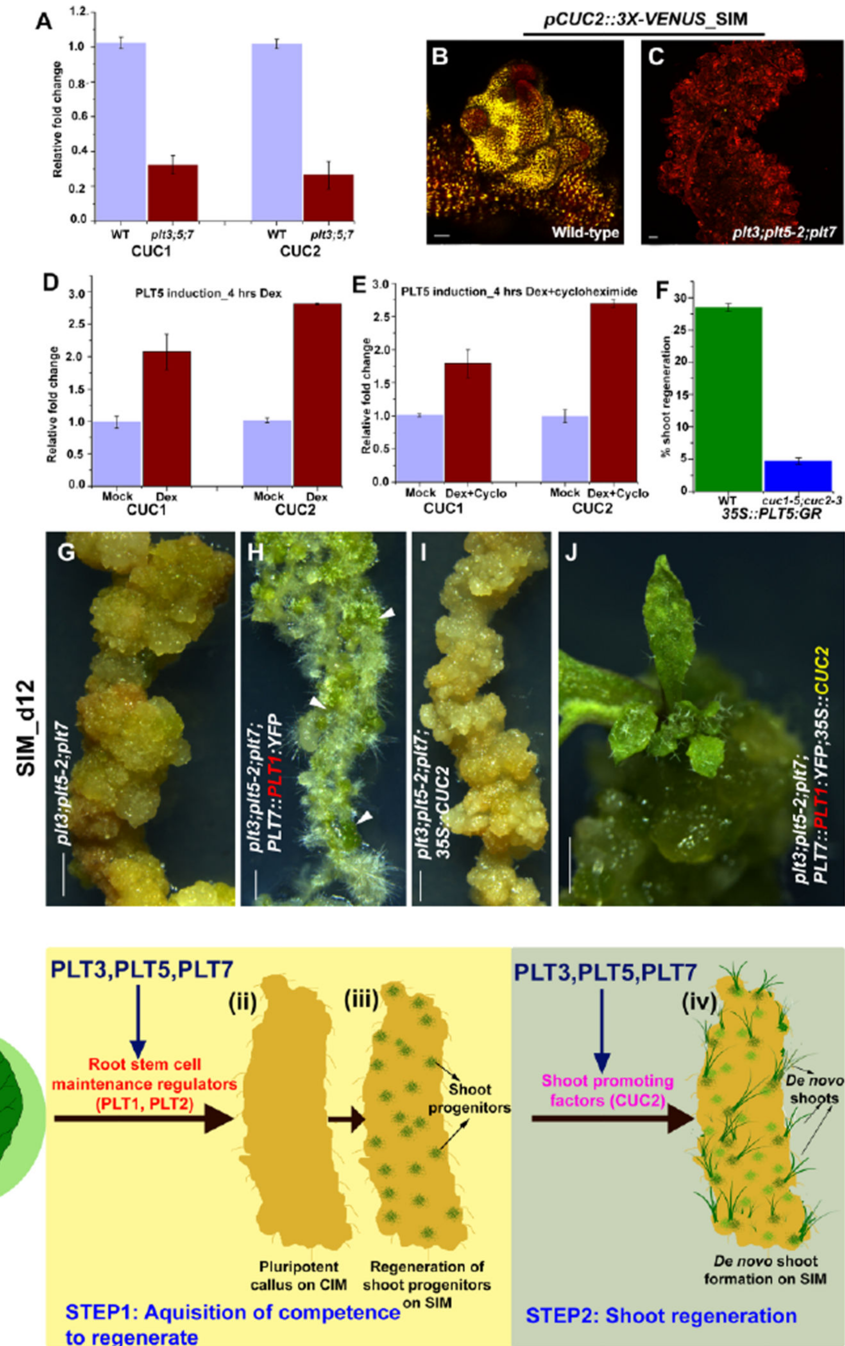

Figure 7. PLT3, PLT5 and PLT7 control *de novo* shoot regeneration by a two-step mechanism

(A) Bar graph showing *CUC1* and *CUC2* expression levels in wild-type and *plt3; plt5-2; plt7* mutant calli after 10 days of induction on SIM, measured by quantitative RT-PCR. (B) *pCUC2::3X-VENUS* was upregulated in wild-type callus on SIM and (C) it was downregulated in *plt3; plt5-2; plt7* callus. (D) *CUC1* and *CUC2* transcripts levels after 4 hrs of *PLT5* induction by DEX and (E) DEX with cycloheximide treatment, measured by quantitative RT-PCR. Expression levels were normalized to *ACTIN2*. (F) Bar graph showing 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) *De novo* shoot regeneration is completely abolished in *plt3; plt5-2; plt7* upon SIM induction. (H) Upon reconstitution of PLT1 expression in *plt3; plt5-2; plt7*, callus cells regain pluripotency and shoot progenitors are regenerated. Arrowheads represent green foci. (I) *De novo* shoot formation is not achieved in *plt3; plt5-2; plt7; 35S::CUC2* upon SIM treatment (J) Ectopic overexpression of *CUC2* in *plt3; plt5-2; plt7;PLT7::PLT1-YFP* leads to complete shoot regeneration 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 enabling 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,E) represent standard error of the mean from three independent biological replicates. Scale bar in (B,C) = 50μm and in (G–I) = 1mm

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