Current Biology
Supplemental Information

PLETHORA Genes Control Regeneration
by a Two-Step Mechanism

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Figure S1. PLT3, PLT5 and PLT7 are upregulated during direct conversion of LRP into shoot (related to Figure 1).

(A) PLT3-YFP, (F) PLT5-YFP and (K) PLT7-YFP expression in untreated LRP. Persistence of expression of (B, C) PLT3 (G, H) PLT5 and (L, M) PLT7 upon SIM-2 treatment during the direct conversion of LRP into shoot. (D, I, N) Upregulation of all three PLTs on the meristem surface and in developing shoot primordia, which are derived directly from LRP after 7 days of induction. (E, J, O) Upregulation of all three PLTs during shoot regeneration on the medium containing cytokinin (2ip) as the sole hormonal supplement. Scale bar: 50µm
Figure S2. PLT3, PLT5 and PLT7 are necessary for shoot regeneration from callus and LRP (related to Figure 2).

(A) Regenerating green foci in wild type callus after 6 days on SIM. (B) First leafy shoot noticed in wild type callus after 10 days of treatment on SIM. (C) Direct conversion of LRP into shoot in wild type after 8 days on SIM-2. (D, E) Neither regenerating foci nor de novo shoots developed in plt3; plt5-2; plt7 calli on SIM. (F) Failure in the conversion of LRP into shoot in plt3; plt5-2; plt7 upon SIM-2 treatment. (G) Regeneration efficiency in calli of various combinations of plt mutants on SIM-2. Number of shoots represents shoots formed per explant (~3 cm). Error bar represents standard error of mean. (H) De novo shoot formation in wild type callus harboring 35S::PLT7:GR after DEX induction on hormone free medium. Arrows represent leafy shoots. (I) No shoot regeneration in wild type callus on hormone free medium with DEX. Scale bar= 1mm.
Figure S3. Auxin responses are disrupted in plt3; plt5-2; plt7 during direct conversion of LRP into shoots (related to Figure 3).

(A, A’) Expression of both PIN1::PIN1::GFP (green) and pDR5rev::3XVENUS-N7 (yellow) in untreated LRP of both wild type and plt3; plt5-2; plt7 mutant. (B, B’) Upregulation of DR5-VENUS signal in LRP and vasculature of both wild type and mutant after two days of treatment on SIM-2. Note the signal intensity is less in the mutant LRP. (C, C’) No detectable PIN1-GFP expression in LRP of both the genotypes until day 4. (D) Upregulation of PIN1::GFP in the shoot meristem formed from the wild type LRP after 5 days of induction. (D’) No detectable PIN1-GFP signal in the cells derived from the mutant LRP. (E) Both PIN1-GFP and DR5-VENUS marking the leaf primordium that emerged from the shoot meristem in wild type after 7 days of culture. (E’) No detectable PIN1-GFP expression in LRP-derived cells of plt3; plt5-2; plt7 after 7 days of culture while DR5-VENUS signal is considerably reduced. (F) Shoot regeneration efficiency, not affected in wild type plants on expression of PIN1-GFP under a DR5 promoter. Number of shoots regenerated was calculated per explant (~3 cm length) after 3 weeks of incubation on SIM. Error bar represent standard error of mean. Scale bar in A-E’ = 50µm
Figure S4. WUS and CLV3 expression is deregulated in plt3; plt5-2; plt7 during regeneration (related to Figure 4).

(A, A’) No WUS::erCFP expression in untreated LRP of both wild type and plt3; plt5-2; plt7. (B) WUS signal (green) in the wild type LRP-derived cells after 2 days of treatment on SIM-2 and (C) gradual increase in the signal intensity after 4 days of SIM-2 induction. (D, E) Localized accumulation of WUS signal at the centre of the shoot meristem converted from LRP in wild type. (B’) A faint expression of WUS reporter in plt3; plt5-2; plt7 LRP-derived cells after 2 days. (C’, D’) Upregulation of WUS signal throughout the LRP-derived cells after 4-5 days. (E’) Persistence of ectopic expression of WUS without confined localization after 7 days of induction. (F, F’) No CLV3::erCFP expression in untreated LRP of both wild type and plt3; plt5-2; plt7 mutant. (G, H, I) Steady up-regulation of CLV3-CFP signal (green) in the LRP-derived cells of both wild type and (G’, H’, I’) mutant after 2-5 days of induction. (J, J’) Confined localization of CLV3 signal at the centre of shoot meristem derived directly from LRP in wild type after 7 days, while the signal remained distributed throughout in the mutant LRP. (K, L) Shoot meristem-localized expression of pWUS::CFP in plt3 single mutant and in plt3; plt5-2 double mutant on SIM. (M, N) pCLV3::CFP expression in the nascent shoot meristem in plt3 single mutant and in plt3; plt5-2 double mutant on SIM. Asterisk in A, A’, F, F’ represents LRP. Scale bar = 50µm
Figure S5. Differential expression of \textit{PLT1-YFP} and \textit{PLT2-YFP} in wild type and \textit{plt3; plt5-2; plt7} calli (related to Figure 5).

(A) \textit{PLT1::PLT1-YFP} expression in wild type callus derived from leaf explants after 3 days and (B) 6 days of induction on CIM. (C, D) No \textit{PLT1} expression in \textit{plt3; plt5-2; plt7} mutant callus derived from leaf explants after 3 and 6 days of induction on CIM. (E) Expression of \textit{PLT2::PLT2-YFP} in wild type callus derived from primary root meristem. (F) \textit{PLT2} expression in callus derived from \textit{plt3; plt5-2; plt7} primary root tip. Scale bar= 50\mu m.
Figure S6. PLT1/PLT2 establishes early competence for shoot regeneration (related to Figure 6).

(A, B) Upregulation of PLT1::PLT1::YFP and PLT2::PLT2::YFP on CIM and (E, F) downregulation on SIM in wild type callus. (C, G) PLT7(1.5kb)::PLT1::YFP displays expression pattern similar to endogenous PLT1 expression in both wild type and (D, H) plt3; plt5-2; plt7 mutant calli. (I) Well organized cellular arrangement in wild type callus on CIM. (J) Highly disorganized cells in plt3; plt5-2; plt7 callus. (K) Regain of cellular morphology similar to wild type by mutant callus after reconstitution of PLT1 expression. (L) Direct shoot regeneration from LRP of wild type after 10 days of induction on SIM-2. (M) LRP of plt3; plt5-2; plt7; PLT7::PLT1::YFP turned green (arrow head), but showed no shoot outgrowth, after 10 days of induction. Inset is green LRP. (N) LRP of plt3; plt5-2; plt7 remained colorless with no sign of regeneration after 10 days of induction. (O) plt3; plt5-2; plt7 callus remained yellowish on SIM. (P) Green callus in plt3; plt5-2; plt7 after transient induction of PLT2. (Q) Constitutive induction of PLT2 under PLT3 promoter in plt3; plt5-2; plt7 even on SIM lead to fate
change and the callus remained yellowish. (R) Regeneration of shoots in plt3; plt5-2; plt7; PLT3::PLT3::YFP where PLT3 was driven by PLT3 promoter. Scale bar in (A-K) = 50µm and in (L-R) = 1mm.

Figure S7. Role of root stem cell regulators and shoot promoting factors in regeneration (related to Figure 7).

(A) CUC1 and CUC2 expression levels after 8 hrs of PLT5 induction by DEX treatment, measured by quantitative RT-PCR. The expression levels were normalized to ACTIN2. Error bars represent standard error of the mean from three independent biological replicates. (B, C) Direct shoot regeneration from LRP is not achieved in plt3; plt5-2; plt7 and in plt3; plt5-2; plt7; 35S::CUC2 on cytokinin rich SIM-2. (D) Regeneration of shoot progenitors (first step) is greatly affected in a plt1; plt2 double mutant. The second step of shoot regeneration is also affected in the mutant (***t-test, p < 0.001). Average length of explants = ~3 cm. Error bar represents standard error of mean. (E) Regeneration of shoot progenitors (first step) is not significantly hindered in cuc1; cuc2 mutants (ns-t-test, p > 0.05, P = 0.076) but the second step of complete shoot formation is reduced (***t-test, p < 0.001). Average length of explants = ~3 cm. Error bar represents standard error of mean. Scale bar in (B, C) = 1mm.
Supplemental Experimental Procedures

Plant Materials

All plants used in this study were in the Col-0 background. The origin of \textit{cuc1-5; cuc2-3} double mutants has been described previously [S1]. The \textit{cuc1-5; cuc2-3; 35S::PLT5:GR} transgenic line was generated by crossing \textit{cuc1-5; cuc2-3/+} and wild type; \textit{35S::PLT5:GR}. To generate \textit{plt3; plt5-2; plt7} mutants expressing \textit{PLT1} along with \textit{CUC2} overexpression, a genetic cross was made between \textit{plt3; plt5-2; plt7; PLT7(1.5kb)::cPLT1:YFP} and \textit{plt3; plt5-2; plt7; 35S::CUC2} (T1 line). Two independent transgenic lines of \textit{plt3; plt5-2; plt7; 35S::CUC2} displaying a strong phenotype of \textit{CUC2} overexpression as previously reported [S1] were crossed with \textit{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 \textit{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 for molecular cloning

Translational fusions, \textit{PLT1::PLT1:vYFP}, \textit{PLT2::PLT2:vYFP} [S2], \textit{PLT3::PLT3:vYFP}, \textit{PLT5::PLT5:vYFP} and \textit{PLT7::PLT7:vYFP} [S3] were described previously. Transgenic lines harbouring the inducible \textit{35S::PLT5:GR} construct [S3], the double marker construct \textit{PIN1::PIN1:GFP; DR5::3XVENUS-N7} [S4] and the transcriptional fusion constructs, \textit{pWUS::erCFP}, \textit{pCLV3::erCFP} [S4] were described previously.

The \textit{pDR5::PIN1:GFP} was constructed by placing \textit{pDR5rev} promoter [S5] upstream to the \textit{PIN1-GFP} [S5] coding region. To generate \textit{pG10-90::WUS} and \textit{pG10-90::ESR2}
constructs, the coding region of \textit{WUS} or \textit{ESR2} including introns amplified from Col-0 genomic DNA was placed under the control of estradiol-inducible \textit{G10-90} promoter [S2, S6]. The transcriptional fusion constructs of \textit{pSCR::H2B:vYFP} and \textit{pWER::H2B:vYFP} were generated by cloning the upstream regulatory sequences of \textit{SCR} [S7] or \textit{WER} [S2, S6] with the coding region of \textit{vYFP} with nuclear localization signal (Histone2B-H2B). \textit{PLT7(1.5kb)::cPLT1:vYFP} (Du and Scheres, unpublished) contains a 1.5 kb truncated upstream regulatory sequences of \textit{PLT7}, cDNA sequence of \textit{PLT1} gene fused in translational frame with the \textit{vYFP}. To generate a \textit{PLT3::PLT2:GR} construct, the genomic sequence of \textit{PLT2} [S2] was fused to the 5’ end of the gene encoding rat glucocorticoid receptor (GR) [S8] and cloned under the control of a heterologous \textit{PLT3} (7.7 kb) promoter [S3]. Coding sequences of \textit{CUC2} including introns was amplified from Col-0 genomic DNA and incorporated between a 35S promoter of \textit{CaMV} and the nopaline synthase terminator to generate the construct of \textit{35S::CUC2}. To generate \textit{pCUC2::3X-VENUS}, a 3.2kb upstream regulatory sequence of \textit{CUC2} was amplified from Col-0 genomic DNA and fused with \textit{3X-VENUS} [S9]. The primers used for PCR amplification are listed in Table S1 in the supplemental information.

**Regeneration conditions**

For indirect shoot 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), 1X Gamborg’s vitamin solution (Sigma) and 0.8% agar. The plant hormones used were 0.5 \(\mu\)g/ml (for root and hypocotyl explants) or 2 \(\mu\)g/ml (for cotyledon and leaf explants) of 2,4-D (Sigma) and 0.05 \(\mu\)g/ml of kinetin.
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 1X MS basal salt mixture, 10 g/l sucrose (Sigma), 0.5 g/l MES (Sigma), 1X 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 [S10], 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 shoot regeneration from lateral root primordium, 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.

**Confocal Microscopy Settings**

Following settings were used for imaging under Leica TCS SP5 II laser scanning microscope. 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 used to excite the signal of propidium iodide were used for the excitation of FM4-64, but the emission signal was collected through a 620-750 nm detection band. Autofluorescence of chlorophyll was excited by any of these wavelengths and we chose a 650-750 nm detection band for collection.

Imaging was also done using a Zeiss LSM 710 Meta confocal microscope. To detect the signal of propidium iodide staining, a 488 nm laser line was used for excitation and a 585-615 nm band-pass filter in conjunction with a 545 nm 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 [S5, S11]. 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.

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Table S1 Oligonucleotide primers used in this study (5'->3')
Supplemental References


