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Correction

In the article by Chatfield *et al.* (2013), the following author details are incorrect:

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They should read:

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The authors wish to apologise for these errors.

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Incipient stem cell niche conversion in tissue culture: using a systems approach to probe early events in *WUSCHEL*-dependent conversion of lateral root primordia into shoot meristems

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SUMMARY

Adventitious shoot organogenesis contributes to the fitness of diverse plant species, and control of this process is a vital step in plant transformation and *in vitro* propagation. New shoot meristems (SMs) can be induced by the conversion of lateral root primorida/meristems (LRP/LRMs) or callus expressing markers for this identity. To study this important and fascinating process we developed a high-throughput methodology for the synchronous initiation of LRP by auxin, and subsequent cytokinin-induced conversion of these LRP to SMs. Cytokinin treatment induces the expression of the shoot meristematic gene *WUSCHEL* (*WUS*) in converting LRP (cLRP) within 24–30 h, and *WUS* is required for LRP → SM conversion. Subsequently, a transcriptional reporter for *CLAVATA3* (*CLV3*) appeared 32–48 h after transfer to cytokinin, marking presumptive shoot stem cells at the apex of cLRP. Thus the spatial expression of these two components (*WUS* and *CLV3*) of a regulatory network maintaining SM stem cells already resembles that seen in a vegetative shoot apical meristem (SAM), suggesting the very rapid initiation and establishment of the new SMs. Our high-throughput methodology enabled us to successfully apply a systems approach to the study of plant regeneration. Herein we characterize transcriptional reporter expression and global gene expression changes during LRP → SM conversion, elaborate the role of *WUS* and *WUS*-responsive genes in the conversion process, identify and test putative functional targets, perform a comparative analysis of domain-specific expression in cLRP and SM tissue, and develop a bioinformatic tool for examining gene expression in diverse regeneration systems.

Keywords: wuschel, meristem, lateral root, cytokinin, regeneration, pluripotent, auxin, *Arabidopsis thaliana*.

INTRODUCTION

Plants are able respond to environmental challenges with impressive metabolic and developmental flexibility. Adaptable development is achieved by controlling the activities of stem cell niches (meristems) in root and shoot, and regulating the growth of tissues produced from them. In addition to primary root and shoot apical meristems (RAMs and SAMs), reiterative development from lateral root primordia (LRP) in the root and axillary meristems in the shoot is regulated in response to diverse cues and signaling inputs (reviewed in McSteen and Leyser, 2005; Scheres, 2007). Remarkably, many plant species also initiate adventitious root and shoot meristems (SMs) *de novo* (reviewed in Steeves and Sussex, 1989; Kerstetter and Hake, 1997) for clonal propagation (e.g. poplar), or to recover from injury. In the root system, adventitious shoots can be initiated from pericycle-derived cells, LRP and RAMs (Bonnet and Torrey, 1966; Peterson, 1970; Spencer-Barreto and Duhoux, 1994).

Artificial induction of adventitious shoots using auxin and cytokinin was first developed over 60 years ago (Skoog, 1950; Skoog and Miller, 1957), and remains a vital step in micropropagation and transformation protocols.

Shoot organogenesis is generally induced from callus by cytokinin treatments. Callus was long considered an essential step in *in vitro* organogenesis, providing a mass of apparently disorganized and undifferentiated cells amenable to 'redifferentiation'. However, recent studies have shown that callus has much in common with LRP, in its proliferation from xylem-pole pericycle cells and gene expression patterns (Che *et al.*, 2007; Atta *et al.*, 2009; Sugimoto *et al.*, 2010). Furthermore, shoot organogenesis has also been induced from LRP/LRP-like organs without an intervening callus stage (Atta *et al.*, 2009).

Shoot organogenesis can also be induced by genetic elevation of endogenous cytokinin levels (Zuo *et al.*, 2002), or misexpression of specific SAM-associated genes, such as *DORNRÖSCHEN* (*DRN*) (Banno *et al.*, 2001) and *WUSCHEL* (*WUS*) (Gallois *et al.*, 2004). In loss-of-function *wus* mutants, stem cells of the SAM are consumed shortly after germination (Laux *et al.*, 1996), and *WUS* is functionally required for shoot organogenesis from callus (Gordon *et al.*, 2007). Within the SAM, *WUS* is expressed in the organizing centre and the protein migrates to overlying cells of the central zone, CZ (Yadav *et al.*, 2011), where it specifies stem cell fate (Mayer *et al.*, 1998). Within the CZ WUS activates *CLAVATA 3* (*CLV3*), which negatively regulates *WUS* expression via the *CLAVATA* pathway, a feedback loop that helps maintain a constant population of stem cells (Fletcher *et al.*, 1999; Brand *et al.*, 2000). When *WUS* is misexpressed in the root, or induced with cytokinin, it also stimulates the expression of *CLV3* in adjacent cells (Gallois *et al.*, 2004; Gordon *et al.*, 2007).

Recent studies have enhanced our understanding of the relationship between *WUS* and cytokinin in SAM function and shoot organogenesis. Type-A *ARABIDOPSIS RESPONSE REGULATORS* (*ARR*s) are negative regulators of cytokinin signaling (To *et al.*, 2004), and are transcriptionally repressed by *WUS* (Leibfried *et al.*, 2005; Busch *et al.*, 2010). In characterizing cytokinin-induced *WUS* expression, Gordon *et al.* (2009) found *CLV*-dependent and -independent mechanisms were involved, primarily mediated through cytokinin receptors *ARABIDOPSIS HISTIDINE KINASE 2* (*AHK2*) and *AHK4*.

In addition to cytokinin-signaling genes, WUS binds at least two different motifs to regulate the transcription of numerous targets (Busch *et al.*, 2010). Furthermore, WUS stimulates transcription of *TOPLESS* (*TPL*) (Busch *et al.*, 2010), which modulates auxin signaling and represses root-specifying genes (Long *et al.*, 2002; Szemenyei *et al.*, 2008; Smith and Long, 2010). Thus *WUS* appears to modulate aspects of cytokinin and auxin signaling, two plant hormones that play key antagonistic roles in root and shoot organogenesis.

In this study we adapt a method for synchronous LRP induction and couple it to cytokinin-mediated *WUS* upregulation to provide a high-throughput system for studying shoot organogenesis via LRP → SM conversion. Expression of transcriptional reporters for *CLV3* and *WUS* identified key stages in LRP → SM conversion, guiding transcriptome analysis and revealing potential targets mediating the process. Furthermore, because there is a functional requirement for *WUS* in SM initiation from callus/LRP, we analyzed the transcriptomes of *wus* loss-of-function mutants, and *WUS* expression domains within cLRP, identifying genes transcriptionally responsive to *WUS* activity (*WUS* responsive) involved in this process. In addition to enhancing our understanding of shoot organogensis, and the role of *WUS*, it is hoped that our work on this intriguing developmental phenomenon will also inform regeneration in natural systems.

RESULTS

Incipient stem cell niche conversion

To study the cytokinin-induced conversion of LRP/LRMs to shoots we first generated synchronous LRP initiation by exposing 3- or 4-day-old seedlings to 10 μM 1-naphthaleneacetic acid (1-NAA), a synthetic auxin used in transcriptomic studies of LRP (Himanen *et al.*, 2004). Seedlings have few LRP prior to NAA treatment, which initiates LRP at every available position along the primary root xylem-pole pericycle. Transfer to cytokinin-enriched media induces the synchronous conversion of induced LRP into SMs, and emerging shoots become visible within 5 days (Figure 1a–c). This simple methodology appears robust, and we have used it to rapidly induce shoots from roots of other brassicas and poplar (Figure 1d).

A high-throughput methodology, adapted from Birnbaum *et al.* (2005), permitted the transfer of hundreds of plants between hormone treatments and rapid sampling (Figure 1a,b). In agreement with previous studies, 24 h after auxin treatment the LRP induced by the treatment reached between three and five cell layers, a stage that precedes commitment to self-sustaining LRMs (Sussex *et al.*, 1995).

WUSCHEL reporters are expressed in cLRP within 30 h

In our experiments we used well-characterized transcriptional reporters for *WUS* and *CLV3*, with expression patterns reflecting mRNA *in situ* hybridization experiments (Reddy and Meyerowitz, 2005; Gordon *et al.*, 2007; Yadav *et al.*, 2009). Within the treated roots, *WUS* reporters were first visible 19 h after transfer from NAA to isopentenyladenine (2iP), and were weakly and sporadically expressed outside the LRP (Figure 2a). *WUS* reporter expression was first seen within cLRP 24–36 h after transfer to 2iP (Figure 2b), but rarely in the outermost layer of cells (2C–E). Expression of this transcriptional reporter for the organizing center of the SAM within former LRP shows that an important change in cell identity, towards that associated with SMs, is already underway just a day after exposure to cytokinin.

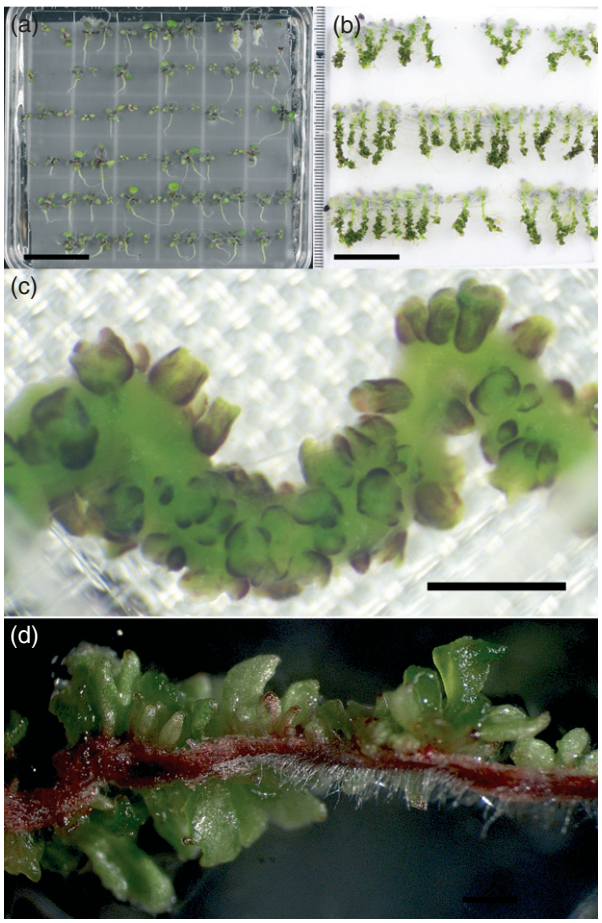


Figure 1. Shoot induction via conversion of lateral root promordia (LRP) to shoot meristems (SMs).
(a, b) High-throughput method: Nitex sheets were used to transfer tens to hundreds of seedlings between treatments. Seedlings were transferred from germination media 3–4 days after imbition to media supplemented with 10 μM 1-naphthaleneacetic acid (1-NAA) for 24 h for rapid LRP induction, and then to 4.4 μM 2iP (a cytokinin) promoting LRP → SM conversion (b). After 6 days of treatment with 2iP, dense shoots were visible.
(c, d) Close-ups of Arabidopsis shoot induction (5 days 2iP; c) and poplar (14 days 2iP; d). Scale bars: 2 cm (a, b); 2 mm (c, d).

A *pCLV3::GFP-ER* reporter appeared 32–48 h after transfer to 2iP marking between two and four cell layers at the apex of cLRP, above and overlapping with populations of small cells expressing *pWUS::DsRED-N7* (Figure 2c,d), and was never seen in the absence of the *WUS* reporter, consistent with previous studies that have found *CLV3* expression to be dependent upon the *WUS* gene product (Laux *et al.*, 1996; Brand *et al.*, 2002). Thus, transcriptional reporter expression of these key regulators of the SAM stem cell population assumed a spatial relationship within cLRP, reflecting that in SAMs. Expression of these two components of a regulatory network responsible for maintaining a shoot meristematic stem cell population constitutes another significant step in the establishment of a new SM.

Transcriptome analysis of LRP–SM conversion reveals changes related to hormone signaling, meristem identity, cell cycle and photosynthesis

By enriching samples with synchronously developing LRP, we hoped to focus on gene expression pertaining to LRP–SM conversion. For transcriptome analysis, key time points in LRP → SM conversion were selected based on the aforementioned reporter analysis. Tissues were sampled after 24 h of exposure to NAA (0 h 2iP), and subsequently after 19, 30 and 48 h of 2iP treatment. The first time point corresponds to saturated LRP development within the primary roots, and 19 h of 2iP treatment corresponds with the initial expression of p*WUS::DsRED-NLS*. After 30 h of treatment with 2iP there is consistent expression of this marker within cLRP prior to the detection of *pCLV3::mGFP-ER*. Treatment with 2iP for 19 h precedes the detectable expression of SAM markers within cLRP, perhaps corresponding to an intermediate state between LRP and initiating SMs. We reasoned that a 30 h time point would reveal gene expression events associated with early SM initiation. After 48 h of treatment with 2iP the *CLV3* marker is expressed within 10–25% of cLRP, reflecting gene expression representing the initial establishment of organized SMs.

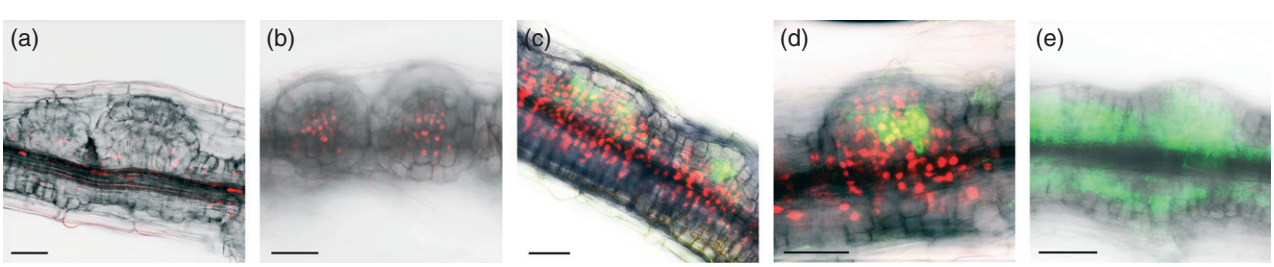


Figure 2. Confocal images of *WUS* and *CLV3* transcriptional reporter expression during conversion of lateral root primordia (LRP) to shoot meristems (SMs).
(a) After 19–24 h of 2iP treatment, sporadic and weak *pWUS::DsRED-NLS* (red nuclei) marks cells peripheral to LRP and scattered cells within the vascular cylinder.
(b) After 30 h of 2iP treatment, *pWUS::DsRED-NLS* is upregulated within cLRP.
(c, d) After 48 h of 2iP, *pCLV3::GFP-ER* (green cells) is seen in small isodiametric cells near the apices of 10–25% of cLRP. The *pCLV3::GFP-ER* domain was found above populations of small cells expressing *pWUS::DsRED-NLS*.
(e) *pWUS::GFP–ER* (green) expression after 30 h of 2iP treatment is also upregulated within the cLRP. Scale bars: 65 μm.

Exogenous auxin and cytokinin were expected to generate marked changes in gene expression. Because expression of key developmental regulators could be relatively small when ranked against this background, lenient criteria were used for the initial selection of differentially expressed genes (DEGs). Using MAS5 processed data, each time point was compared pairwise with every other time point, applying a fold-change threshold of 1.75, a *P*-value cut-off of <0.05 and the rejection of mean expression values <50 units.

Figure 3 provides an overview of DEGs identified in each comparison (also Table S1). As expected, large numbers of DEGs (2399–3472) were identified in comparisons between 0 h and 2iP time points. There was also a marked overlap in the DEGs from each of these comparisons, with 1700 DEGs identified in all three (Figure 3). For comparisons between cytokinin treatment periods, 340 and 642 DEGs were identified between 19 and 30 h, and between 19 and 48 h, respectively. Interestingly, although the 30 and 48 h time points of 2iP treatment separate important changes in reporter gene expression, no DEGs were found in this comparison.

The expression of positive cell-cycle regulators and RM-associated genes is decreased by cytokinin, whereas the expression of photosynthetic and SAM-associated genes is increased

Consistent with the presumed changes in identity occurring as 2iP promotes LRP → SM conversion, reduced expression of many RM/LRP-associated genes and increased expression of SM-associated genes were observed (Table S2). However, several key SAM-associated genes, including *CLV3*, did not pass the expression value cut-off. Misexpression of *CLV3* has been shown to precipitate consumption of the RAM (Fiers *et al.*, 2005), but *CLV3* reporter expression, and low expression values of *CLV3*, suggest it does not play a key role in the initial loss of LRM identity.

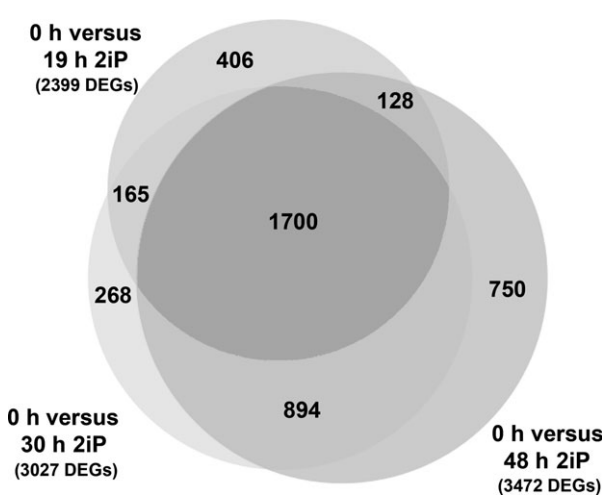


Figure 3. Distribution of differentially expressed genes (DEGs) during conversion of lateral root promordia (LRP) to shoot meristems (SMs) in a pairwise comparison of three different durations of cytokinin treatment (4.4 μM 2iP). '0 h' corresponds to 24 h of LRP-induction using 10 μM 1-naphthaleneacetic acid (1-NAA). Overlap in DEGs between time points is high, suggesting substantial common transcriptional responses to auxin → cytokinin transfer.

Unsurprisingly, transferring seedlings from high-auxin to high-cytokinin media is reflected in the increased expression of many cytokinin-responsive signaling genes, and in the reduced expression of auxin-induced signaling and metabolic genes (Tables S3 and S4, and over-represented gene ontology, GO, categories in Table S1), suggestive of an involvement in maintaining hormone signaling or metabolic homeostasis.

Cytokinins and certain cytokinin-signaling components promote differentiation of chloroplasts and expression of photosynthetic genes (Schmulling *et al.*, 1997; Argyros *et al.*, 2008), and all photosynthesis-related plant-encoded DEGs were found to be increased in steady-state transcript levels by exogenous 2iP in our study (over-represented GO categories in Table S1).

The reduced expression of type-A and -B cyclins and cyclin-dependent kinases, and the increased expression of three cyclin-dependent kinase inhibitors (Table S5), suggests a reduction in cell division/numbers of dividing cells on 2iP. Reduced expression of other positive regulators of cell division, such as *EF2a*, and decreased expression of many histones (e.g. S-phase marker *HIS4*; Table S5) support this interpretation. These observations are consistent with studies showing that cytokinins inhibit cell division in RMs and LRP founder cells (Werner *et al.*, 2003; Li *et al.*, 2006; Dello Ioio *et al.*, 2007).

Gene ontology (GO) enrichment analysis revealed transcription factors were over-represented amongst DEGs in most pairwise comparisons (Table S1). This category contained 61 differentially expressed homeobox genes, some of which have known roles in meristem and organ initiation. For example, *WOX13* is dynamically expressed during RAM/LRP initiation, and showed decreased expression on 2iP. Conversely, *ARABIDOPSIS THALIANA HOMEOBOX 1* (*ATH1*) and *PENNYWISE* (*PNY*) interact with *SHOOT MERISTEMLESS 1* (*STM1*) in the SAM, and their expression was increased.

Comparison with callus-based regeneration reveals an overlap with DEGs identified in LRP→SM conversion

To identify potential key regulators of shoot regeneration common to different *in vitro* systems, we compared targets identified in studies of shoot organogenesis from callus with LRP → SM conversion. Che *et al.* (2006) analysed transcriptome changes during root or shoot organogenesis from callus, and described the 'top-20' DEGs with increased/decreased expression during callus induction, or subsequent shoot or root induction. Of the

top-20 DEGs identified during presumed commitment to shoot organogenesis, 11 and 12 of the genes with increased and decreased expression, respectively, also appeared amongst the DEGs identified in our study, with similar patterns of expression (Table S6).

We then surveyed genes previously identified as affecting shoot organogenesis for differential expression during LRP → SM conversion (Table S7). One of these, *HLS1/COP3*, is a negative regulator of callus-based shoot regeneration (Chatfield and Raizada, 2008), and exhibits >14-fold decrease in expression levels on 2iP in our study (Table S7). Interestingly, *RSM1* overexpression phenocopies loss of *HLS1* function (Hamaguchi *et al.*, 2008), and *RSM1* expression was increased more than eightfold on 2iP.

WUS is required for LRP → SM conversion in the high-throughput system

Misexpression of *WUS* is sufficient to generate ectopic shoots in root tissue (Gallois *et al.*, 2004), and is functionally required for shoot organogenesis from root-derived callus (Gordon *et al.*, 2007). We have tested the effect of three *wus* loss-of-function mutant alleles (Methods S1) in the high-throughput LRP–SM system, and found an absolute requirement for a functional copy of the gene (Figure 4). Our data also suggest a positive gene dosage effect of *WUS* on the numbers of SMs generated (Figure 4).

Transcriptome analysis of *wus* mutants identifies *WUS*-responsive DEGs

To further examine the role of *WUS* in LRP → SM conversion, we compared transcriptomes of loss-of-function *wus* mutants with the wild type (WT). We first compared a reported *wus* null allele, SAIL_150_G06 (McElver *et al.*, 2001; Sonoda *et al.*, 2007), with WT using the aforementioned 2iP treatment periods. Three biological replicates were recorded with Affymetrix ATH1 microarrays, and MAS5 processed data analysed using the LIMMA (linear models for microarrays) package (Smyth, 2005). A significance cut-off of $P < 0.05$, a minimum fold change >1.5 and a minimum expression value of 50 yielded 543 DEGs in total.

Figure 5 shows the distribution and overlap of the DEGs identified, and a summary of over-represented GO categories (details Tables S8 and S9). Using our lenient selection criteria, between 63 and 121 genes were found to have increased or decreased steady-state transcript levels in the mutant at each time point. So, although the developmental consequences of *wus* loss of function are dramatic, the perturbation of gene expression was small compared with that associated with the hormone treatments used to induce LRP and conversion. Additionally, unlike our WT time course, very little overlap was found between time points in terms of DEGs identified, suggesting that examining downstream consequences of *wus* loss of function successfully focused on transcriptome changes relevant to discrete stages in *WUS*-dependent development.

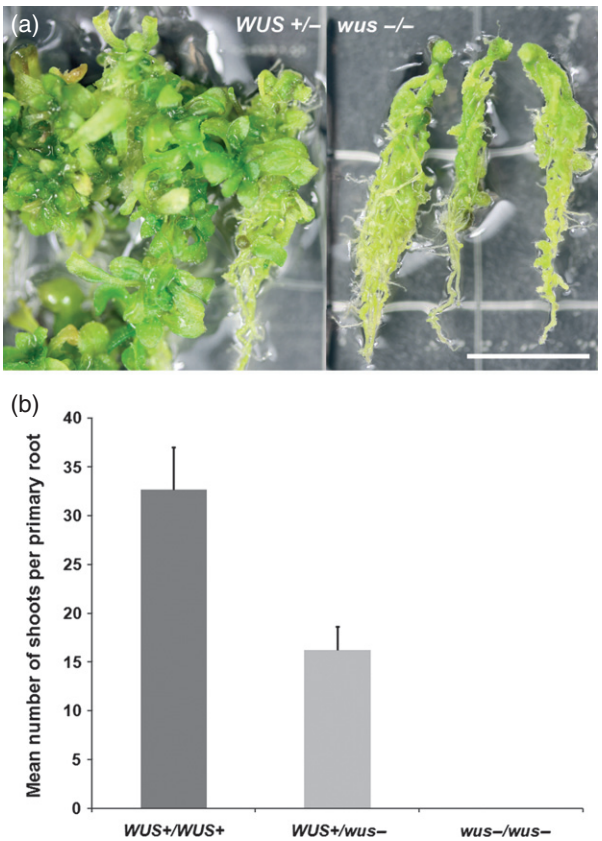


Figure 4. Shoot induction via conversion of lateral root promordia (LRP) to shoot meristems (SMs) is dependent upon *WUS*.
(a) A 10-day-old seedlings transferred to 10 μM 1-naphthaleneacetic acid (1-NAA) for 24 h, and then to 4.4 μM 2iP for 21 days. *WUS* homozygous and heterozygous plants (on left) regenerated a mass of shoots, whereas homozygous *wus1* mutants did not generate any shoots. Scale bar: 10 mm.
(b) *WUS* gene dosage and LRP → SM conversion, wild type (WT) versus *wus* loss-of-function alleles. The number of shoots induced on the primary root of seedlings after 7 days of treatment with 4.4 μM 2iP was measured ($n = 35$). Error bars indicate standard errors.

After 30–48 h of 2iP treatment, the *WUS* reporter was consistently expressed in WT cLRP and the most relevant transcriptome differences were anticipated at these times. Amongst the DEGs identified in these comparisons, the most over-represented GO categories included the biological processes of post-embryonic development, apotosis, post-translational protein modification, glucoside biosynthesis, phenylpropanoid biosynthesis and responses to light. This output resembles the groups of GO categories described by Busch *et al.* (2010) in a genomic study identifying *WUS*-responsive genes: namely, the regulation of development (including meristem and cell death), metabolism (including glucosinolate) and response to stimuli. Furthermore, the distribution of *WUS*-repressed and -induced genes described by Busch *et al.* (2010) amongst

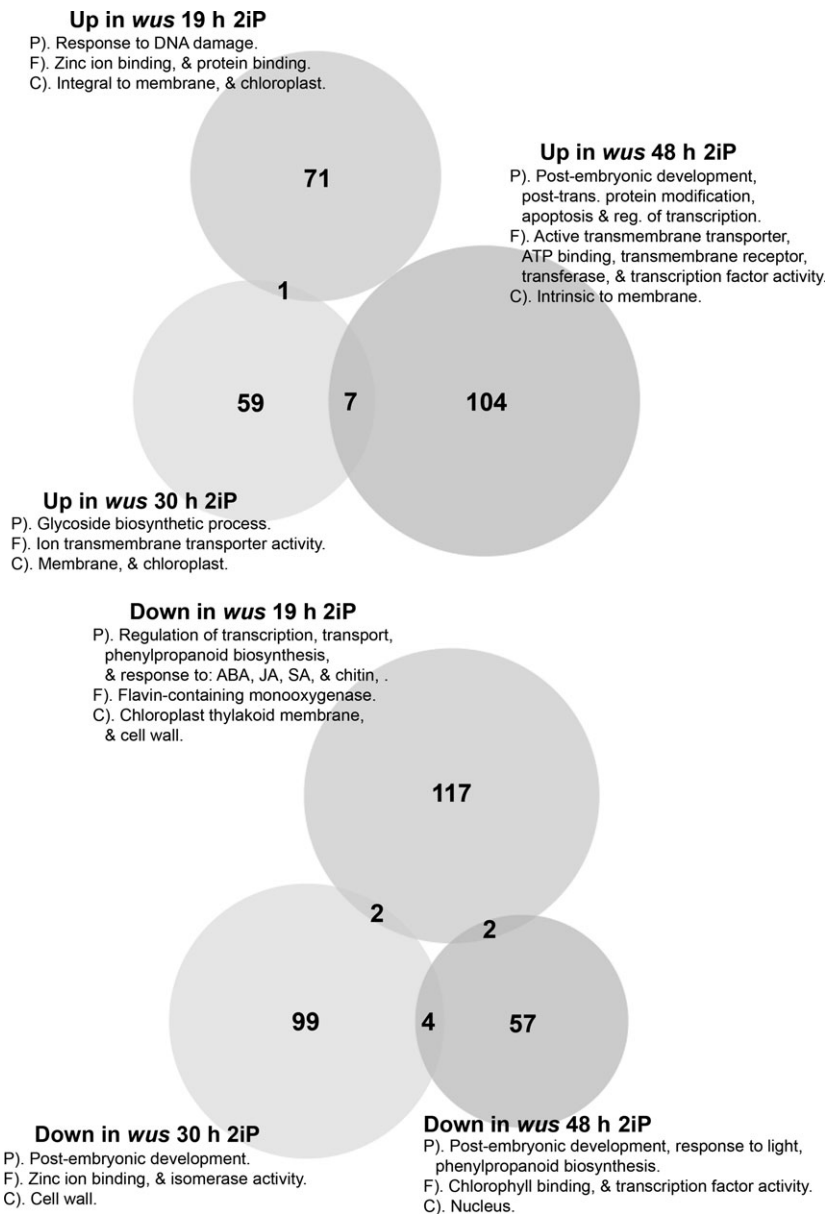


Figure 5. Distribution of differentially expressed genes (DEGs) in a *wus* loss-of-function mutant during cytokinin-induced conversion of lateral root promordia (LRP) to shoot meristems (SMs) and select gene ontology (GO) terms enriched at each time point.
Compared with the transcriptome changes associated with the treatments driving LRP → SM conversion (Figure 3), those associated with loss of *WUS* function during this process were small, and the overlap in DEGs between time points is relatively low. This suggests we have identified discrete patterns of *WUS*-dependent gene expression associated with each developmental stage.

the DEGs we identified (Figure 6) suggests that our approach has been successful in identifying a subset of *WUS*-responsive DEGs during LRP → SM conversion. After 19 h of 2iP treatment, *WUS* reporter expression is weak and sporadic in tissues around cLRP, and the ratio of *WUS*-repressed and -induced genes amongst DEGs suggests *WUS* loss of function has not yet directly affected the transcription of targets (Figure 6). Conversely, after 30 h of 2iP treatment the proportion of *WUS*-repressed genes amongst the DEGs with higher expression in *wus* increases to more than fivefold that of *WUS*-induced genes, suggesting that the loss of *WUS* has permitted elevated expression of these genes. Conversely, although the number of *WUS*-repressed genes has decreased amongst DEGs with reduced expression in *wus* at this time, they outnumber *WUS*-induced genes. However, after a further 18 h, *WUS* expression within WT cLRP indicates that >11% of the DEGs with reduced expression in *wus* belong to the *WUS*-induced group, and none to the *WUS*-repressed group (Figure 6). Furthermore, after 48 h of 2iP treatment

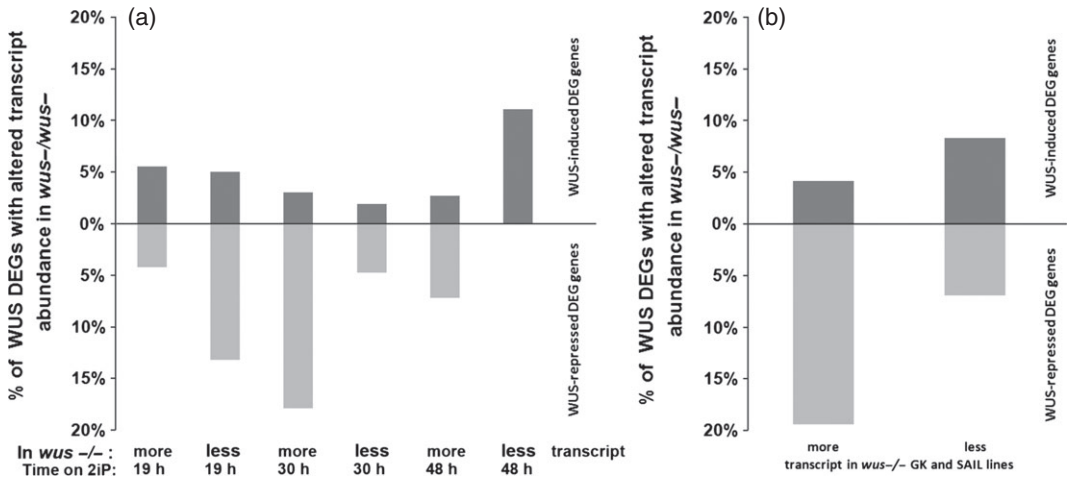


Figure 6. Proportions of *WUS*-repressed and *WUS*-induced genes (Busch *et al.*, 2010) amongst differentially expressed genes (DEGs) identified in transcriptome analysis of *wus* loss-of-function mutants during conversion of lateral root promordia (LRP) to shoot meristems (SMs) are consistent with rapid transcriptional responses to *WUS* expression in cLRP.

(a) A comparison of a *wus* loss-of-function mutant (SAIL_150_G06) with the wild type (WT). *WUS* reporter expression was confined to scattered cells outside cLRP after 19 h of treatment with 2iP, and ratios of *WUS*-induced and *WUS*-repressed genes amongst the DEGs suggests *WUS*-responsive gene expression has not been perturbed. After 30 h of treatment with 2iP, *WUS* reporter expression was found within cLRP, and *WUS*-repressed genes now represent the majority of DEGs with increased expression in *wus* mutants, and made a reduced contribution to DEGs with lower expression in the mutant. After 48 h of treatment with 2iP, no *WUS*-repressed genes were found amongst DEGs with lower expression in *wus*.

(b) In an additional comparison of two *wus* loss-of-function mutants with WT after 30 h of treatment with 2iP, *WUS*-repressed genes again outnumbered *WUS*-induced genes amongst DEGs with increased expression in *wus*, and *WUS*-induced genes outnumber *WUS*-repressed genes amongst DEGs with lower expression in *wus*.

the proportion of *WUS*-repressed genes amongst DEGs with increased expression in *wus* remained higher than *WUS*-induced genes. In addition, we surveyed promoter and intron sequences of DEGs for *cis*-element sequences bound by WUS (Lohmann *et al.*, 2001; Busch *et al.*, 2010), and found them to be over-represented amongst DEGs after 48 h of 2iP. Two or more instances of the 6-bp sequence CACGTG (Busch *et al.*, 2010) were found within 500 bp upstream of 5.4% (1.7% expected, $P = 0.003$) of the DEGs with increased expression in *wus*, and two or more instances of the sequence TTAATSS (Lohmann *et al.*, 2001) were found within the introns of 7.94% (3.45% expected) of DEGs with decreased expression in *wus*, although the latter observation was not deemed significant ($P = 0.055$).

Thus, for a subset of genes the effect of *WUS* upregulation within cLRP seems broadly consistent with published findings on the regulation of gene expression by *WUS*. In addition, we have identified many targets not previously identified as *WUS* responsive that are differentially regulated in the loss-of-function mutant under the specific conditions associated with LRP → SM conversion (Table S8, and GO analysis in Table S9).

As might be expected, DEGs included those associated with developmental processes in SMs (Table S8), including *CUP-SHAPED COTYLEDONS 1* and *3* (*CUC1* and *CUC3*) and *BLADE ON PETIOLE 2* (*BOP2*). However, many meristem-associated genes were expressed at low levels, and despite enrichment of samples with cLRP, SAM-associated genes positively regulated by *WUS* (e.g. *CLV3*; Brand *et al.*, 2002; Yadav *et al.*, 2011), or repressed by *WUS* (e.g. *CLV1*; Busch *et al.*, 2010), were not identified as DEGs. It may be that the relevant cell types still represent an insubstantial fraction of samples, or that our sampling precedes the significant upregulation of many meristem-associated genes.

An important role of *WUS* in meristem function is believed to be the regulation of cytokinin-inducible *ARRs*. Leibfried *et al.* (2005) used inducible misexpression to isolate *WUS*-responsive genes, and identified four type-A *ARR* genes (*ARR5*, *ARR6*, *ARR7* and *ARR15*) as *WUS*-repressed. In contrast, no *ARR* genes were amongst DEGs with higher expression in *wus*, and few cytokinin-related targets were identified as differentially expressed after 30–48 h of treatment with 2iP (Table S8), from which it is difficult to infer an outcome upon cytokinin-signaling output. This discrepancy may reflect different tissues sampled, and cytokinin treatments masking the impact of *WUS* on *ARR* expression in our study.

To identify associations and putative functional relationships between DEGs, cluster analysis was performed and each cluster analyzed for over-represented GO terms. The DEGs were grouped into 18 clusters by *k*-means clustering, seven of which yielded statistically over-represented GO terms ($P < 0.05$ Hochberg false discovery rate; Figure S1; Table S10). Although many GO categories overlapped with those identified in our time-point comparison (Table S9), several interesting new groups were highlighted. These included: lipase activity (cluster 5), nuclear protein import (cluster 6) and, endo-1,4-β-xylanase activity (cluster 7). Endo-1,4-β-xylanases are associated with cell expansion and shape changes, and inclusion of three (of five on the

ATH1 microarray) within cluster 7 suggests *WUS,* or dependent processes, reduce these activities.

After 30 h of treatment with 2iP, we hoped to identify early events in *WUS*-dependent LRP → SM conversion. To provide greater resolution of *WUS*-related DEGs at this time point, we examined the transcriptome of another *wus* mutant allele (GABI_870H12). GABI-KAT constructs were designed for activation tagging, but in this line an intragenic insertion appears to drive elevated expression of a truncated non-functional product (Methods S1). Heterozygotes yield loss-of-function *wus* phenotypes in approximately 25% of progeny, and these homozygous mutants are unable to undergo LRP → SM conversion. For comparison of the two alleles with the WT, processed data were filtered to remove genes absent in one *wus* mutant allele, but not the other. Using the LIMMA package of BIOCONDUCTOR, a *P*-value threshold of <0.05 and a minimum fold change of 1.5 in one genotype, 144 DEGs similarly regulated in both *wus* mutant alleles were identified (Table S11). Of these initial DEGs, 21% overlapped with those identified in our *wus* SAIL/WT comparison. Applying the fold change cut-off to both alleles increased overlap to 37%, comprising 25 and 48% of DEGs up- or downregulated in a *wus* mutant background, respectively. The observed differences in overlap between genes with increased or decreased levels of expression could reflect differences in the function of the mutant gene products, but as both mutant alleles seem functionally similar in terms of LRP → SM conversion, the subset of mutual DEGs appears to offer stronger candidates for genes mediating *WUS*-dependent LRP → SM development. Consistent with this view, the proportion of *WUS*-induced to *WUS*-repressed genes amongst DEGs with reduced expression in *wus* increased in this two-allele comparison (Figure 6).

Insertional knock-outs in WUS-responsive candidates affect LRP → SM conversion

To explore the roles of putative *WUS*-responsive targets identified by transcriptome analysis, we refined selection criteria to test insertional knock-outs of promising targets. To enrich for potential direct targets of WUS, we surveyed <1 kb upstream of the *wus* mutant DEGs for two or more instances of sequences corresponding to putative WUS-binding *cis*-elements: CACGTG and TTAATSS. As functional TTAATSS sequences were originally identified within an intron, we included DEGs with two or more instances within introns. These candidates were then surveyed with the Arabidopsis eFP Browser (Winter *et al.*, 2007) for genes displaying differential expression in the SAM (Yadav *et al.*, 2009) or embryo (Casson *et al.*, 2005). Initially, 32 *wus* mutant DEGs were selected, of which 28 possessed corresponding T-DNA insert lines. Homozygous lines were assayed for LRP → SM conversion by scoring the numbers of shoots initiated on seedling roots treated with 4.4 or 2.2 μM 2iP for 5–7 days. The lower cytokinin concentration was included to screen for enhanced shoot-induction rates. A total of 39 homozygous insertion lines, corresponding to 27 genes, were tested in at least two replicates (Figure 7; Table S12). Four of these lines, corresponding to four different DEGs with reduced expression in *wus* during LRP → SM conversion, showed a consistent reduction in shoot initiation rates (Figure 7). All mutants appear phenotypically normal, apart from their deficit in shoot initiation. However, further work will be required to determine the relevance of the mutations to *WUS*-dependent processes and LRP → SM conversion.

Cell-specific profiling of the *WUSCHEL* domain of cLRP

Cell-specific expression profiling has been used to explore gene expression within specific domains of RMs (Birnbaum *et al.*, 2003; Brady *et al.*, 2007; Gifford *et al.*, 2008) and SMs (Yadav *et al.*, 2009), with improved sensitivity and resolution in relating transcriptome changes to development and responses to stimuli. Although our samples were enriched with cLRP, the relative contribution by key cell types/domains may be insufficient to resolve key genes, illustrated by low *WUS* expression values. We therefore isolated and profiled *pWUS::mGFP5-ER* cells to compare expression in the *WUS* domain of cLRP with the *WUS* domain of established SMs.

Tissues harvested after 30 h of treatment with 2iP were protoplasted rapidly (1 h) and cells expressing *pWUS::mGFP5-ER* were isolated with a fluorescence-activated cell sorter (FACS). Isolated RNA underwent two cycles of amplification and transcriptomes were recorded with Affymetrix ATH1 arrays. Previous studies, using RM and SM cells, have identified genes that respond to protoplasting with changes in expression (Birnbaum *et al.*, 2003; Yadav *et al.*, 2009), and these targets were removed from comparisons.

The mean expression value of *WUS* in *pWUS::mGFP5-ER* cells was about sevenfold higher than the whole root, suggesting successful enrichment for *WUS*-expressing cells, but was lower than that obtained for the *WUSp* domain of SAMs (Yadav *et al.*, 2009). This latter observation was expected because the expression of transcriptional reporters for *WUS* only began in cLRP at this time.

Pearson correlation coefficients of 0.97–0.99 for comparisons indicate a high level of reproducibility between our *pWUS::mGFP5-ER* replicates (Table S13). Lower correlation coefficients (0.513–0.596) were found between *pWUS::mGFP5-ER* cells in our experiments and those isolated by Yadav *et al.* (2009). In addition to the likely differences in expression arising from harvesting *WUS*-expressing cells from different organs, low correlation values probably reflect differing culture conditions, particularly the high concentrations of hormones used in our experiments.

Next we identified genes differentially expressed within *pWUS::mGFP5-ER* cells from cLRP compared with whole

(a)

AGI ID	Annotation	Lines tested	*WUS* cis-elements	Expression	SAM / embryonic expression?
At2g28090	Heavy metal transport/detoxification superfamily protein	SALK_028133C (exon)	2 TTAATSS 500 bp upstream (375 and 460 bp)	Down in *wus* SAIL and GK after 30 h 2iP	Embryonic expression. Globular stage basal and torpedo stage root (Casson *et al*. 2005)
At1g04230	Protein of unknown function (DUF2361)	SALK_006107C (promoter)	2 TTAATSS in introns, 1 TTAATSS 1 kb upstream (867 bp),	Down in *wus* SAIL and GK after 30 h 2iP	Higher expression in rib-meristem and stem cell zones. (pCLV3. Yadav *et al*. 2009)
At1g01260	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	SALK_150281C (promoter)	2 TTAATSS in 1 kb up stream (68 and 566 bp) and 2 CACGTG in 500 bp upstream (238 & 249 bp)	Down in *wus* SAIL and GK after 30 h 2iP	Higher expression in rib-meristem (pWUS. Yadav *et al*. 2009)
At3g62600	ATERDJ3B_ERDJ3B__ DNAJ heat shock family protein	SALK_055599C (exon)	1 CACGTG (900 bp up stream). 3 TTAATSS within 1 kb upstream (743, 807 & 938 bp). 2 TTAATSS in introns	Down in *wus* SAIL after 19 & 30 h 2iP	Not meristem or embryo associated expression.

(b)

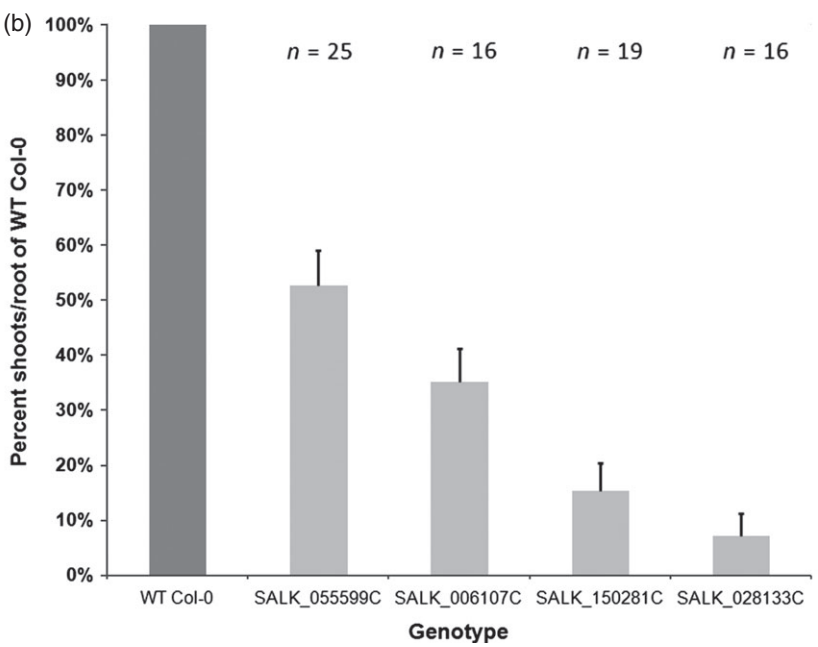
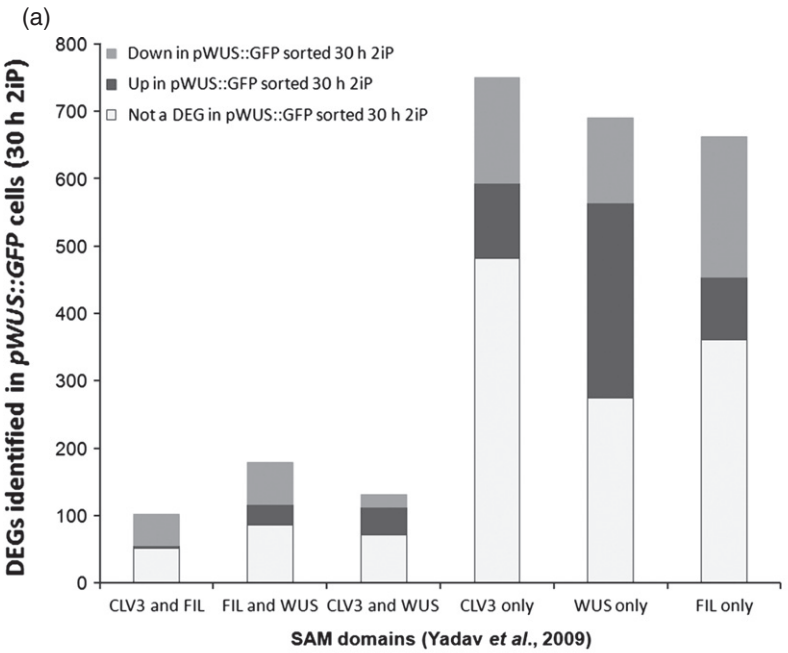


Figure 7. T-DNA insertion lines corresponding to four candidate *WUS*-responsive targets identified from transcriptome analysis of *wus* loss-of-function alleles show reduced conversion of lateral root promordia (LRP) to shoot meristems (SMs) compared with the wild type (WT), as scored by shoot induction rates. (a) Homozygous T-DNA insertional mutants were also chosen based on the presence of potential WUS-binding *cis*-elements, and differential expression in SMs or embryonic domains.
(b) Shoot induction from seedling primary roots after 24 h of treatment with 10 μM 1-naphthaleneacetic acid (1-NAA), followed by 6 days of 4.4 μM 2iP. Shoots were scored after 5–7 days of cytokinin treatment.

root samples, and examined how these genes were distributed amongst those assigned to SAM domains (Yadav *et al*., 2009). Figure 8 shows that of the genes previously assigned to each SAM domain, the highest proportion of overlap with the DEGs from *pWUS*::*mGFP5-ER* cells is the *WUSp* domain of the SAM at 60.3%, compared with 35.7 and 45.5% for *CLV3p* and *FILAMENTOUS FLOWER* (*FILp*) domains, respectively. Moreover, a higher proportion of the overlapping genes in the *WUS* SAM domain are DEGs, with higher expression in *pWUS*::*mGFP5-ER* cells from cLRP: 42.0%, compared with 14.8 and 14.1% in *CLV3* and *FIL* domains, respectively. This suggests that within hours of initiating *WUS* reporter expression in cLRP, the transcriptome of these cells began to resemble the*WUSp* domain of an SAM.

Examining the distribution of sequences associated with *WUS cis*-elements we found the 6-bp sequence CACGTG was significantly over-represented 500–1000 bp upstream



(b)

	Total DEGs	1 or more CACGTG 1 kb upstream		2 or more CACGTG 500 bp upstream		2 or more TTAATSS within introns		2 or more TTAATSS 500 bp upstream		1 or more TTAATSS 1 kb upstream	
		Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected
Up in *pWUS::mGFP5-ER 30 h 2iP*	3927	457	326.24	53	36.27	75	72.35	27	25.58	537	549.93
		$P = 4.5E{-}13$		$P = 4.5E{-}3$		NS		NS		NS	
Down in *pWUS::mGFP5-ER 30 h 2iP*	2097	738	611.04	82	67.92	157	135.67	56	48.1	1011	1029.85
		$P = 2.81E{-}7$		NS ($P = 0.087$)		NS ($P = 0.056$)		NS		NS	

Figure 8. (a) Distribution of genes assigned to shoot meristem (SM) domains (Yadav *et al.*, 2009) amongst differentially expressed genes (DEGs) from *pWUS::mGFP5-ER* sorted cells from converting lateral root primordia (cLRP; 30 h 2iP). The greatest overlap is with the *WUSp* domain of the SM (60.3%), compared with *CLV3p* (35.7%) and *FILp* (45.5%) domains.
(b) Occurrence of potential WUS-binding *cis*-element-associated sequences, TTAATSS (Lohmann *et al.*, 2001) and CACGTG (Busch *et al.*, 2010), within promoters and introns of DEGs indentified in sorted *pWUS::mGFP-ER* cells from cLRP. Significant enrichment for CACGTG sequences was found within 1 kb upstream of DEGs.

of DEGs from *pWUS::mGFP5-ER* cells in cLRP (Figure 8). Two or more TAATTSS sequences within introns were also observed at a higher frequency than expected, but the cut-off of $P < 0.05$ was not met ($P = 0.056$). Enrichment of DEGs from this domain with elements mediating transcriptional responses to *WUS* is consistent with rapid transcriptional responses to *WUS* expression within cLRP.

We then examined the distribution *WUS*-responsive genes identified in our transcriptome analysis of *wus* mutants, and those identified by Busch *et al.* (2010), amongst DEGs from *pWUS::mGFP5-ER* cells in cLRP. Interestingly, genes found to have increased expression in *pWUS::mGFP5-ER* cells were enriched amongst those found to have increased expression in *wus* (Figure 9a). Conversely, those with decreased expression in *pWUS::mGFP5-ER* cells were enriched amongst those with decreased expression in *wus* (Figure 9a). Similarly, *WUS*-induced and *WUS*-repressed genes (Busch *et al.*, 2010) were found to be enriched amongst those with decreased and increased expression, respectively, in *pWUS::mGFP5-ER* cells (Figure 9b). These findings mirror those of Busch *et al.* (2010), who found *WUS*-repressed genes were enriched within the *WUS* domain of the SAM, whereas *WUS*-induced genes were enriched among transcripts expressed in the combined *CLV3* and *WUS* domains, and combined *CLV3* and *FIL* domains. It was suggested that this finding reflected a contribution by both direct and indirect targets amongst *WUS*-responsive genes, and linked the prevalence of transcripts with reduced expression in the *WUS* domain to evidence that WUS acts primarily as a transcriptional repressor (Leibfried *et al.*, 2005; Ikeda *et al.*, 2009), modulating target gene expression. Our findings are in line with these hypotheses, and may also reflect the duration of *WUS* expression in cLRP and WUS non-cell autonomous activity (Mayer *et al.*, 1998; Gallois *et al.*, 2004; Yadav *et al.*, 2011).

To characterize transcriptome differences between *WUS* domains within established SAMs and within cLRP we compared *pWUS::mGFP5-ER* cells in our experiment with

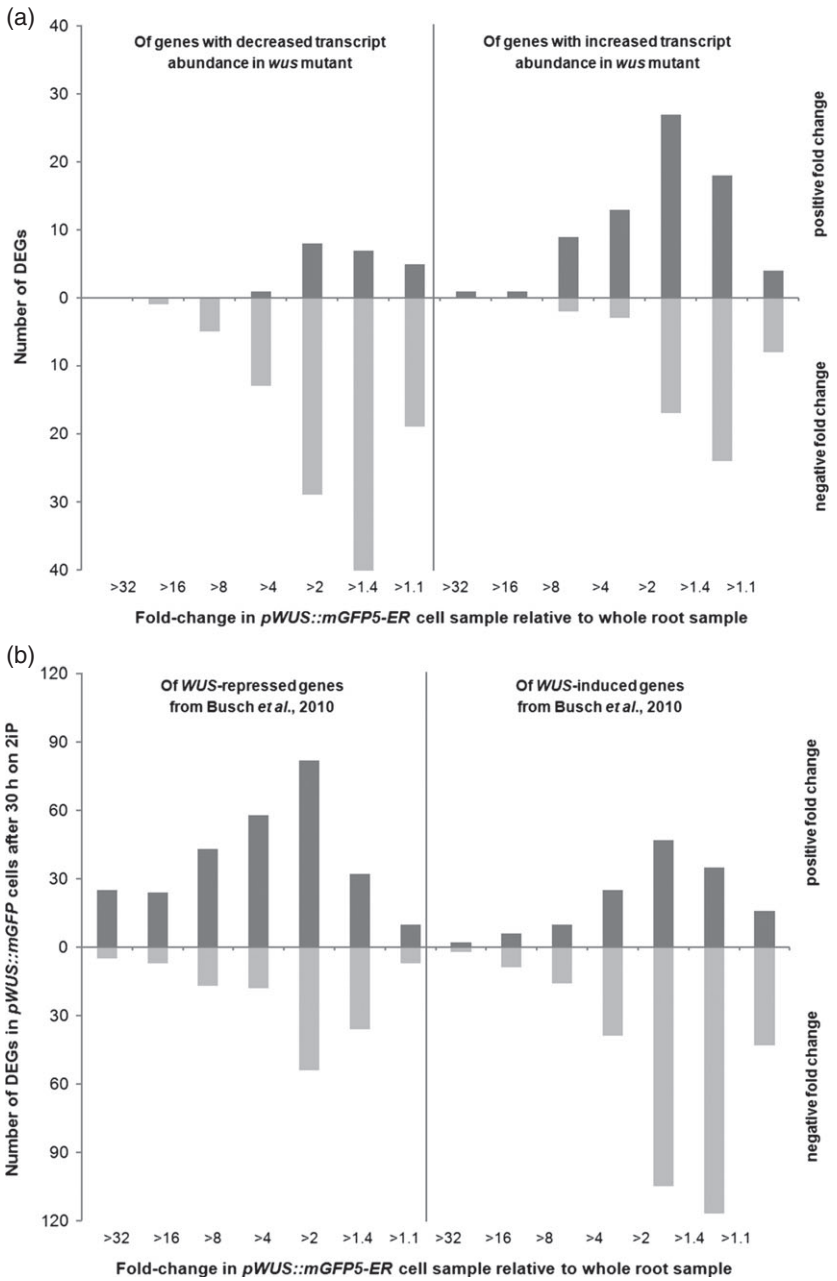


Figure 9. *WUS*-responsive genes amongst differentially expressed genes (DEGs) identified in *pWUS*::*mGFP5-ER* cells from converting lateral root primordia (cLRP).
(a) Genes with decreased expression in *pWUS*::*mGFP5-ER* cells were enriched amongst those with decreased transcript levels in *wus* mutants. Conversely, genes found to have increased expression in *pWUS*::*mGFP5-ER* cells were enriched amongst those found to have increased transcript levels in the *wus* mutants.
(b) Distribution of DEGs from *pWUS*::*mGFP5-ER* cells in cLRP amongst *WUS*-repressed and *WUS*-induced genes (Busch *et al.*, 2010). *WUS*-repressed genes were enriched amongst DEGs with increased expression in *pWUS*::*mGFP5-ER* cells, whereas *WUS*-induced genes were enriched amongst DEGs with decreased expression in *pWUS*::*mGFP5-ER* cells. Fold-change categories are not cumulative.

WUSp SAM cells (Yadav *et al.*, 2009). *pWUS*::*mGFP5-ER*-associated DEGs with more than twofold difference in expression comprised 1224 DEGs with elevated expression in cLRP, compared with *WUSp* SAM, and 1452 DEGs with lower expression (Table S14). GO analysis (Tables 1 and S15) revealed the enrichment of various 'response to stimuli' terms, including the responses to hormones (ABA, ethylene, cytokinin and auxin), which may reflect the exogenous hormones applied in our study. Differential over-representation of root versus shoot developmental terms (Table 1) amongst DEGs from cLRP is consistent with continuing conversion of the treated root tissues. Over-representation of the GO categories for meristem initiation, and meristem structural organization amongst DEGs with

Table 1 Selected enriched gene ontology (GO) terms amongst differentially expressed genes (DEGs) identified in the transcriptome of fluorescence-activated cell sorted (FACS) cells expressing *pWUS::mGFP-ER* from lateral root primordia (LRP) undergoing conversion to shoot meristems (SMs; after 30 h of treatment with 2iP), compared with the *WUSp* expression domain of *apetala1/cauliflower* double mutant SMs (Yadav *et al.*, 2009). GO analysis was performed using AGRIGO (http://bioinfo.cau.edu.cn/agriGO)

GO acc	Term type	Term	Query item	Query total	bg item	bg total	*P* value	Yekutieli FDR
Selected enriched GO terms. DEG >2 fold UP in *pWUS::GFP* 30 h 2iP versus SAM *WUSp* (Yadav *et al.*, 2009)								
GO:0042221	Biological process	Response to chemical stimulus	188	1224	1684	22 479	1.90E-18	7.40E-15
GO:0006950	Biological process	Response to stress	175	1224	1766	22 479	6.60E-13	6.50E-10
GO:0009407	Biological process	Toxin catabolic process	17	1224	44	22 479	5.90E-11	3.30E-08
GO:0010876	Biological process	Lipid localization	10	1224	15	22 479	5.60E-10	2.40E-07
GO:0009725	Biological process	Response to hormone stimulus	73	1224	687	22 479	1.10E-07	2.50E-05
GO:0016137	Biological process	Glycoside metabolic process	15	1224	101	22 479	4.00E-04	4.70E-02
GO:0000302	Biological process	Response to reactive oxygen species	10	1224	52	22 479	4.50E-04	5.20E-02
GO:0009791	Biological process	Post-embryonic morphogenesis	5	1224	26	22 479	1.20E-02	5.40E-01
GO:0007568	Biological process	Aging	9	1224	71	22 479	1.50E-02	6.20E-01
GO:0048364	Biological process	Root development	15	1224	170	22 479	4.60E-02	1.00E+00
Selected enriched GO terms. DEG >2 fold DOWN in *pWUS::GFP* 30 h 2iP versus SAM *WUSp* (Yadav *et al.*, 2009)								
GO:0009791	Biological process	Post-embryonic development	83	1452	501	22 479	1.20E-14	5.90E-11
GO:0001510	Biological process	RNA methylation	5	1452	6	22 479	6.40E-06	5.30E-03
GO:0048367	Biological process	Shoot development	34	1452	240	22 479	1.70E-05	8.90E-03
GO:0034660	Biological process	ncRNA metabolic process	23	1452	136	22 479	2.30E-05	9.40E-03
GO:0051641	Biological process	Cellular localization	52	1452	450	22 479	5.20E-05	1.60E-02
GO:0048366	Biological process	Leaf development	24	1452	156	22 479	7.50E-05	1.80E-02
GO:0010014	Biological process	Meristem initiation	5	1452	9	22 479	1.10E-04	2.20E-02
GO:0060918	Biological process	Auxin transport	10	1452	44	22 479	4.20E-04	5.00E-02
GO:0009933	Biological process	Meristem structural organization	9	1452	40	22 479	8.70E-04	8.20E-02
GO:0034470	Biological process	ncRNA processing	14	1452	83	22 479	8.70E-04	8.20E-02
GO:0009908	Biological process	Flower development	30	1452	257	22 479	1.40E-03	1.20E-01
GO:0016570	Biological process	Histone modification	9	1452	46	22 479	2.50E-03	1.70E-01
GO:0007049	Biological process	Cell cycle	25	1452	275	22 479	3.30E-03	2.10E-01
GO:0009765	Biological process	Photosynthesis, light harvesting	5	1452	18	22 479	4.80E-03	2.60E-01
GO:0010228	Biological process	Veg. to reprod. phase transition of meristem	11	1452	71	22 479	5.80E-03	2.90E-01
GO:0009790	Biological process	Embryonic development	37	1452	376	22 479	8.20E-03	3.60E-01

Singular enrichment analysis was used with Fisher's statistical test, Yekutieli multi-test adjustment and a minimum significance level of $P < 0.05$.

lower expression in the *WUS* domain of cLRP, may also reflect the transitional state of these organs.

Meristem initiation/organization includes targets interacting with WUS, or transcriptionally modulated by it. *TOPLESS* (*TPL*) and *TOPLESS-RELATED 4* (*TPR4*) encode for transcriptional co-repressors that interact with WUS (Kieffer *et al.*, 2006). Expression of *TPL* is enhanced by WUS (Busch *et al.*, 2010), and directly targets *PLETHORA 1* and 2 (*PLT1* and *PLT2*) genes (Smith and Long, 2010), which are master regulators promoting basal/root fate (Aida *et al.*, 2004; Galinha *et al.*, 2007). Two other *WUS*-induced meristematic genes, *STM1* and a *CYCLIN-DEPENDENT KINASE B2;1* (*CDKB2;1*) were also found to have lower expression in the newly initiated *WUS*-reporter domain. *CLV1* is a direct target of WUS, which represses *CLV1* expression (Busch *et al.*, 2010). If factors that positively regulate the expression of *CLV1* within the SAM are absent in cLRP this might account for the lower expression of *CLV1* in the *WUS* domain of these organs. As a shoot stem cell population, marked by *CLV3* reporter expression, has not developed at this time, it is unsurprising that expression of other elements of the *WUS–CLV* pathway have yet to be established, including *CLV1* and *POLTERGEIST* (*POL*) (Song *et al.*, 2006).

Other interesting enriched biological processes amongst DEGs with lower expression in cLRP included: non-coding RNA processing, RNA methylation and histone modification. The establishment of a functional shoot stem cell niche can be expected to involve coordinated regulation of transcription, and the stability and functional output of numerous interacting targets. The categories histone modification, RNA methylation and ncRNA categories may include genes mediating these processes in initiating or established SAMs that have yet to be upregulated in LRP.

DISCUSSION

In vitro shoot regeneration and adventitious shooting in diverse natural systems can occur through the conversion of LRP or RMs (Bonnet and Torrey, 1966; Peterson, 1970;

Spencer-Barreto and Duhoux, 1994; Atta *et al.*, 2009; Sugimoto *et al.*, 2010). To investigate this phenomenon we used a high-throughput methodology for synchronous LRP → SM conversion, enabling us to apply a systems approach to further understanding the process.

Characterizing reporter expression for *WUS* and *CLV3* identified important stages in the conversion of LRP into SMs, permitting transcriptome analysis to elaborate the critical role played by *WUS* in the process. The appearance of *WUS* reporter expression, prior to the initiation of a shoot stem cell population marked by *pCLV3::GFP-ER*, suggests that the upregulation of *CLV3* does not play a primary role in inhibiting LRP development and initial stages of conversion. Cell-specific profiling and comparison of *WUSp* domains in cLRP and SMs indicated that the expression of many shoot meristematic genes, including other elements of the *CLV* pathway, are not yet induced when *WUS* reporter expression is initiated in cLRP. However, the over-representation of sequences associated with *cis*-elements bound by WUS amongst DEGs suggests that upregulation of *WUS* within cLRP drives rapid changes in the transcriptional activity during conversion. Similarly rapid changes in gene expression associated with the re-specification of cell identity have been shown during regeneration of RAMs (Sena *et al.*, 2009).

Transcriptome analysis of *wus* loss-of-function mutants and cell-specific profiling permitted us to remove the background of gene expression perturbed by exogenous hormone treatments, and focus upon the DEGs reflecting important developmental transitions and the role of *WUS* in them. These studies revealed differential regulation of known *WUS*-responsive genes and identified new putative *WUS*-responsive targets associated with LRP → SM conversion. In addition to reflecting our specific culture conditions and ectopic expression of *WUS* in root tissues, it is hoped that some of these new targets may be uniquely associated with the *WUS*-dependent conversion process.

A scan for candidates with a functional role in LRP → SM conversion was enriched for potential WUS-interacting genes by cross-referencing our putative *WUS*-responsive targets with available embryo and SAM-related transcriptome data, and for enrichment with WUS-binding *cis*-element sequences. This approach identified four homozygous mutant lines that negatively impacted shoot induction rates via LRP → SM conversion. Further work will be required to determine whether the associated genes do indeed make a functional contribution to LRP → SM conversion downstream of *WUS*. Because high-throughput LRP → SM conversion is likely to be dependent upon appropriate responses to auxin and cytokinin, and other response networks affecting growth in culture, a relatively high proportion of mutations may affect the process.

Overall, we have laid important groundwork in characterizing key transitions early in the conversion of incipient root stem cell niches to SMs, and have identified targets affecting the process. The approach offers a tractable system to investigate questions pertaining to pluripotency, cell fate reprogramming and stem cell niche patterning. Historically, *in vitro* organogenesis has provided an invaluable tool for research and biotechnology. To facilitate further comparative analysis of regeneration systems, and provide a resource for the community, we have constructed a 'Regeneration' eFP browser (http://bar.utoronto.ca/efp_arabidopsis/cgi-bin/efpWeb.cgi?dataSource=Regeneration; see Figure S2 for an example visualization) for visualizing gene expression in our data sets alongside those obtained in studies of callus-based systems (Che *et al.*, 2006; Sugimoto *et al.*, 2010), cell-specific profiling (Yadav *et al.*, 2009) and RAM regeneration (Sena *et al.*, 2009).

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Arabidopsis thaliana ecotypes Columbia (CS1092), Landsberg *erecta* (CS20), line WUS_{pro}*:GFP-ER* (CS23897; Jonsson *et al.*, 2005), line WUS_{pro}*:DsRed-N7* $CLV3_{pro}$*:GFP-ER* (CS23895; Gordon *et al.*, 2007), *wus-1* (CS15) and *wus* null allele SAIL_150_G06 (CS807292), and all SALK and SAIL T-DNA mutant lines (Figure 7; Table S12), were provided by the Arabidopsis Biological Resource Center (ABRC, http://abrc.osu.edu), and *wus* GABI-KAT allele (GABI_870H12) was provided by the Nottingham Arabidopsis Stock Centre (NASC, http://arabidopsis.info).

Seedlings were grown aseptically on 200-μm Nitex mesh sheets (Sefar, http://www.sefar.com) upon phytogel (Sigma-Aldrich, http://www.sigmaaldrich.com) solidified media (2 g L^{-1}) containing half-strength MS salts, 4.5 mM 2-(*N*-morpholine)-ethanesulphonic acid (MES) and 1% sucrose, pH 5.7. Hormone induction media contained full-strength Gamborg's B5 with vitamins (*Phyto*Technology Laboratories, http://www.phytotechlab.com), 20 g L^{-1} glucose, 0.5 g L^{-1} MES and 2 g L^{-1} Phytagel, pH 5.8, amended with 1000 × stocks of either 1-NAA or 2iP.

Seedlings were transferred on Nitex from half-strength MS plates at 3–4 days after germination to plates containing 10 μM NAA for 24 h to induce LRP, then transferred to 4.4 or 2.2 μM 2iP to promote LRP → SM conversion. For screening mutants and testing *wus* alleles, the numbers of shoots were scored after 5 and 7 days of 2iP treatment. Shoots were defined as three or more leaves initiated in a radial pattern around a presumed SM.

Microscopy

Images were acquired with a Leica upright DM 6000CS microscope connected to a TCS SP5 system (Leica, http://www.leica.com). Argon (50 mW) and GreenHeNe (1.2 mW) lasers were used for excitation. Maximal projections of *z*-stacks are shown.

Protoplasting and fluorescence-activated cell sorting (FACS)

Protoplasting and sorting were performed according to the methodology described by Birnbaum *et al.* (2005) using the enzyme mix described by Yadav *et al.* (2009). Approximately 250 seedling roots were harvested within 5 min, sliced and placed in protoplasting solution for 1 h. Between 1000 and 25 000 protoplasts were sorted

into 350 μl of reverse transcriptase (RT) buffer (RNA Easy kit; Qiagen, http://www.qiagen.com) within 10 min, and flash frozen.

Microarray experiments and analysis

The WT, mutant analyses and cell-specific experiments were performed in triplicate, and RNA was extracted from 250 treated seedling roots pooled for each sample. Roots were removed with a scalpel blade across the Nitex mesh and flash frozen. Time points sampled included: 0, 19, 30 and 48 h 2ip treatment. RNA was extracted from seedling roots or sorted protoplasts using an RNA Easy kit (Qiagen). RNA extracted from sorted protoplasts underwent amplification using the GeneChip® IVT Express Kit (Affymetrix, http://www.affymetrix.com). For each sample, 5 μg of total RNA was reverse transcribed (SuperScript II; Invitrogen, http://www.invitrogen.com), labelled and hybridised to the Arabidopsis ATH1 Genome Array (Affymetrix). Data were pre-processed using MAS5/GCOS (Hubbell *et al.*, 2002) implemented in R (R Development Core Team, 2011) and BIOCONDUCTOR (Gentleman *et al.*, 2004), with a TGT value of 100. Expression data were filtered to remove probe sets reporting low transcript abundances (mean expression value <50, approximately 2.5-fold higher than the background). Differentially expressed genes were identified by raw *P* value and fold change, or by contrasts made using linear models for microarrays (LIMMA) (Smyth, 2005) implemented in R/BIOCONDUCTOR (Gentleman *et al.*, 2004). See Methods S1 for the quantitative real-time PCR (qRT-PCR) validation of arrays.

GO enrichment analysis

Singular enrichment analysis of DEGs was performed using agriGO (http://bioinfo.cau.edu.cn/agriGO; Zhou *et al.*, 2010), using Fisher as the statistical test method, Hochberg (false-discovery rate) or Yekutieli (false-discovery rate under dependency) as the multi-test adjustment method, and minimum mapping entries were set at three or five, and ATH1 was used as the background.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Patterns of relative mean expression values in the wild type (WT) and *wus* mutant (accession number SAIL_150_G06) during LRP–SM conversion, corresponding to nine clusters (of 18 generated by *k*-means clustering) significantly enriched for different GO terms.

Figure S2. The 'Regeneration' eFP Browser, a bioinformatic tool for comparing gene expression patterns in different *in vitro* regeneration systems.

Methods S1. Quantitative real-time PCR (qRT-PCR) validation of ATH1 arrays.

Table S1. Over-represented gene ontology (GO) categories amongst genes differentially expressed in wild type (Col-0) during cytokinin-induced LRP → SM conversion.

Table S2. Expression of selected root and shoot meristematic genes in seedling primary roots treated to promote LRP → SM conversion.

Table S3. Cytokinin signaling and cytokinin metabolic genes differentially expressed during LRP → SM conversion.

Table S4. Auxin-related genes differentially expressed during LRP → SM conversion.

Table S5. Cell cycle-related and chromatin-related DEGs identified in transcriptomic analysis of cytokinin-induced LRP → SM conversion.

Table S6. Common targets identified in transcriptome analyses by Che *et al.* (2006) (regeneration from root-derived callus) and our study of cytokinin-induced LRP → SM conversion.

Table S7. Expression of genes previously identified as modulating *in vitro* shoot regeneration during LRP → SM conversion.

Table S8. Genes differentially expressed in a *wus* mutant (accession no. SAIL_150_G06) during cytokinin-induced LRP → SM conversion.

Table S9. Over-represented GO terms amongst DEGs identified in transcriptome comparison between the homozygous *wus* (SAIL_150_G06) mutant and the wild type during cytokinin-induced LRP → SM conversion.

Table S10. Significantly enriched GO terms corresponding to seven clusters (of 18 generated by *k*-means clustering) derived from genes differentially expressed in a *wus* loss-of-function background (accession no. SAIL_150_G06) during LRP → SM conversion.

Table S11. Genes differentially expressed in the root of two *wus* alleles compared with wild type during cytokinin-induced LRP → SAM conversion, after 30 h of treatment with 2iP.

Table S12. Candidate homozygous T-DNA insertion lines tested (two or more replicates) for differences in LRP → SM conversion rates and found not to differ significantly from the wild type.

Table S13. Pearson correlation coefficients for comparisons between arrays used in cell-specific profiling studies.

Table S14. Genes differentially expressed in FACS-sorted cells expressing *pWUS::mGFP-ER* from LRP undergoing conversion to SMs (after 30 h of treatment with 2iP), compared with the *WUSp* expression domain of *apetala1/cauliflower* double mutant SAMs (Yadav *et al.*, 2009).

Table S15. Enriched GO terms amongst DEGs identified in the transcriptome of FACS-sorted cells expressing *pWUS::mGFP-ER* from LRP undergoing conversion to SMs (after 30 h of treatment with 2iP), compared with the *WUSp* expression domain of *apetala1/cauliflower* double-mutant SMs (Yadav *et al.*, 2009).

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