



**A Genetic Framework for the Control of Cell
Division and Differentiation in the Root Meristem**

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targets against which novel therapeutic agents can be developed.

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Supporting Online Material

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A Genetic Framework for the Control of Cell Division and Differentiation in the Root Meristem

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Plant growth and development are sustained by meristems. Meristem activity is controlled by auxin and cytokinin, two hormones whose interactions in determining a specific developmental output are still poorly understood. By means of a comprehensive genetic and molecular analysis in *Arabidopsis*, we show that a primary cytokinin-response transcription factor, ARR1, activates the gene *SHY2/IAA3* (*SHY2*), a repressor of auxin signaling that negatively regulates the *PIN* auxin transport facilitator genes; thereby, cytokinin causes auxin redistribution, prompting cell differentiation. Conversely, auxin mediates degradation of the *SHY2* protein, sustaining *PIN* activities and cell division. Thus, the cell differentiation and division balance necessary for controlling root meristem size and root growth is the result of the interaction between cytokinin and auxin through a simple regulatory circuit converging on the *SHY2* gene.

Postembryonic root growth is sustained by the root meristem. Stem cells in the root meristem generate transit-amplifying cells, which undergo additional division in the proximal meristem, and differentiate at the meristem transition zone that encompasses the boundary between dividing and expanding cells in the different cell files (Fig. 1A). For meristem maintenance, the rate of cell differentiation must equal the rate of generation of new cells: How this balance is achieved is a central question in plant development.

Classic plant tissue culture experiments showed that auxin and cytokinin are key signaling mol-

ecules controlling meristems activity because they antagonistically affect, in vitro, shoot and root organogenesis (1, 2). Recently, the in vivo importance of the cytokinin and auxin antagonistic interaction has been proven during *Arabidopsis* root meristem size determination (3) and for embryonic root stem cell niche specification (4), but the genetic and molecular basis of this interaction remains to be clarified.

The expression domains of the genes encoding the two-component cytokinin signaling pathway, the AHK3 receptor kinase and the ARR1 and ARR12 transcription factors, and the root meristem phenotype of *ahk3*, *arr1*, and *arr12* mutants demonstrate that cytokinin acts at the transition zone to control cell differentiation rate (3, 5, 6). Furthermore, the expression patterns of cytokinin biosynthesis genes (7) and experiments of tissue- and spatial-specific cytokinin depletion in the root meristem showed that cytokinin specifically acts at the vascular tissue transition zone, where it controls the differentiation rate of all the other root cells by antagonizing a non-cell-autonomous signal that we suggested may be

auxin (3). On the other hand, experiments of exogenous application of auxin and the effects of mutations in the *PIN* auxin efflux facilitators are consistent with a role of auxin in controlling cell division (3, 8). Thus, the size of the root meristem may be established by a balance between the antagonistic effects of cytokinin, which mediates cell differentiation, and auxin, which mediates cell division.

In order to dissect the cytokinin-auxin interaction in the root meristem, we sought to identify the gene(s) immediately responsive to cytokinin signaling. Cytokinin control of root meristem size is mediated by both ARR1 and the ARR12 transcription factors, specifically expressed at the root transition zone and acting, respectively, early and late during meristem development (3). Whereas the root meristems of *arr12* mutants were larger than those of wild type but eventually stopped growing, *arr1* mutant meristems kept increasing in size over time (3). ARR1 seems therefore to have a critical role in determining root meristem size, and thus we set to verify whether this transcription factor alone would be sufficient to control cell differentiation and root meristem size. To this aim, we checked the root phenotype of plants harboring a glucocorticoid-inducible construct of ARR1 (35S::ARR1:ADDK:GR) (9). As in the case of exogenous cytokinin applications (3), root meristems of 35S::ARR1:ADDK:GR plants were significantly reduced after 8 hours of dexamethasone (a glucocorticoid derivative) induction, suggesting that ARR1 is capable alone of controlling root meristem size (fig. S1, A and B).

The same 35S::ARR1:ADDK:GR construct had been previously used to identify a number of putative direct-target genes of the ARR1 transcription factor (10). Among the 23 putative targets of ARR1 (10), there is notably *SHY2*, a member of the auxin/indole-3-acetic acid inducible (*Aux/IAA*) family of genes (11) that act as auxin-response inhibitors by forming heterodimers with the ARF (auxin response factor) transcription factors, thereby preventing activation of auxin-responsive genes (among which are the *Aux/IAA* genes) by these latter (12, 13). Auxin causes the

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degradation of the Aux/IAA proteins via the SKIP-CULLIN-FBOX and transport inhibitor response 1 (SCF^{TIR1}) ubiquitin-ligase complex, thus releasing the ARFs from inhibition (13). Degradation of the Aux/IAA proteins and consequent ARF activity depend on auxin concentration: High concentration of auxin causes Aux/IAA degradation, whereas at low auxin concentration these proteins are stable and interact with the ARFs (13).

The presence of *SHY2* among the putative targets of ARR1 suggests that, in determining root meristem size, cytokinin antagonizes auxin by modulating auxin signaling.

To verify this hypothesis, we analyzed the root-meristem phenotype of *shy2* gain- and loss-of-function mutants (14, 15). The *shy2-2* gain-of-function mutant allele displayed a shorter root (14, 15) (fig. S1D) and a reduced root meristem size already 2 days after germination (Fig. 1, C and D), which phenocopied the effects of application of cytokinin and of induction of 35S::ARR1ΔDDK:GR. On the contrary, the *shy2-31* loss-of-function mutant allele (15) showed an increased number of

meristematic cells and kept accumulating cells in the meristem 5 days after germination, vastly exceeding the fixed number of cells of wild-type meristems (Fig. 1, B and D). The increased size of the root meristem correlated with an enhanced rate of root growth and resulted in a longer root (fig. S1D). Because the *shy2-31* roots closely resembled *arr1* mutant roots, we constructed and analyzed *arr1,shy2-2* and *arr1,shy2-31* double mutant plants. The root meristem sizes of both *arr1,shy2-2* and *arr1,shy2-31* double mutants were indistinguishable from that of the *arr1* mutant (fig. S1E), supporting the notion that the *SHY2* protein may act downstream of the ARR1 transcription factor.

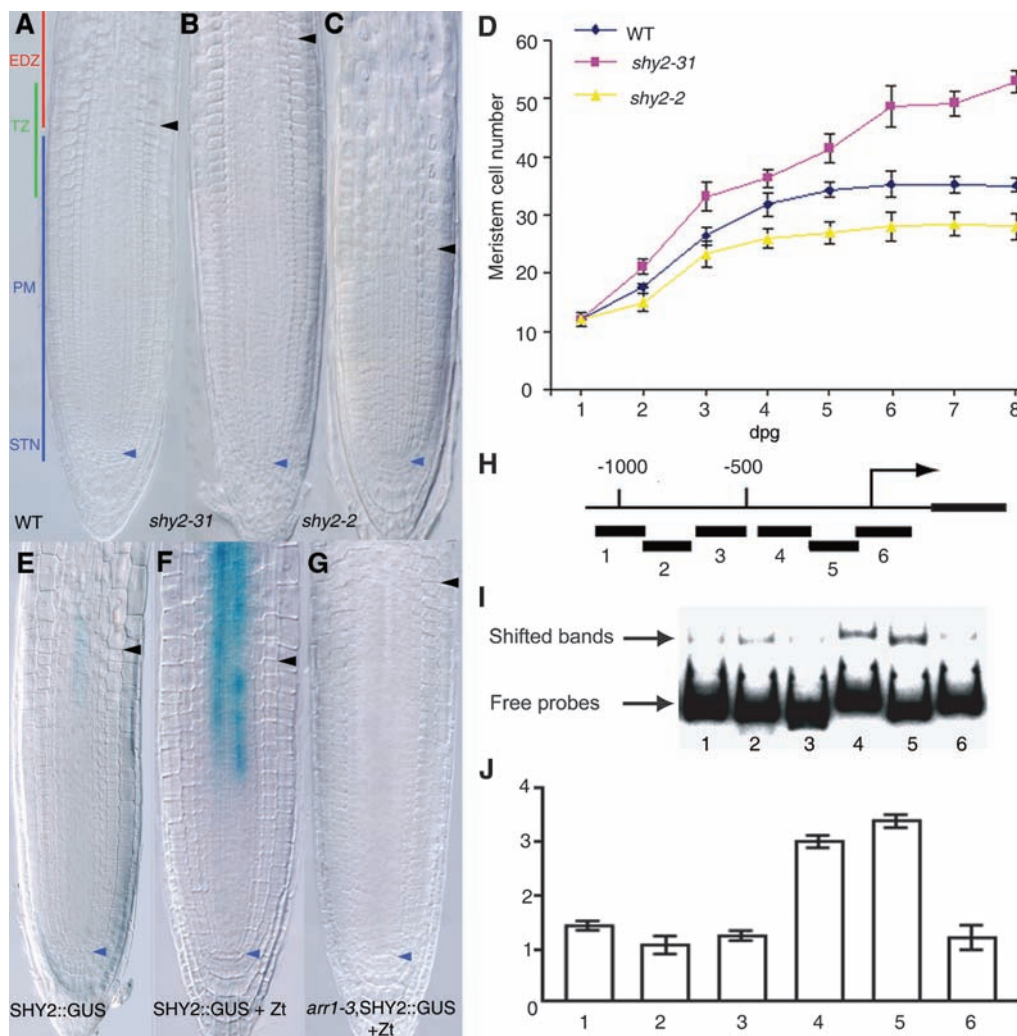
To substantiate the suggested regulatory role of ARR1 on the *SHY2* gene, we compared the expression of this gene in wild-type and *arr1* mutant roots by using a *SHY2*::GUS promoter fusion (11, 16). In wild-type root meristems, the activity of this fusion was specifically detected at the vascular tissue transition zone (16) (Fig. 1E and fig. S4J) and was strongly induced by cytokinin treatment (Fig. 1F). This induction of *SHY2*

promoter activity was matched by a corresponding accumulation of the *SHY2* protein, as visualized by Western blot analysis of a transgenic line harboring a Myc-epitope-tagged *SHY2* protein (16) (fig. S3A). In contrast, in the *arr1* mutant background *SHY2*::GUS activity was hardly detectable and did not show any increase upon cytokinin treatment (Fig. 1G), confirming that activation of the *SHY2* promoter in roots depends on ARR1.

To assess whether *SHY2* is necessary and sufficient to mediate the cytokinin control of root meristem size, we analyzed the root phenotype of the *shy2-31* loss-of-function mutant harboring the 35S::ARR1ΔDDK:GR construct. The *shy2-31*, 35S::ARR1ΔDDK:GR roots did not show any reduction in size after 8 hours of dexamethasone induction (fig. S1, A to C), suggesting that *SHY2* alone mediates the effect of ARR1 in response to cytokinin to control root meristem size.

The genetic evidence provided establishes a regulatory interaction between ARR1 and *SHY2*. To confirm the physical interaction between the ARR1 protein and the *SHY2* promoter in vivo, we

Fig. 1. The *SHY2* gene controls root meristem size and is a direct regulatory target of ARR1. (A to C) Five-days-postgermination (dpg) root meristems of wild-type (A), *shy2-31* loss-of-function mutant (B), and *shy2-2* gain-of-function mutant (C). Root meristem size is expressed as the number of cortex cells in a file extending from the quiescent center (blue arrowheads) to the first elongated cortex cell (black arrowheads). STN indicates stem cell niche, PM, proximal meristem; EDZ, elongation-differentiation zone; and TZ, transition zone. The transition zone is different for each cell type, giving a jagged shape to the boundary between dividing and expanding cells. (D) Root meristem cell number of wild-type, *shy2-2*, and *shy2-31* mutants depicted in (A) to (C), measured over time. (E to G) Expression of the *SHY2*::GUS construct in wild-type roots (E), in roots treated 3 hours with 5 μ M transzeatin (Zt) and stained for 6 hours (F), and in the *arr1-3* mutant treated 3 hours with 5 μ M Zt and stained for 6 hours (G). (H) Schematic representation of the *SHY2* promoter region. Thick and thin lines correspond to coding and non-coding regions, respectively. The bent arrow indicates the transcription start site. Bars with numbers illustrate the DNA fragments used in both ChIP (J) and gel mobility shift analyses (I). (I) Gel mobility shift analysis. Labeled DNA fragments were subjected to gel mobility shift analysis with a fusion protein consisting of GST and ARR1 DNA binding domain. (J) ChIP analysis. Chromatin preparations from ARR1:GFP and wild-type (negative control) roots were coimmunoprecipitated with anti-GFP antibody. An enrichment fold of each fragment was obtained by normalizing the recovery rate for the ARR1:GFP preparation against that for the negative control preparation (24). Error bars indicate SE.



performed chromatin immunoprecipitation (ChIP) assays using roots expressing an ARR1:GFP (green fluorescent protein) fusion protein under the control of the *ARR1* promoter. Two DNA fragments in the *SHY2* promoter region (fragments 4 and 5 in Fig. 1, H and J) but not those in the promoter of a negative control gene, *TUA4* (fig. S2, A and B), were significantly enriched by co-immunoprecipitation with anti-GFP using fragmented chromatin prepared from ARR1:GFP roots as compared with the preparation from wild-type roots. As in the case of the promoter region of *ARR6*, a direct target-gene of ARR1 (9), the enrichment of DNA fragments was correlated with the binding affinity of the fragments to the ARR1 DNA binding domain in an in vitro gel mobility shift analysis (Fig. 1, I and J, and fig. S2, C and D), supporting the evidence that ARR1 specifically bound to the *SHY2* promoter in vivo. The interaction was confirmed to be sequence-specific by gel mobility shift analysis with specific and nonspecific competitors and

deoxyribonuclease (DNase) I footprinting analysis (fig. S2, E to G).

These data confirm that *SHY2* is a direct regulatory target of the ARR1 transcription factor and provide support for the suggestion that cytokinin antagonizes auxin at the vascular tissue transition zone by modulating auxin signaling and thus determines root meristem size.

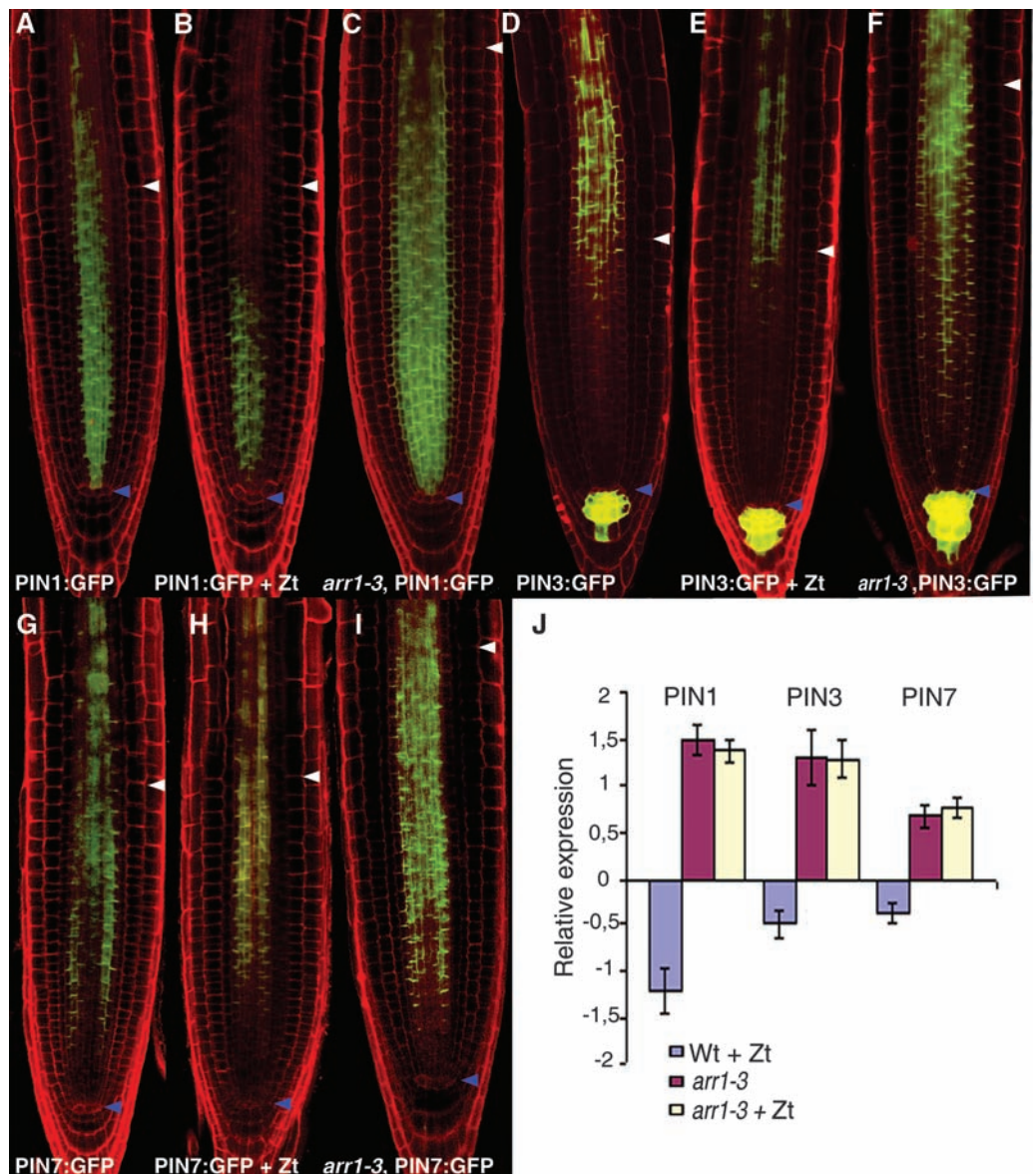
The existence of a positive feedback loop between auxin and the PIN auxin-transport facilitators had been established (17), and we had previously reported that cytokinin, acting from the vascular tissue transition zone, requires polar auxin transport to determine the transition zone of all other tissues (3). Thus, we asked whether *SHY2* activation by cytokinin, and the resulting reduction of auxin response at the vascular tissue transition zone, would interfere with the activity of the *PIN* genes in order to control root meristem size.

We first monitored the effect of application of exogenous cytokinin on the expression of the

PIN1, *PIN3*, and *PIN7* genes. These particular *PIN* genes were chosen because they are expressed in the vascular tissue transition zone (17) and because a role for these genes in controlling root meristem size has already been established (3, 7); in fact, the triple *pin1.pin3.pin7* mutant displays a short root meristem (7) and is cytokinin-resistant (fig. S3B). Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) showed that 4 hours of cytokinin treatment were sufficient to significantly reduce expression of *PIN1*, *PIN3*, and *PIN7* (Fig. 2J), whereas cytokinin-mediated reduction in root meristem size was first detectable only after 12 hours of exposure to cytokinin (fig. S3B). Notably, as visualized by PIN:GFP translational reporter fusions (8, 18), down-regulation of *PIN* genes was particularly evident in the vascular tissue transition zone (Fig. 2, B, E, and H).

To assess whether this cytokinin-dependent down-regulation of the *PIN1*, *PIN3*, and *PIN7* genes was mediated by the AHK3/ARR1 sig-

Fig. 2. Cytokinin perception at the vascular tissue transition zone controls root meristem size restricting *PIN1*, *PIN3*, and *PIN7* expression domain. (A, D, and G) Five-dpg wild-type root meristems expressing, respectively, *PIN1*:GFP, *PIN3*:GFP, and *PIN7*:GFP. (B, E, and H) Reduction of PIN expression in cytokinin-treated roots (5 μ M Zt, 6 hours) expressing, respectively, *PIN1*:GFP, *PIN3*:GFP, and *PIN7*:GFP. No reduction in root meristem size is still detectable. (C, F, and I) PIN expression in the *arr1-3* mutant root meristem expressing, respectively, *PIN1*:GFP, *PIN3*:GFP, and *PIN7*:GFP. In the *arr1-3* mutant, the PIN expression domain at the vascular tissue transition zone is expanded (white arrowheads). Blue arrowheads indicate quiescent center cells; white arrowheads indicate cortex transition zone. (J) qRT-PCR confirm *PINs* gene mRNA down-regulation after 4 hours of cytokinin treatment (5 μ M Zt) in wild-type roots, up-regulation in the *arr1-3* mutant background, and no down-regulation in the *arr1-3* mutant upon cytokinin treatment. Relative expression is normalized to *ACTIN*, and 0 corresponds to *PINs* mRNA amount in untreated wild-type roots. Error bars, SD; $P < 0.5$; Student's *t* test; $n = 3$.



naling pathway, we monitored the expression and distribution of *PIN* mRNAs in the *ahk3* and *arr1* mutant backgrounds. The expression of the three *PIN* mRNAs was higher in the *ahk3* and *arr1* mutants (Fig. 2J and fig. S4K) and the *PIN* expression domains in the vascular tissue transition zone were expanded as compared with the wild type (Fig. 2, C, F, and I, and fig. S4, B, E, and H). Furthermore, no reduction in root meristem size (fig. S3B) and no down-regulation of *PIN1*, *PIN3*, and *PIN7* were observed in the *ahk3* and *arr1* mutant backgrounds in response to cytokinin treatment (Fig. 2J and fig. S4, C, F, I, and K). Thus, perception of cytokinin at the root vascular tissue transition zone, mediated by the AHK3/ARR1 signaling pathway, down-regulates the *PIN1*, *PIN3*, and *PIN7* genes and restricts their expression domain.

To assess whether this *PIN* down-regulation depended on *SHY2* activation by cytokinin, we monitored the expression and distribution of *PIN1*, *PIN3*, and *PIN7* mRNAs in the *shy2-31* loss-of-function mutant background. *PIN::GFP*

fusion analysis (Fig. 3, B, E, and H) and real-time qRT-PCR (Fig. 3J) showed that, as in the case of the *ahk3* and *arr1* mutants described above, the expression of *PIN1*, *PIN3*, and *PIN7* in the *shy2-31* mutant was increased and expression domains expanded at the vascular tissue transition zone as compared with the expression of wild type. Furthermore, no down-regulation of the *PIN* genes and no root meristem reduction were observed in the *shy2-31* mutant in response to cytokinin (Fig. 3, C, F, I, and J, and fig. S3B). On the contrary, the expression of *PIN1*, *PIN3*, and *PIN7* mRNAs was lower in the *shy2-2* gain-of-function mutant (fig. S5H).

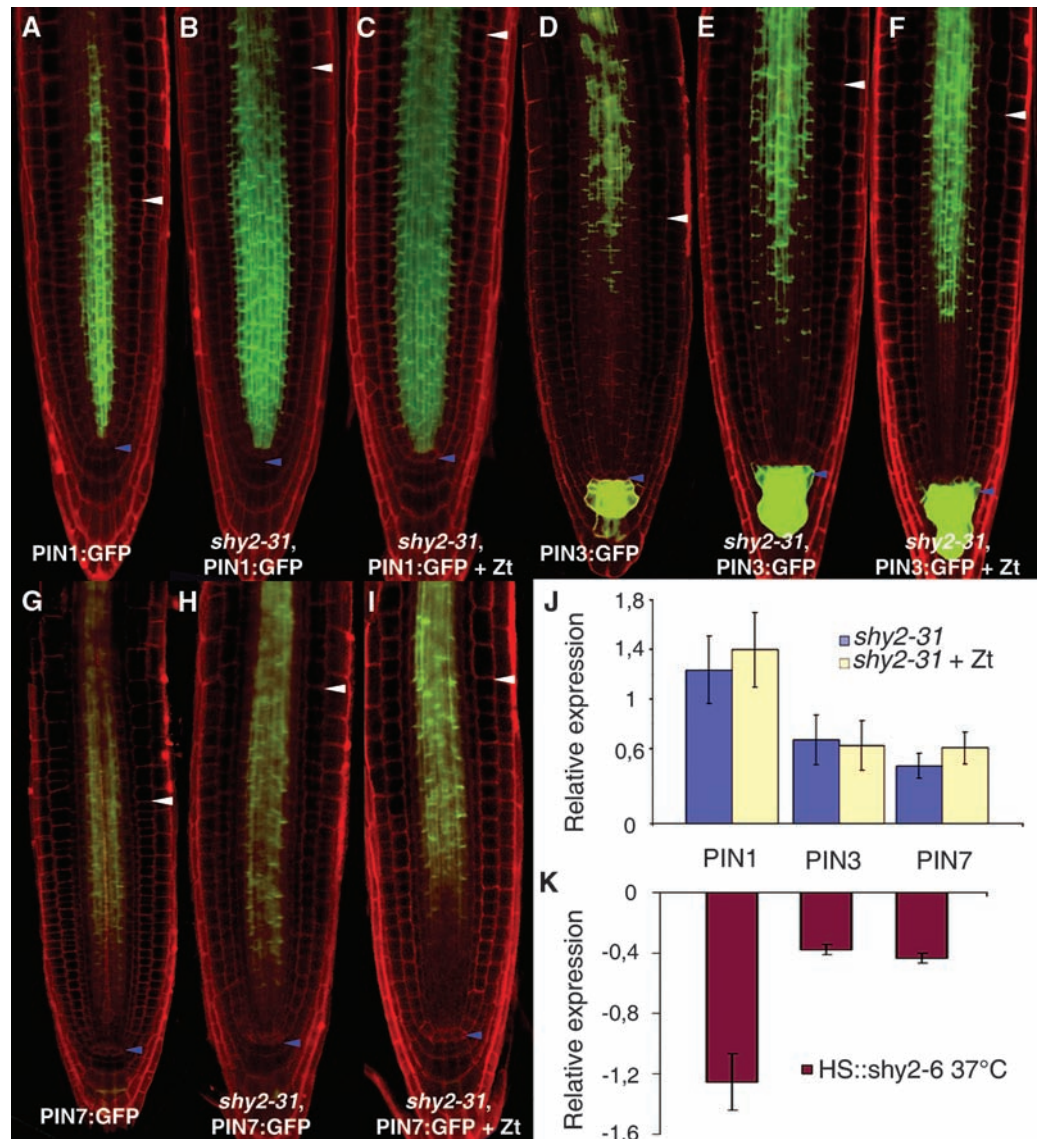
To further substantiate the regulatory role of the *SHY2* protein on the expression of the *PIN* genes, we transiently expressed a nondegradable version of the *SHY2* protein under a heat-shock promoter (*HS::shy2-6*) (15) and recorded changes in *PIN1*, *PIN3*, and *PIN7* gene expression by real-time qRT-PCR. Induction of *HS::shy2-6* for 1 hour was sufficient to reduce the expression of *PIN1*, *PIN3*, and *PIN7* (Fig. 3K), and the consequent

reduction of root meristem size was observed after 3 hours (fig. S5, E to G). These data suggest that the activation of *SHY2* by cytokinin at the vascular tissue transition zone restricts the expression of *PIN1*, *PIN3*, and *PIN7* to the vascular tissue transition zone and that this is necessary to position the transition zone of all other tissues of the root.

It had been shown that auxin controls root meristem size, sustaining the activity of the *PIN* genes as well as cell division (3, 8). Accordingly, exogenous application of auxin to wild-type roots during growth caused an increase in meristem size detectable after 24 hours of exposure to auxin (fig. S5, A, B, and G), whereas 12 hours of auxin treatment were sufficient to induce the expression of *PIN1*, *PIN3*, and *PIN7* (fig. S5H).

We then asked whether this up-regulation was the result of the auxin-dependent *SHY2* protein degradation via the SCF^{TIR1} ubiquitin-ligase complex (fig. S3A) (13, 19). No up-regulation of the *PIN* genes and no increase of meristem size (i.e., cell division) upon application of auxin were

Fig. 3. Cytokinin-mediated *SHY2* gene induction at the vascular tissue transition zone controls root meristem size restricting the *PIN1*, *PIN3*, and *PIN7* expression domain. (A, D, and G) Five-dpg wild-type root meristem expressing, respectively, *PIN1::GFP*, *PIN3::GFP*, and *PIN7::GFP*. (B, E, and H) Five-dpg *shy2-31* root meristems expressing, respectively, *PIN1::GFP*, *PIN3::GFP*, and *PIN7::GFP*. *PIN* expression domain at the vascular tissue transition zone is expanded (white arrowheads). (C, F, and I) No change in *PIN* expression in cytokinin-treated *shy2-31* mutant root meristem (5 μ M Zt, 12 hours) expressing, respectively, *PIN1::GFP*, *PIN3::GFP*, and *PIN7::GFP*. No changes in root meristem size are detectable. Blue arrowheads indicate quiescent center cells; white arrowheads indicate cortex transition zone. (J) qRT-PCR confirms up-regulation of the *PIN* gene mRNAs in the *shy2-31* mutant and no down-regulation in the *shy2-31* mutant upon cytokinin treatment (5 μ M Zt, 12 hours). Relative expression is normalized to *ACTIN*, and 0 corresponds to *PIN* mRNA levels in untreated wild-type roots. Error bars, SD; $P < 0.5$; Student's *t* test; $n = 3$. (K) qRT-PCR showing *PIN* down-regulation in root of *HS::shy2-6* plants induced at 37°C for 1 hour. Relative expression is normalized to *ACTIN*, and 0 corresponds to *PIN* mRNA amounts in wild-type roots kept for 1 hour at 37°C. Error bars, SD; $P < 0.5$; Student's *t* test; $n = 3$.



observed in the gain-of-function mutant *shy2-2* in which the interaction of SHY2 with the F-box protein TIR1 is affected (19) (fig. S5, C, D, G, and H).

The cytokinin biosynthetic gene adenosine triphosphate/adenosine diphosphate isopentenyl-transferase 5 (AtIPT5), involved in controlling root meristem size (3) and specifically expressed in the columella and at the vascular tissue transition zone (7) (fig. S6, A to E), is rapidly induced by auxin (7). To establish whether the control of auxin on cytokinin biosynthesis is mediated by SHY2, we analyzed the expression of an IPT5::GUS promoter fusion (7) in the *shy2-2* gain-of-function mutant and in the *shy2-31* loss-of-function mutant. Although IPT5::GUS activity was higher and more sensitive to auxin in the *shy2-31* mutant than in the wild type, auxin-dependent IPT5::GUS activity was totally lost in the *shy2-2* gain-of-function mutant (fig. S6, A to E).

We propose a model where cytokinin and auxin antagonistically interact at the vascular tissue transition zone to balance cell differentiation with cell division, thus determining root meristem size, by controlling in opposite ways the abundance of the SHY2 protein. Cytokinin reduces auxin response by activating, via the AHK3/ARR1 two-component signaling pathway, transcription of the SHY2 gene; the SHY2 protein in turn regulates negatively the expression of the PIN genes (fig. S7). The resulting redistribution of auxin leads to cell differentiation of all the other tissues, thus reducing root meristem size. Conversely, auxin controls root meristem growth by directing degradation of the SHY2 protein, thus sustaining the

activity of the PIN genes and cell division (fig. S7). The SHY2 protein negatively controls auxin transport on the one hand and cytokinin biosynthesis on the other, thus conferring robustness to the feedback loop we propose.

This model is consistent with the low concentration of auxin at the transition zone observed by other authors (20) and with our previous demonstration that the vascular tissue transition zone is the developmental domain of cytokinin action (3).

It is tempting to hypothesize that in root meristem size determination the PLETHORA (PLT) (21) proteins mediate the effect of cytokinin by responding to changes in polar auxin transport caused by cytokinin. It has been shown that the gradient of auxin in the root meristem creates a gradient of PLT proteins that direct different outputs in response to auxin in different regions of the meristem (22). Changes in PIN proteins activity affect the gradient distribution of auxin and of the PLT proteins (8, 23). Interestingly, we observed that the *PLT1* and *PLT2* genes are down-regulated by cytokinin (fig. S6F).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/322/5906/1380/DC1
Materials and Methods
Figs. S1 to S7
References

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Chromosome Alignment and Transvection Are Antagonized by Condensin II

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Polytene chromosome structure is a characteristic of some polyploid cells where several to thousands of chromatids are closely associated with perfect alignment of homologous DNA sequences. Here, we show that *Drosophila* condensin II promotes disassembly of polytene structure into chromosomal components. Condensin II also negatively regulates transvection, a process whereby certain alleles are influenced transcriptionally via interallelic physical associations. We propose that condensin II restricts trans-chromosomal interactions that affect transcription through its ability to spatially separate aligned interphase chromosomes.

Interphase chromosomal trans-interactions occur in many species and impact chromosome structure and gene expression (1–4). As evidenced in *Drosophila*, trans-interactions can lead to polytene chromosomes, where all maternal and paternal chromatids are aligned in precise register (5). The *Drosophila* ovarian nurse cells disassemble their polytene chromosomes into unpaired homologs and chromatid fibers during mid-oogenesis

(Fig. 1A and fig. S1) (6). We used this system to isolate two noncomplementing mutations in a predicted condensin II subunit, *Cap-H2* (7–9), that cause failure in nurse cell polytene disassembly. Polyteny instead persists in the trans-heterozygous combinations of *Cap-H2*^{Z3-0019}/*Cap-H2*^{Z3-5163} (Fig. 1B and fig. S2A) and when either allele is in trans to a deletion of its genomic locus (Fig. 1, C and D). This was corroborated through fluores-

cence in situ hybridization (FISH) labeling to a specific locus in stage 7 egg chambers, where wild-type polytenes disassembled, yet 92.9% of mutant nuclei had all maternal and paternal chromatids aligned in register (fig. S3) (7). Polytene persistence in *Cap-H2* mutants likely does not occur indirectly through altered cell cycle progression or DNA replication patterns because neither the length of S phase nor ploidy were detectably different in homozygous polytene mutants versus heterozygous controls (figs. S4 and S5) (7). This result instead suggests that *Cap-H2* function is necessary to disassemble nurse cell polytene chromosomes.

Metazoa have two condensin complexes that are referred to as condensin I and II. Each uses the adenosine triphosphatases SMC2 and SMC4, but forms complexes with different non-SMC subunits Cap-H, Cap-G, and Cap-D2 or Cap-H2, Cap-G2, and Cap-D3, respectively (10, 11). Condensins function in the condensation of chromosomes, facilitate proper anaphase segregation, and

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