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LETTERS

# Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis

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**Plant stem-cell pools, the source for all organs, are first established during embryogenesis. It has been known for decades that cytokinin and auxin interact to control organ regeneration in cultured tissue[1]. Auxin has a critical role in root stem-cell specification in zygotic embryogenesis[2,3], but the early embryonic function of cytokinin is obscure[4–6]. Here, we introduce a synthetic reporter to visualize universally cytokinin output *in vivo*. Notably, the first embryonic signal is detected in the hypophysis, the founder cell of the root stem-cell system. Its apical daughter cell, the precursor of the quiescent centre, maintains phosphorelay activity, whereas the basal daughter cell represses signalling output. Auxin activity levels, however, exhibit the inverse profile. Furthermore, we show that auxin antagonizes cytokinin output in the basal cell lineage by direct transcriptional activation of *ARABIDOPSIS RESPONSE REGULATOR* genes, *ARR7* and *ARR15*, feedback repressors of cytokinin signalling. Loss of *ARR7* and *ARR15* function or ectopic cytokinin signalling in the basal cell during early embryogenesis results in a defective root stem-cell system. These results provide a molecular model of transient and antagonistic interaction between auxin and cytokinin critical for specifying the first root stem-cell niche.**

Cytokinins are adenine-derived signalling molecules that have many essential roles in postembryonic growth and development. However, the role of cytokinin signalling in early embryogenesis remains unclear[4–6]. To visualize cytokinin's signalling output *in vivo*, we aimed to design a synthetic reporter that overcame the limitations of current reporters, typically immediate-early cytokinin target genes. The discrete expression patterns of these markers[7,8] indicate that they integrate unknown secondary input that reflects cytokinin-independent regulation.

Cytokinin signalling is mediated by a multistep two-component circuitry through histidine and aspartate phosphorelay[9]. Nuclear B-type response regulators mediate transcriptional activation in response to phosphorelay signalling activity, whereas A-type response regulators repress signalling in a negative-feedback loop. The DNA-binding domains of diverse B-type response regulator family members are conserved and bind a common DNA-target sequence (A/G) GAT(T/C) *in vitro*[10–12]. This motif is significantly enriched in the *cis*-regulatory region of immediate-early cytokinin target genes[13], suggesting its *in vivo* relevance. To generate a universal cytokinin reporter, we tested and optimized synthetic reporter designs using luciferase (LUC) activity in *Arabidopsis* mesophyll protoplast assays[14,15]. The resulting synthetic reporter, *TCS::LUC* (two-component-output-sensor), harboured the concatemerized B-type Arabidopsis response regulator (ARR)-binding motifs[10–12] and a minimal 35S promoter[14]. Only cytokinins activated *TCS::LUC*, whereas other plant hormones such as auxin, abscisic acid and gibberellic acid, had no effect (Fig. 1a, b). All three known cytokinin receptors contributed to its cytokinin-dependent induction *in vivo*, as cells isolated from double cytokinin receptor mutants were compromised in their ability to induce *TCS::LUC* expression (Fig. 1c). The extent of this reduction correlated with the in planta contribution of the different receptors[6]. B-type ARR family members promoted strong *TCS::LUC* induction in a co-transfection assay[14] (Fig. 1d). Conversely, coexpression of A-type ARR family members inhibited cytokinin-dependent *TCS::LUC* activity (Fig. 1e). *TCS::LUC* displayed concentration-dependent activation by cytokinin from as low as 100 pM up to about 1 μM. Furthermore, *TCS* mediated significantly higher induction compared with the native *ARR6* promoter[14] (Fig. 1f). Addition of a viral translational enhancer (Ω)[16] amplified the response further (Fig. 1f). Taken together, these findings suggest that *TCS::LUC* could specifically report even low levels of phosphorelay output triggered by any of

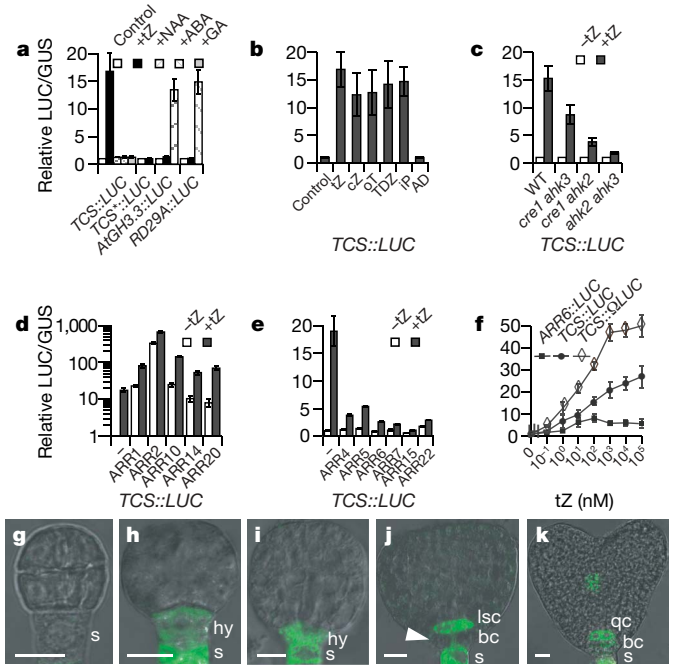


**Figure 1 | Sensitive and specific response of *TCS*.** **a**, *TCS::LUC* is induced by 100 nM trans-zeatin (tZ), but not 1 μM auxin (NAA), 100 μM abscisic acid (ABA), or 50 μM gibberellic acid (GA). *TCS*::LUC* is a negative control. **b**, *TCS::LUC* is induced by (all at 100 nM): cis-zeatin (cZ), ortho-topolin (oT), thidiazuron (TDZ) and $N^6$-($\Delta^2$-isopentenyl)adenine (iP). Adenine (AD) is a negative control. **c**–**e**, *TCS::LUC* induction by trans-zeatin is reduced in double mutant combinations of *ahk2-2*, *ahk3-3* and *cre1-1* (ref. 6) (**c**); stimulated by B-type ARRs (**d**); and reduced by A-type ARRs (**e**). **f**, Dose responses are shown. **g**–**k**, Embryonic *TCS::GFP* activity. Closed arrowhead in (j) points to *TCS::GFP* downregulation in the basal cell lineage. bc, basal cell lineage; hy, hypophysis; lsc, lens-shaped cell; qc, quiescent centre; s, suspensor. Error bars are s.d. ($n = 3$); scale bars represent 10 μm.

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the three endogenous cytokinin receptors and relayed to any response regulator tested.

To determine the expression pattern in planta, we generated transgenic *Arabidopsis* plants carrying the green fluorescent protein (GFP) reporter controlled by the *TCS* synthetic promoter. The activity of *TCS::GFP* in the seedling was consistent with cytokinin actions previously documented, for example, in cotyledons[7] (Supplementary Fig. 1b), shoot meristem[7,17] (Supplementary Fig. 1c), root tip[7,17,18] and root vasculature[19] (Supplementary Fig. 1d), as well as in emerging lateral root base and primordia[20] (Supplementary Fig. 1e, f). Seedlings subjected to a short-term incubation with the cytokinin-synthesis inhibitor lovastatin[21] abolished *TCS::GFP* expression (Supplementary Fig. 2b, e). Notably, *TCS::GFP* expression was restored by the co-administration of a cytokinin together with lovastatin (Supplementary Fig. 2c, f). The analyses validated the physiological response of the novel synthetic cytokinin reporter in intact plants.

To uncover new roles of phosphorelay signalling *in vivo*, we followed *TCS::GFP* expression during early embryogenesis (Fig. 1g–k). As cytokinins have long been implicated in shoot regeneration[1], we were surprised to detect the first distinct signal in the founder of the root stem cells, the hypophysis, at the 16-cell stage (Fig. 1h). By the transition stage, the hypophysis has undergone asymmetrical cell division (compare Fig. 1i with Fig. 1j, and Fig. 2b with Fig. 2c). The resulting large basal daughter cell and its descendants repressed *TCS::GFP* expression, whereas the apical lens-shape cell retained its expression (Figs 1j and 2c). By the heart stage, a second phosphorelay output had appeared near the shoot stem-cell primordium (Fig. 1k).

To identify the signalling components involved in embryonic phosphorelay activity, we determined the transcription levels of all the two-component genes at the transition stage. A subset of candidates for each signalling step was transcribed (Fig. 2a). We were interested in the expression patterns of the A-type ARR genes, as they represent direct targets commonly used as markers of cytokinin signalling. *ARR7* messenger RNA was abundant at the transition stage. *ARR7::GFP* transgenic lines displayed initial GFP activity at the late globular stage, just after the asymmetric division of the hypophysis. Notably, *ARR7::GFP* activity was high in the basal daughter cell but lower in the lens-shape cell and its descendants (Figs 2d and 3c), inverse to *TCS::GFP* levels (Figs 2c and 3a). mRNA *in situ* hybridization confirmed a similar endogenous *ARR7* expression pattern (Fig. 3s). *ARR15*, the sister gene of *ARR7*, is expressed in the comparable domain, albeit at much lower levels (Figs 2a, e and 3d). Therefore, *ARR7* and *ARR15* expression only partially reflected the phosphorelay output (compare Fig. 2d, e with Fig. 2c, and Fig. 3c, d, s with Fig. 3a). These results suggest that additional input might control *ARR7* and *ARR15* expression.

Auxin signalling output visualized by the synthetic reporter *DR5::GFP* (ref. 2) was highest in the hypophysis-derived basal cell (Fig. 2f), similar to *ARR7* and *ARR15* expression. This raised the possibility that auxin could induce transcription of the cytokinin repressors *ARR7* and *ARR15*, which in turn prevented phosphorelay output in the basal cell lineage (Fig. 4m). To test the effect of ectopic auxin signalling, *TCS::GFP, DR5::GFP, ARR7::GFP* and *ARR15::GFP* transgenic embryos (Fig. 3a–d) were treated with the synthetic auxin analogue 2,4-dichlorophenoxyacetic acid (2,4-D; ref. 2) (Fig. 3g–j). Incubation with 2,4-D resulted in expansion of the *DR5::GFP* expression domain (Fig. 3h)[2]. The levels of *ARR7::GFP* and *ARR15::GFP* expression also increased (Fig. 3i, j, t) and their domain widened (Fig. 3i, j), whereas *TCS::GFP* activity was abolished (Fig. 3g). Exogenous cytokinin caused a broad expansion of *TCS::GFP* expression (Fig. 3m), but left *DR5::GFP* expression unaffected (Fig. 3n). Both *ARR7::GFP* and *ARR15::GFP* expression was increased (Fig. 3o, p, t), reflecting their documented status as direct cytokinin target genes[17]. These experiments revealed that besides cytokinin, auxin regulated *ARR7* and *ARR15* transcription; supporting a model where high endogenous auxin activity suppresses cytokinin output through the stimulation of *ARR7* and *ARR15* transcription (Fig. 4m).

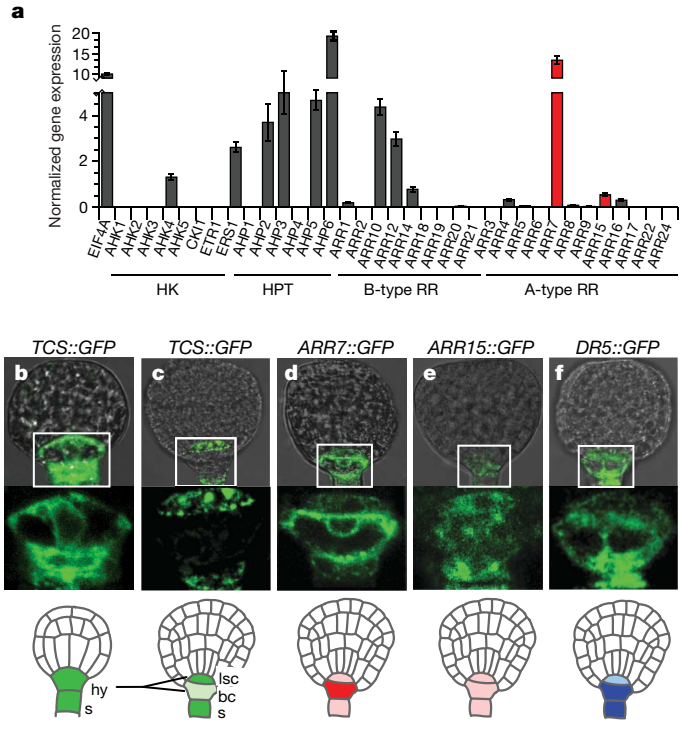


**Figure 2 | Inverse correlation between cytokinin and auxin signalling.** **a**, Two-component gene transcription by qRT–PCR at transition stage. Error bars represent s.d. ($n = 3$). **b**, *TCS::GFP* in hypophysis (hy) at early globular stage. **c**, Downregulation in basal cell lineage (bc) at late globular stage. **d**, First *ARR7::GFP* expression peaks are in the basal cell lineage. **e**, *ARR15::GFP* expression. **f**, *DR5::GFP* activity is highest in the basal cell lineage. The boxed sections are magnified in the middle panel; a schematic interpretation is shown in the bottom panel. CKI1, cytokinin independent 1; ERS1, ethylene response sensor 1; ETR1, ethylene response 1; HK/AHK, *Arabidopsis* histidine kinases; HPT/AHP, histidine-phosphotransfer proteins; lsc, lens-shaped cell; RR/ARR, response regulators; s, suspensor.

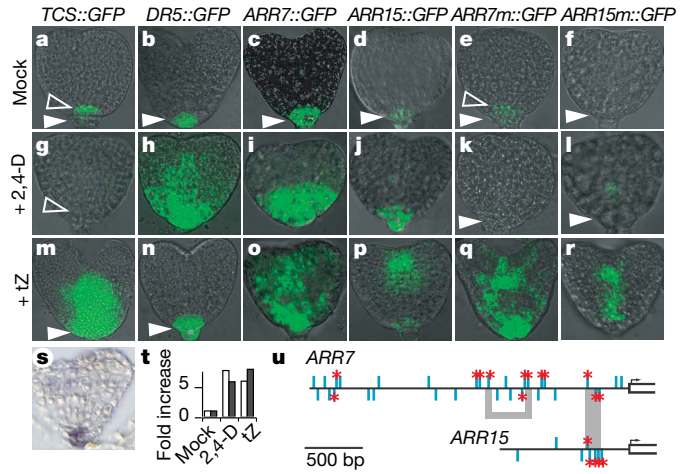


**Figure 3 | *ARR7* and *ARR15* mediate auxin control of cytokinin signalling.** **a**–**r**, Overnight treatment with water (top row), 50 μM 2,4-D (middle row) or trans-zeatin (tZ; lower row). *ARR7m::GFP* (**e**, **k**, **q**) and *ARR15m::GFP* (**f**, **l**, **r**) are explained in **u**. Filled arrowheads denote basal cell lineage; open arrowheads quiescent centre. **s**, *In situ* hybridization to *ARR7* mRNA. **t**, *ARR7* (open bar) and *ARR15* (filled bar) mRNA induction assayed by qRT–PCR after 4-h treatment of embryos with either 1 μM trans-zeatin or 2,4-D. **u**, Illustration of *ARR7* and *ARR15* promoters. TGTC on sense and antisense strands as blue bars; red asterisks denote point mutations TGTC→TGGC. Grey shading connects conserved regions. See text for details.

To explore the possibility that auxin signalling directly induced transcription of *ARR7* and *ARR15*, we analysed their *cis*-regulatory regions for motifs that might mediate auxin input. Auxin response elements (AuxRE) have been defined as TGTCTC. However, careful *in vitro* analysis demonstrated that only the first four nucleotides are essential for auxin response factor (ARF) binding[22] and we therefore screened the promoters of *ARR7* and *ARR15* for the TGTC motif. We found 32 occurrences in the *ARR7*, and 8 in the *ARR15* upstream region (Fig. 3u). Previously characterized functional *AuxRE* have been categorized as 'simple' (defined by repetitive motifs) or 'composite' (where the motif is flanked by a cofactor-binding site)[23]. These criteria guided us in focusing on putative functional TGTC hits. Point mutations shown to abolish *ARF* binding[22] were introduced specifically in TGTC motifs occurring at least twice in a 30-base-pair (bp) window or flanked by sequence conserved within or between the *ARR7* and *ARR15* promoters (Fig. 3u). The resulting mutated reporters *ARR7m::GFP* and *ARR15m::GFP* (Supplementary Table 2) showed strongly reduced expression in the auxin-signalling domain (Fig. 3e, f, filled arrowhead). Furthermore, ectopic auxin signalling was unable to stimulate their expression (Fig. 3k, l, arrowheads). By contrast, they retained responsiveness to cytokinin (Fig. 3q, r). Notably, uncoupled from auxin input, *ARR7m::GFP* showed an expression pattern similar to that contributed by the cytokinin reporter *TCS::GFP* (compare Fig. 3e with Fig. 3a). Consequently, exogenous auxin application caused repression of *ARR7m::GFP* expression (Fig. 3k, l), probably due to higher endogenous *ARR7* and *ARR15* expression (Fig. 3i, j, t) that prevents cytokinin response (Fig. 3g). These results suggest that auxin signalling directly induces transcription of *ARR7* and *ARR15* through conserved TGTC elements. The sensitivity of the *ARR7* and *ARR15* promoters to auxin seemed to be confined to early embryogenesis, as expression of *ARR7::GFP* and *ARR15::GFP* in the root tip was undetectable by the upturned-U stage (Supplementary Fig. 3), whereas localized auxin signalling persisted.

A question remained as to the function of *ARR7* and *ARR15*, expressed early in embryogenesis under the control of auxin. No embryo defect was observed in the *arr7* or *arr15* single mutants[8] (Fig. 4a–f, Supplementary Fig. 6a–f and data not shown). The *arr7 arr15* double mutants were reported to cause female gametophytic lethality[8], precluding analysis of embryonic function. We therefore generated conditional double loss-of-function *arr7 arr15* embryos by expressing an ethanol-inducible[24] RNA interference

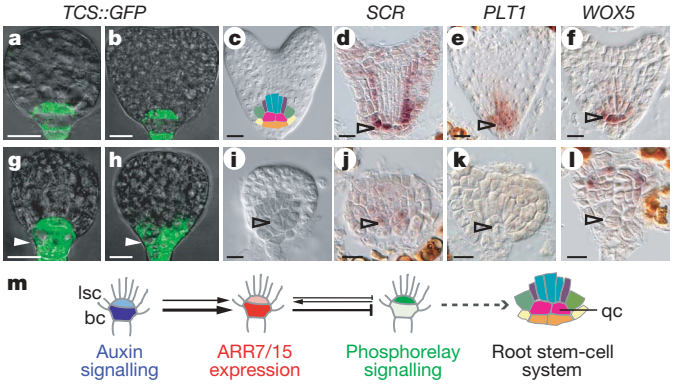


**Figure 4 | Function of differential phosphorelay output for root stem-cell establishment.** **a–l**, Embryos are *arr15*, *RPS5A::AlcA/AlcR::ARR7(RNAi)*. **a–f**, Control embryos with no ethanol. **g–l**, Embryos after *ARR7(RNAi)* induction in the *arr15* mutant background. Induction was for 10 h (**g**), 36 h (**h**) and 60 h (**i–l**). **d–f, j–l**, *In situ* hybridizations. Artificial colours (**c**) denote stem-cell identity, with quiescent centre (qc) in pink. Cells shaded in grey (**i**) have unclear identity. Filled arrowheads point to basal cell lineage (bc); open arrowheads to qc (**d–f, j–l**) or missing qc (**i–l**). **m**, Model for auxin-dependent phosphorelay downregulation in the basal cell lineage. Scale bars represent 10 μm.

construct against *ARR7* (*ARR7(RNAi)*) (Supplementary Fig. 4) in an *arr15* background with or without the *TCS::GFP* reporter (Fig. 4). Control experiments were performed with single *arr15* mutant embryos carrying uninduced *ARR7(RNAi)* (Fig. 4a–f) and ethanol-induced ARR7(*RNAi*) mutant embryos (Supplementary Fig. 6a–f). Ten hours after *ARR7(RNAi)* transgene induction in *arr15* embryos, ectopic phosphorelay output, revealed by *TCS::GFP* expression, was observed in the basal cell lineage (Fig. 4g). After 36 h, in addition to ectopic cytokinin signalling, cell shapes and number became irregular (Fig. 4h). After 60 h, the morphology of the root stem-cell system was severely distorted (Fig. 4i–l), and the attribution of stem-cell identity based on shape and position was ambiguous in the double mutant (Fig. 4i). Furthermore, the expression of key transcription factors required for root stem-cell specification and function, *SCARECROW* (*SCR*)[25], *PLETHORA 1* (*PLT1*)[26] and *WUSCHEL-RELATED-HOMEOBOX 5* (*WOX5*)[27], was abolished or severely reduced (Fig. 4j–l). Eventually, embryo development arrested (not shown). The single mutant control embryos (Fig. 4a–f and Supplementary Fig. 6a–f) did not show any of these phenotypes. These results suggest that loss of both *ARR7* and *ARR15* causes ectopic cytokinin signalling in the basal cell lineage (Fig. 4g), which interferes with the stereotypical cell division pattern (Fig. 4h) and prevents the establishment of normal embryonic pattern, in particular the root stem-cell system, as judged by morphology (Fig. 4i–l) and expression of key marker genes (Fig. 4j–l).

To determine whether directly activating cytokinin signalling in the basal cell lineage also affects stem-cell development, we used the *DR5* promoter to direct the expression of a constitutively active variant of the B-type *ARR10*, most abundantly expressed in early embryos (Fig. 2a), in auxin-signalling cells. Mutation of the aspartate residue at position 69 to glutamate (D69E) mimics the phosphorylated, active state of *ARR10* (ref. 28). Indeed, early embryonic expression of *ARR10(D69E)* in auxin-maximum cells resulted in a phenotype comparable to loss of *ARR7* and *ARR15* function (Supplementary Fig. 6g). Finally, we addressed the requirement of phosphorelay signalling in early embryo development. It has been reported that mutations in three cytokinin receptors have no obvious effect on embryonic pattern formation[5–7]. Residual activity, or phosphorelay activity independent of known cytokinin receptors, might still occur in these conditions. We chose to interfere dominantly with transcriptional activation executed by B-type ARR proteins and converted the abundant (Fig. 2a) positive regulator *ARR10* into a potent dominant-acting transcriptional repressor by adding an EAR repression domain[29] (Supplementary Fig. 5). Induced ubiquitous expression of *ARR10-EAR* in early globular embryos led to strong pattern defects (Supplementary Fig. 6h). As the lens-shaped cell is prominently marked by *TCS::GFP* expression during early embryogenesis (Fig. 2c), the result suggests that its phosphorelay activity is also important for stem-cell specification. Notably, manipulations of cytokinin signalling initiated later, at embryonic heart stage, had no effect on root stem-cell organization (Supplementary Fig. 7). Thus, differential phosphorelay output seems to be transiently required for successful development of the hypophysis-derived daughter cells into an operational root stem-cell system (Fig. 4m).

By combining a new visualization tool and inducible genetic manipulations, we have uncovered a locally and temporally defined antagonistic interaction between auxin and cytokinin that controls the establishment of the first root stem-cell niche. In the prevailing view, A-type response regulators such as *ARR7* and *ARR15* act in the negative-feedback loop to cytokinin signalling. As a result, A-type response regulator levels are in balance with signalling levels, and their expression domains are centred on the pathway output. By contrast, cytokinin activity will be reduced or eliminated where other signals induce A-type response regulators. Thus, gaining control of feedback regulators represents a simple yet effective mechanism to define the output domain of other pathways, and enables dynamic

and quantitative interactions among signalling pathways to promote the complex plant developmental programmes.

## METHODS SUMMARY

**Plasmid constructs.** *TCS* contains six direct repeats of AAAATCTACAA-AATCTTTTTGGATTTTGTGGATTTTCTAGC (core B-type ARR pentamers[10–12] are underlined); negative control *TCS** has six repeats of AAAATGTA-CAAAATGTTTTTGCATTTTGTGCATTTTCTAGC. *TCS* was cloned in front of a minimal 35S promoter with a TATA box[30], followed by the *LUC*, Ω*LUC* or Ω*GFP*[30] coding regions. The integrated reporter construct with the Ω translational enhancer[16] was designated *TCS::GFP*. The constructs *RPS5A::AlcR/AlcA::ARR7*(*RNAi*) and *DR5rev::AlcR/AlcA::ARR10*(*D69E*) were cloned based on a binary vector received from E. Lam (personal communication). For the *ARR7*(*RNAi*) construct, the first exon and intron of the *ARR7* gene were cloned in sense orientation followed by the first exon in antisense orientation.

**Plant material and treatment.** Plants were of the Columbia background and grown at 12 h light/23 °C and 12 h dark/20 °C cycle. *In vitro* embryo culture was performed as described[2]. Ethanol (0.5–1.0%), which had no effect on normal embryogenesis, was used to induce transgene expression. The *arr15* (WISCDSLOX334D02) mutant harbours a T-DNA insertion in the first exon of the *ARR15* gene, 73 bp from the translation start (details in Supplementary Fig. 8). The corresponding seed stock CS851593 was obtained from the *Arabidopsis* Biological Resource Centre (ABRC, USA). *Arabidopsis* protoplasts were isolated and transfected as described previously[14,15].

**Gene expression analysis.** RNA was extracted and amplified from ten pooled embryos, using PicoPure RNA isolation and RiboAMP RNA amplification kits (Arcturus). For expression in wild-type transition-stage embryos, three biological replicates were processed. Quantitative PCR with reverse transcription (qRT–PCR) was performed as described[8] and the amplification of *EIF4A* and *TUB4* served as standards. Primer sequences are provided in Supplementary Table 1.

***In situ* hybridization.** *In situ* hybridization was performed as described previously[8].

**Full Methods** and any associated references are available in the online version of the paper at www.nature.com/nature.

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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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**Author Contributions** B.M. initiated the project, performed the experiments and analysed the data; B.M. and J.S. discussed the results, planned the experiments and wrote the manuscript.

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## METHODS

**Plasmid constructs.** Reporter and effector plasmids used for protoplast transient assays are as previously described[14,15]. The coding regions of *ARR15*, *ARR22* and *ARR20* were obtained from an *Arabidopsis* complementary DNA library by PCR. The *ARR14* gene, the *cis*-regulatory regions of *ARR7* (3 kb), *ARR15* (1.2 kb) and *RPS5A* (1.7 kb)[31] were generated by PCR from genomic DNA. *ARR14*, *ARR15*, *ARR20* and ARR*22* were then cloned into an expression vector as described[14]. Point mutations in the *ARR10* coding region resulting in an aspartate 69 to glutamate mutation, and in the *ARR7* and *ARR15 cis*-regulatory sequences (various TGTC to TGGC mutations) were introduced using the QuikChange Multi Site-Directed Mutagenesis Kit from Stratagene. *ARR7::GFP* and *ARR15::GFP* reporter genes were subcloned into the minibinary vector pCB302 (ref. 32) for plant transformation. To increase the expression levels of reporters, a TMV leader sequence (Ω) stimulating translation[16] was added before the GFP start codon of all GFP constructs. The minimal 35S promoter, Ω and GFP sequence (for *TCS::GFP*), and Ω and GFP sequence (for all other GFP reporters) was amplified by PCR using the *DR5::GFP* (ref. 30) plasmid as a template. The sequences of oligonucleotides used for cloning are provided in Supplementary Table 2. In the ethanol-inducible vector *35S::AlcR/AlcA::*gene of interest (pDM7, gift from E. Lam), the 35S promoter was replaced by the *RPS5A* (ref. 31) or *DR5::Ω* (ref. 30) promoters. After the *AlcA* promoter, *ARR10D69E-GFP*, *ARR10-EAR-GFP* or *ARR7*(*RNAi*) was cloned. The sequences of *ARR7*(*RNAi*), *ARR10*(*D69E*)*-GFP* and *ARR10-EAR-GFP* are provided in Supplementary Table 2. All plasmids were sequenced to ensure that no unwanted mutations were introduced.

**Transgenic plants, embryo and seedling analyses.** Of fifteen independent transgenic *TCS::GFP* lines screened, three lines with consistent and relatively high expression in embryonic root stem cells were chosen for detailed analysis. Of each stage, at least ten embryos per line were analysed with no variations in expression pattern observed. Typically, transgene silencing in *TCS::GFP* transformed lines was observed beginning in the second generation after transformation, leading to an increased fraction of embryos with reduced or absent GFP activity. Five lines showed very weak expression in embryonic root stem cells whereas seven had no detectable expression in root stem cells. At least six transgenic lines for each GFP construct (*ARR7::GFP*, *ARR15::GFP*, or mutated derivatives) were screened. Two *ARR7::GFP* lines showed relatively weak expression, four lines were intermediate and one line showed stronger expression in embryonic root stem cells. An intermediate line was used for the detailed experiments. Three *ARR7m::GFP* lines had no detectable expression in the root stem cells, two exhibited an expression pattern as reported in this work, and one line exhibited stronger expression. The lines with visible expression were tested for auxin-inducibility as shown in Fig. 3k. None of them showed an increase in expression like the *ARR7::GFP* lines. All of the *ARR15::GFP* lines, six in total, similarly showed very weak GFP expression in root stem cells. All seven of the *ARR15m::GFP* lines had undetectable expression in root stem cells. For *in vitro* embryo culture, a few ovules from each silique were dissected to analyse the stage of the embryos before incubation. The remaining ovules were equally distributed between different treatments and control. All tissue culture plates were sealed with parafilm and kept in the dark overnight for hormone treatment, or up to 60 h for ethanol treatment. To assess the consequences on viability, unopened siliques were incubated up to 10 days in medium containing 0.5× Murashige–Skoog, 0.35% phytagar, 2% sucrose, pH 5.7. Ovules were collected in fixative for mRNA *in situ* hybridizations. Embryos were dissected from ovules and mounted in phosphate buffer to analyse GFP activity, cleared and mounted with chloral hydrate to score phenotypes, or collected in extraction buffer for RNA isolation. Ovules from four independent transgenic lines for *RPS5A::AlcR/AlcA::ARR7*(*RNAi*) in wild-type or *arr15* background were treated with 1% ethanol or incubated without ethanol and assayed in parallel. Most of the *ARR7*(*RNAi*) *arr15* embryos (69%) showed strong defects in root stem cells after 60 h treatment when the ethanol induction started at early globular stage ($n = 71$). About 11% of *ARR7*(*RNAi*) embryos ($n = 55$) and 9% of wild-type ovules ($n = 43$) after 60 h treatment with ethanol showed mild aberrations in the root pole, similar to phenotypes reported previously[33]. The low percentage of mild aberrations was due to embryo culture condition but not ethanol treatment (data not shown). Loss of *PLT1*, *SCR* and *WOX5* was only observed in sections derived from ethanol-induced *ARR7*(*RNAi*) *arr15* embryos. For detailed analysis and crosses to *TCS::GFP*, one of the three *TCS::GFP* lines with high expression in root stem cells was chosen. Ovules from four independent transgenic lines of *RPS5A::AlcR/AlcA::ARR10*(*D69E*), *RPS5A::AlcR/AlcA::ARR10-EAR* and *DR5::AlcR/AlcA::ARR10*(*D69E*) were treated with 0.5% ethanol. Strong phenotypes were observed in 80% of embryos analysed ($n > 40$). Treatment with 1% ethanol increased the severity of the phenotypes in the mutants but not wild-type embryos. To reduce endogenous cytokinin production, lovastatin, a potent inhibitor of the mevalonate pathway[20,34], was prepared as described previously[35] and added to seedlings grown in liquid culture medium (half-strength Murashige–Skoog medium, 1% sucrose, pH 5.7).

***In situ* hybridizations.** The *SCR*, *PLT1* and *WOX5* riboprobes were as described[26,27,36]; the *ARR7* probe comprised the complete *ARR7* translated sequence. Ovules were fixed at 4 °C with 4% paraformaldehyde in PBS for 8 h after vacuum infiltration. The tissue was dehydrated and embedded in paraplast plus. Eight-micrometre sections were placed on SuperFrost-Plus slides. Paraplast was removed by immersion in Histoclear. Sections were rehydrated, incubated for 30 min at 37 °C with 1 μg ml$^{-1}$ proteinase K in TE (50 mM Tris-HCl pH 8, 50 mM EDTA), 10 min in 4% paraformaldehyde in PBS and 10 min in 0.5% acetic anhydride in 0.1 M triethanolamine, pH 8. After dehydration by an ethanol series, slides were air-dried before application of the hybridization solution. Per slide, 50–200 ng labelled riboprobe (probe was hydrolysed in case of *ARR7*) was applied in 80 μl hybridization solution. After incubation in a humidified box at 58 °C overnight (72 h for *ARR7*), slides were washed twice with 2×SSC in 50% formamide for 1 h at 58 °C. Slides were then washed twice in NTE (500 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) at 37 °C for 5 min each, immersed in preheated (37 °C) buffer 1 (100 mM Tris-HCl pH 7.5, 150 mM NaCl) and then cooled to room temperature. Antibody solution (anti-digoxigenin-alkaline-phosphatase-coupled antibody, diluted 1:2,000 in buffer 1 with 1% blocking reagent) was applied for 2 h. Slides were washed twice for 10 min with 100 mM Tris pH 9.5, 100 mM NaCl. 200 μl of fresh staining solution (10% (w/v) polyvinylalcohol 70–100 kDa, 5 mM MgCl, 0.2 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.2 mM nitroblue tetrazolium salt, 100 mM Tris pH 9.5 and 100 mM NaCl) was added to each slide. Staining occurred over 4 h (72 h for *ARR7* probe) in a humidified box in the dark. Slides were finally washed in water, de- and re-hydrated in ethanol series, and then mounted in 50% glycerol.

**Microscopy and imaging.** GFP expression was recorded in parallel with transmitted light using a Leica SP2 confocal scanning microscope. Signals were combined in Adobe Photoshop CS3. On the basis of qRT–PCR analysis, the *ARR15* transcript was about 20-fold lower than *ARR7* transcript in transition-stage embryos (Fig. 2a). To visualize the low levels of *ARR15::GFP*, we maximised the sensitivity of the confocal microscope by increasing the signal gain. Embryo sections and cleared whole-mount preparations were recorded with a Leica DFC500 digital camera mounted to a Leica DM5000 microscope.


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