Published in final edited form as:
Nature. 2008 June 19; 453(7198): 1094–1097. doi:10.1038/nature06943.

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

Bruno Müller and **Jen Sheen**
Department of Molecular Biology, Massachusetts General Hospital, Department of Genetics, Harvard Medical School, Boston, Massachusetts 02114, USA

Abstract

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 plays a critical role for 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 universally visualise cytokinin output in vivo. Surprisingly, the first embryonic signal is detected in the hypophysis, the founder cell of the root stem-cell system. Its apical daughter cell, precursor of the quiescent centre, maintains phosphorelay activity, whereas the basal daughter cell represses signalling output. Intriguingly, auxin activity levels exhibit the inverse profile. We show that auxin antagonizes cytokinin output in the basal cell-lineage by direct transcriptional activation of *ARABIDOPSIS RESPONSE REGULATOR (ARR)* 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. The 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 play many essential roles in postembryonic growth and development. However, the role of cytokinin signalling in early embryogenesis remained unclear[4-6]. To visualise cytokinin's signalling output in vivo, we aimed at designing a synthetic reporter that overcomes 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 (RRs) mediate transcriptional activation in response to phosphorelay signalling activity, while A-type RRs repress signalling in a negative-feedback loop. The DNA-binding domains of diverse B-type RR 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 optimised 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 concatemerised B-type ARR-binding motifs[10-12] and a minimal *35S* promoter[14]. Only cytokinins activated *TCS-LUC*, while other plant

Correspondence and requests for materials should be addressed to J.S. (e-mail: sheen@molbio.mgh.harvard.edu) and B.M. (e-mail: mueller@molbio.mgh.harvard.edu).

The authors declare no competing interests.

hormones, 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 was 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, co-expression 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 to the native *ARR6* promoter[14] (Fig. 1f). Addition of a viral translational enhancer (Ω)[16] amplified the response further (Fig. 1f). Taken together, *TCS-LUC* could specifically report even low levels of phosphorelay output triggered by any of 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 that were subjected to a short-term incubation with the cytokinin-synthesis inhibitor lovastatin[21] abolished *TCS::GFP* expression (Supplementary Fig. 2b,e) Importantly,*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. Since 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 32-cell stage (Fig. 1h). By the transition stage, the hypophysis has undergone an asymmetrical cell division. Intriguingly, the resulting large basal daughter cell and its descendants repressed *TCS::GFP* expression, while the apical lens-shape cell retained its expression (Fig. 1j). 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 especially interested in the expression patterns of the A-type *ARRs*, as they represent direct targets commonly used as markers of cytokinin signalling. *ARR7* mRNA was abundant at the transition stage. *ARR7::GFP* transgenic lines displayed first GFP activity at the late globular stage, just after the asymmetric division of the hypophysis. Surprisingly, *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 hybridisation 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). The results suggested that additional input might control *ARR7* and *ARR15* expression.

Interestingly, auxin signalling output visualised by the synthetic reporter *DR5::GFP*[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*, that 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 were treated with the synthetic auxin analogue 2,4-D[2] (Fig. 3g–l). Incubation with 2,4-D caused 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), while *TCS::GFP* activity was abolished (arrowhead, 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, and supported a model where high endogenous auxin activity suppressed cytokinin output via stimulation of *ARR7* and *ARR15* transcription (Fig. 4m).

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 suggested that only the first four nucleotides are essential for auxin response factor (ARF) binding[22]. Therefore, we screened the promoters of *ARR7* and *ARR15* for the TGTC motif. We found 32 occurrences for *ARR7* and 8 for *ARR15* (Fig. 3u). Previously characterised 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 30bp 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* showed strongly reduced expression in the auxin signalling domain (filled arrowhead Fig. 3e, f). Furthermore, ectopic auxin signalling was unable to stimulate their expression (arrowheads Fig. 3k, l). In contrast, they retained responsiveness to cytokinin (Fig. 3q, r). Intriguingly, 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* (Fig. 3k, l), presumably due to higher endogenous *ARR7* and *ARR15* expression (Fig. 3i, j, t) that prevents cytokinin response (Fig. 3g). The results suggested that auxin signalling directly induces transcription of *ARR7* and *ARR15* via 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 localised auxin signalling persisted.

What is 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 (ref. 8›Supplementary Fig. 6a–f, Fig. 4a–f, and data not shown). The *arr7 arr15* double mutants were reported to cause female gamethophytic lethality[8], precluding analysis of embryonic function. Therefore, we generated conditional double loss-of-function *arr7 arr15* embryos by expressing an ethanol-inducible[24] RNA interference construct against *ARR7* (*ARR7-i*) (Supplementary Fig. 4) in *arr15* background with or without the *TCS::GFP* reporter (Fig. 4). Control experiments were performed with single *arr15* mutant embryos carrying un-induced *ARR7-i* (Fig. 4a–f) and ethanol-induced *ARR7-i* mutant embryos (Supplementary Fig. 6a–f). Ten hours after *ARR7-i* transgene induction in *arr15* embryos, ectopic phosphorelay output, revealed by *TCS::GFP* expression, was observed in the basal cell lineage (Fig. 4g). After 36 hours, in addition to ectopic cytokinin signalling, cell shapes and number became irregular (Fig. 4h). Later, after 60 hours, 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], *PLETHORA1* (*PLT1*)[26] and *WUSCHEL-RELATED-HOMEOBOX5* (*WOX5*)[27], was abolished or severely reduced (Fig. 4j–l). Eventually, embryo development arrested (not shown). The single mutant control embryos (Fig. 4a–f, Supplementary Fig. 6a–f), did not show any of these phenotypes. These results suggested 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 test 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*[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 had no obvious effect on embryonic pattern formation[5-7]. Residual, or phosphorelay activity independent of known cytokinin receptors might still occur in these conditions. We chose to dominantly interfere 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 suggested its phosphorelay activity is also important for stem cell specification. Interestingly, manipulations of cytokinin signalling initiated later, at embryonic heart stage, had no obvious effect on root stem cell organization (Supplementary Fig. 7). Thus, differential phosphorelay output seems transiently required for successful development of the hypophysis-derived daughter cells into an operational root stem-cell system (Fig. 4m).

By combining a new visualisation 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 RRs such as *ARR7* and *ARR15* act in the negative feedback loop to cytokinin signalling. As a result, A-type RR levels are in balance with signalling levels, and their expression domains are centred on the pathway output. In contrast, cytokinin activity will be reduced or eliminated where other signals induce A-type RR. 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 programs.

METHODS SUMMARY

Plasmid constructs

TCS contains 6 direct repeats of aaAATCTacaaAATCTttttGGATT-ttgtGGATTttctagc (core B-type ARR pentamers[10-12] in capital letters), negative control *TCS** has 6 repeats of aaAATgTacaaAATgTttttGcATTttg-tGcATTttctagc. *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-i* and $DR5rev\text{-}AlcR/AlcA\text{-}ARR10^{D69E}$, were cloned based on a binary vector received from E. Lam (personal communication). For

the *ARR7*-i 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 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 in ref. [2]. Ethanol (0.5-1.0%) with 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, after bp 73 from the translation start (details in Supplementary Fig. 8). The corresponding seed stock CS851593 was obtained from the Arabidopsis Biological Resource Center (ABRC, USA). Arabidopsis protoplasts were isolated and transfected as described in refs. [14,15].

Quantitative RT-PCR

RNA was extracted and amplified from ten pooled embryos, using PicoPure RNA isolation and RiboAMP RNA amplification kits (Arcturus, Mountain View, CA, USA). For expression in wild-type transition-stage embryos, three biological replicates were processed. qRT-PR was performed as described in ref. [8]. Amplification of *EIF-4A* and *TUB4* served as standards. Primer sequences are provided in Supplementary Information (Table 1).

In situ hybridisation

In situ hybridisation was performed as in ref. [8].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank T. Kakimoto and C. Ueguchi for providing *ahk* mutant seeds, J. Friml for the *DR5-GFP* plasmid and *DR5::GFP* seeds, E. Lam for providing the AlcA/AlcR vector, A. Jazwinska for help with mRNA in-situ hybridisations, and S. Riku for help with plant growth and protoplast experiments. We are grateful to C. Ping and S. Howell, to Y. Guo, Y. Tan and G. Selvaraj, T. Mizuno, J. Zuo; D. Jackson, and to J. To for sharing unpublished results. This work was supported by a Fellowship for Prospective Researchers by the Swiss National Science Foundation (SNF), a Long Term Fellowship of the International Human Frontier Science Program (HFSP) organization to B.M., and grants from the National Science Foundation and National Institutes of Health to J.S.

References

1. Skoog F, Miller CO. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp Soc Exp Biol 1957;54:118–130. [PubMed: 13486467]
2. Friml J, Vieten A, Sauer M, Weijers D, Schwarz H, Hamann T, Offringa R, Jürgens G. Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. Nature 2003;426:147–153. [PubMed: 14614497]
3. Weijers D, Jürgens G. Auxin and embryo axis formation: the ends in sight? Curr Opin Plant Biol 2005;8:32–37. [PubMed: 15653397]
4. Riefler M, Novak O, Strnad M, Schmülling T. Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell 2006;18:40–54. [PubMed: 16361392]
5. Nishimura C, Ohashi Y, Sato S, Kato T, Tabata S, Ueguchi C. Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in Arabidopsis. Plant Cell 2004;16:1365–1377. [PubMed: 15155880]
6. Higuchi M, et al. In planta functions of the Arabidopsis cytokinin receptor family. Proc Natl Acad Sci U S A 2004;101:8821–8826. [PubMed: 15166290]

7. To JP, Haberer G, Ferreira FJ, Deruere J, Mason MG, Schaller GE, Alonso JM, Ecker JR, Kieber JJ. Type-A Arabidopsis response regulators are partially redundant negative regulators of cytokinin signaling. Plant Cell 2004;16:658–671. [PubMed: 14973166]
8. Leibfried A, To JP, Busch W, Stehling S, Kehle A, Demar M, Kieber JJ, Lohmann JU. WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature 2005;438:1172–1175. [PubMed: 16372013]
9. Müller B, Sheen J. Advances in cytokinin signaling. Science 2007;318:68–69. [PubMed: 17916725]
10. Sakai H, Aoyama T, Oka A. Arabidopsis ARR1 and ARR2 response regulators operate as transcriptional activators. Plant J 2000;24:703–711. [PubMed: 11135105]
11. Hosoda K, Imamura A, Katoh E, Hatta T, Tachiki M, Yamada H, Mizuno T, Yamazaki T. Molecular structure of the GARP family of plant Myb-related DNA binding motifs of the Arabidopsis response regulators. Plant Cell 2002;14:2015–2029. [PubMed: 12215502]
12. Imamura A, Kiba T, Tajima Y, Yamashino T, Mizuno T. In vivo and in vitro characterization of the ARR11 response regulator implicated in the His-to-Asp phosphorelay signal transduction in Arabidopsis thaliana. Plant Cell Physiol 2003;44:122–131. [PubMed: 12610214]
13. Rashotte AM, Carson SD, To JP, Kieber JJ. Expression profiling of cytokinin action in Arabidopsis. Plant Physiol 2003;132:1998–2011. [PubMed: 12913156]
14. Hwang I, Sheen J. Two-component circuitry in Arabidopsis cytokinin signal transduction. Nature 2001;413:383–389. [PubMed: 11574878]
15. Yoo SD, Cho YH, Sheen J. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc 2007;2:1565–1572. [PubMed: 17585298]
16. Gallie DR. The 5'-leader of tobacco mosaic virus promotes translation through enhanced recruitment of eIF4F. Nucleic Acids Res 2002;30:3401–3411. [PubMed: 12140325]
17. D'Agostino IB, Deruere J, Kieber JJ. Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. Plant Physiol 2000;124:1706–1717. [PubMed: 11115887]
18. Aloni R, Langhans M, Aloni E, Ullrich CI. Role of cytokinin in the regulation of root gravitropism. Planta 2004;220:177–182. [PubMed: 15365840]
19. Mähönen AP, et al. Cytokinin signaling and its inhibitor AHP6 regulate cell fate during vascular development. Science 2006;311:94–98. [PubMed: 16400151]
20. Lohar DP, Schaff JE, Laskey JG, Kieber JJ, Bilyeu KD, Bird DM. Cytokinins play opposite roles in lateral root formation, and nematode and Rhizobial symbioses. Plant J 2004;38:203–214. [PubMed: 15078325]
21. Orchard CB, et al. Tobacco BY-2 cells expressing fission yeast cdc25 bypass a G2/M block on the cell cycle. Plant J 2005;44:290–299. [PubMed: 16212607]
22. Ulmasov T, Hagen G, Guilfoyle TJ. Dimerization and DNA binding of auxin response factors. Plant J 1999;19:309–319. [PubMed: 10476078]
23. Guilfoyle T, Hagen G, Ulmasov T, Murfett J. How does auxin turn on genes? Plant Physiol 1998;118:341–347. [PubMed: 9765520]
24. Roslan HA, et al. Characterization of the ethanol-inducible alc geneexpression system in Arabidopsis thaliana. Plant J 2001;28:225–235. [PubMed: 11722766]
25. Sabatini S, Heidstra R, Wildwater M, Scheres B. SCARECROW is involved in positioning the stem cell niche in the Arabidopsis root meristem. Genes Dev 2003;17:354–358. [PubMed: 12569126]
26. Aida M, et al. The PLETHORA genes mediate patterning of the Arabidopsis root stem cell niche. Cell 2004;119:109–120. [PubMed: 15454085]
27. Sarkar AK, Luijten M, Miyashima S, Lenhard M, Hashimoto T, Nakajima K, Scheres B, Heidstra R, Laux T. Conserved factors regulate signalling in Arabidopsis thaliana shoot and root stem cell organizers. Nature 2007;446:811–814. [PubMed: 17429400]
28. Hass C, et al. The response regulator 2 mediates ethylene signalling and hormone signal integration in Arabidopsis. EMBO J 2004;23:3290–3302. [PubMed: 15282545]
29. Hiratsu K, Matsui K, Koyama T, Ohme-Takagi M. Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in Arabidopsis. Plant J 2003;34:733–739. [PubMed: 12787253]

30. Ottenschläger I, Wolff P, Wolverton C, Bhalerao RP, Sandberg G, Ishikawa H, Evans M, Palme K. Gravity-regulated differential auxin transport from columella to lateral root cap cells. Proc Natl Acad Sci U S A 2003;100:2987–2991. [PubMed: 12594336]

31. Weijers D, Franke-van Dijk M, Vencken RJ, Quint A, Hooykaas P, Offringa R. An Arabidopsis Minute-like phenotype caused by a semi-dominant mutation in a RIBOSOMAL PROTEIN S5 gene. Development 2001;128:4289–4299. [PubMed: 11684664]

32. Xiang C, Han P, Lutziger I, Wang K, Oliver DJ. A mini binary vector series for plant transformation. Plant Mol Biol 1999;40:711–717. [PubMed: 10480394]

33. Sauer M, Friml J. In vitro culture of Arabidopsis embryos within their ovules. Plant J 2004;40:835–843. [PubMed: 15546365]

34. Laureys F, Dewitte W, Witters E, Van Montagu M, Inze D, Van Onckelen H. Zeatin is indispensable for the G2-M transition in tobacco BY-2 cells. FEBS Lett 1998;426:29–32. [PubMed: 9598972]

35. Laule O, Furholz A, Chang HS, Zhu T, Wang X, Heifetz PB, Gruissem W, Lange M. Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in Arabidopsis thaliana. Proc Natl Acad Sci U S A 2003;100:6866–6871. [PubMed: 12748386]

36. Di Laurenzio L, Wysocka-Diller J, Malamy JE, Pysh L, Helariutta Y, Freshour G, Hahn MG, Feldmann KA, Benfey PN. The SCARECROW gene regulates an asymmetric cell division that is essential for generating the radial organization of the Arabidopsis root. Cell 1996;86:423–433. [PubMed: 8756724]

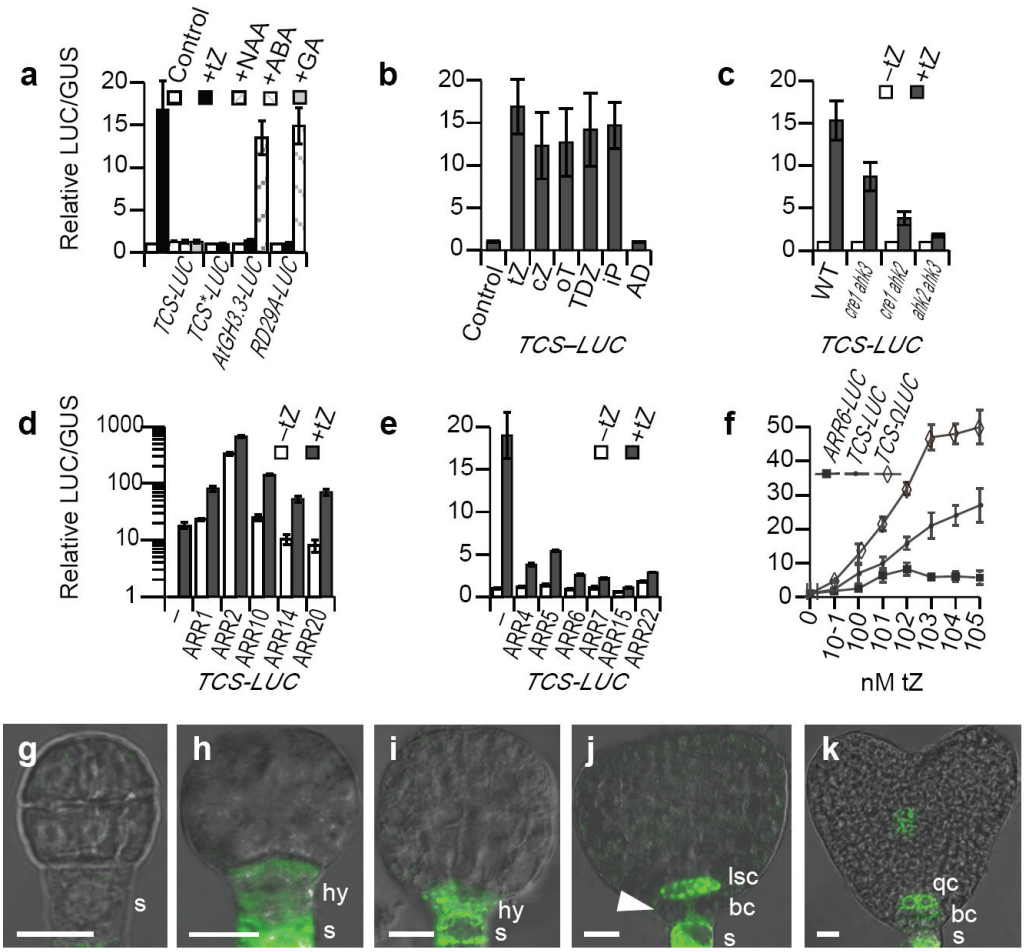

Figure 1. Sensitive and specific response of *TCS*

a, 100 nM trans-zeatin (tZ) induces *TCS-LUC*, not 1 μM auxin (NAA), 100μM abscisic acid (ABA), or 50 μM gibberellic acid (GA). *TCS**-*LUC* negative control. **b**, *TCS-LUC* induced by cZ, cis-zeatin; oT, ortho-topolin; TDZ, thidiazuron; iP, N^6-(Δ^2-isopentenyl)adenine; AD, adenine (negative control), all at 100 nM. **c–e**, *TCS-LUC*induction by tz (**c**) reduced in double-mutant *ahk2–2, ahk3–3, cre1–1*[6] Combinations, (**d**) stimulated by B-type ARRs, (**e**) reduced by A-type ARRs. **f**, Dose responses. **g–k**, Embryonic *TCS::GFP* activity. s, suspensor; hy, hypophysis; lsc, lens-shaped cell; bc, basal cell lineage; qc, quiescent centre. Error bars s.d. (n=3), scale bars 10μM.

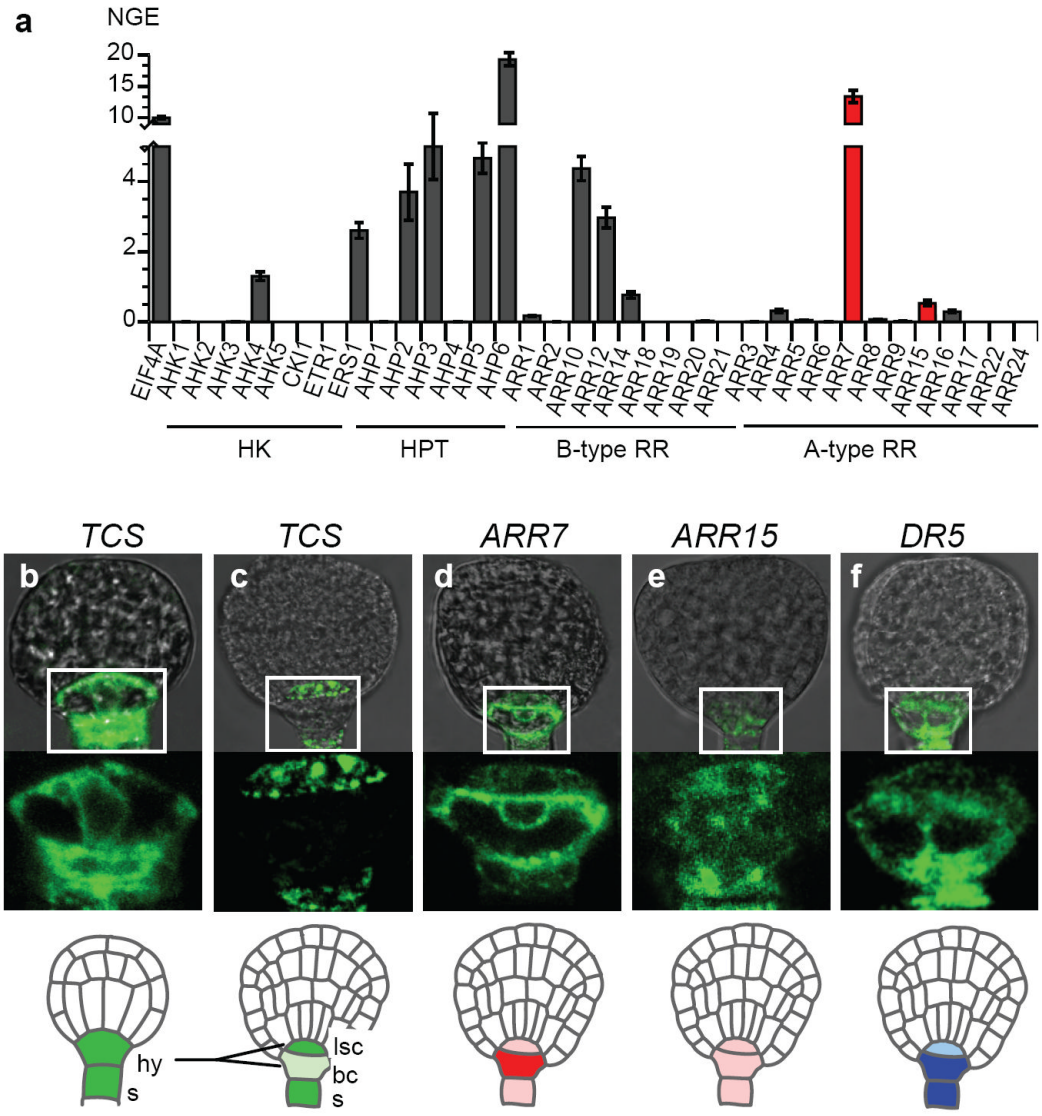

Figure 2. Inverse correlation between cytokinin and auxin signalling

a, Two-component gene transcription by quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR) at transition stage. Error bars s.d. (n=3). **b**, *TCS::GFP* in hy at early globular stage. **c**, Downregulation in bc at late globular stage. **d,** First *ARR7::GFP* expression peaks in bc. **e**, *ARR15::GFP* expression. **f**, *DR5::GFP* activity highest in bc. Zoomed view in middle row. Schematic interpretation in bottom row. NGE, normalized gene expression; HK/AHK, Arabidopsis histidine kinases; HPT/AHP, histidine-phosphotransfer proteins; RR/ARR, response regulators; CKI1, cytokinin independent 1; ETR1, ethylene response 1; ERS1, ethylene response sensor 1.

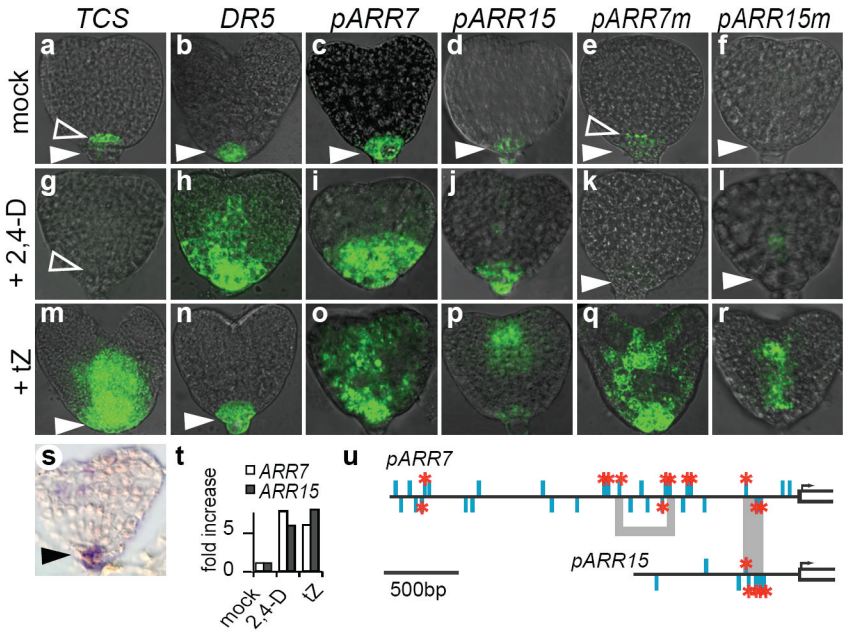

Figure 3. *ARR7* and *ARR15* mediate auxin control of cytokinin signalling

a–r, Over-night treatment with water, 50 μM 2,4-D or tZ. *pARR7m* (**e, k, q**) *pARR15m* (**f, l, r**) explained in (**u**). Filled arrowheads denote bc, open arrowheads qc. **s**, In situ hybridisation to *ARR7* mRNA. **t**, *ARR7* and *ARR15* mRNA induction assayed by qRT-PCR after 4-hour treatment of embryos with 1 μM tZ or 2,4-D. **u**, *ARR7* and *ARR15* promoters. TGTC on sense and antisense strands as blue bars. Red asterisks: TGTC -> TGgC. Grey shade connects conserved regions; see text for detail.

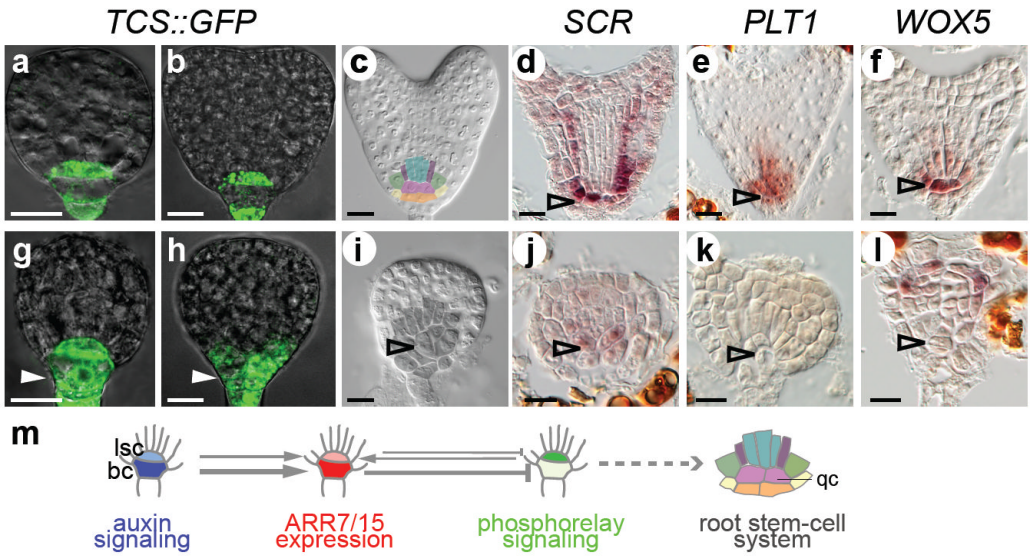

Figure 4. Function of differential phosphorelay output for root-stem cell establishment

a–l, Embryos are *arr15*, *RPS5a::AlcA AlcR::ARR7-i*. **a–f**, Control embryos, no ethanol. **g–l**, Embryos after *ARR7-i* induction in the *arr15* mutant background. Induction for 10 h (**g**), 36 h (**h**) and 60 h (**i–l**). **d–f**, **j–l**, In situ hybridisations. Artificial colours (**c**) denote stem-cell identity, with qc in pink. Cells shaded in grey (i) have unclear identity. Filled arrowheads point to bc, open arrowheads to qc (**d–f, j–l**), or missing qc (**i–l**). **m**, Model for auxin-dependent phosphorelay downregulation in bc. Scale bars 10 μm.