METHODS

Plasmid constructs. Reporter- and effector plasmids used for protoplast transient assays are described in refs. 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*[31] were generated by PCR from genomic DNA. *ARR14, ARR15, ARR20* and ARR*22* were then cloned into an expression vector as described in ref. 14. Point mutations in the *ARR10* coding region from aspartate 69 to glutamate, and in the *ARR7* and *ARR15* cis-regulatory sequences from various TGTC to TGgC 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[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, GFP sequence (for all other GFP reporters) was amplified by PCR using the DR5-GFP[30] plasmid as a template. The sequences of oligonucleotides used for cloning are provided in Supplementary Table 2. In the ethanol-inducible vector *p35S-AlcR, pAlcA-gene of interest* (pDM7, gift from E. Lam), the 35S promoter was replaced by the *pRPS5a*[31] or *DR5-Ω*[30] promoters. After the *pAlcA* promoter, *ARR10D69E-GFP*, *ARR10-EAR-GFP* or *ARR7*-i was cloned. Sequence of the *ARR7*-i, *ARR10*^{D69E}*-GFP* and *ARR10-EAR-GFP* are provided in Supplementary Table 2. All plasmid were sequenced to ensure 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 while 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 weak expression, four lines were intermediate and one line showed strong 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 meristem, two exhibited an expression pattern as reported in this work, and one line exhibited somewhat stronger expression. The lines with visible expression were tested for auxin-inducablity as shown Fig. 3k. None of them showed an increase in expression like the *ARR7::GFP* lines. All of *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 analyze the stage of the embryos prior to 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 0.5x MS, 0.35% Phytagar, 2% sucrose, pH adjusted to 5.7. Ovules were collected in fixative for mRNA in-situ hybridizations. Embryos were dissected from ovules and mounted in phosphate buffer to analyze 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-i in wild-type or *arr15* background were treated with 1% ethanol or incubated without ethanol and assayed in parallel. The majority of *ARR7-i arr15* embryos (69%) after 60 h treatment showed strong defects in root stem cells when the ethanol induction started at early globular stage (n=71). About 11% *ARR7-i* 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 in ref. 33. The low percentage of mild aberrations were 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-i 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-ARR10EAR, DR5rev-AlcR/AlcA-ARR10*^{D69E} were treated with 0.5% ethanol. Strong phenotypes were observed in 80% of embryos analyzed (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 in ref. 35 and added to seedlings grown in liquid culture medium (half-strength Murashige and Skoog medium, 1% sucrose, pH 5.7).

In situ hybridisations. The *SCR, PLT1* and *WOX5* riboprobes were the same as in references [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 hr after vacuum infiltration. The tissue was dehydrated and embedded in Paraplast Plus. Eight-micrometer sections were placed on SuperFrost-Plus slides. Paraplast was removed by immersion in Histoclear. Sections were rehydrated, incubated 10 min with 1 µg/ml Proteinase K in TE (50 mM Tris-HCl [pH 8], 50 mM EDTA) at 37°C, 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 humid box at 58°C overnight (72 h for *ARR7*), slides were washed twice in 2x SSC/50% formamide for 1 hr at 58°C. Slides were again washed in NTE (500 mM NaCl; 10 mM Tris-HCl, pH7.5; 1 mM EDTA) at 37°C, 2 times for 5 min each, then immersed in preheated (37°C) buffer 1 (100 mM Tris-HCl; pH 7.5; 150 mM NaCl) and cooled to room temperature. Antibody solution (buffer1, 1% blocking reagant, Anti-Digoxigenin-alkaline-phosphatase-coupled antibody, diluted 1/2000) was applied for 2 h. Slides were washed twice for ten minutes with 100mM Tris (pH 9.5), 100 mM NaCl. 200µl of fresh staining solution (10% [w/v] polyvinylalcohol 70 – 100 kD; 5 mM MgCl; 0.2 mM 5-bromo-4-chloro- 3-indolyl phosphate [BCIP]; 0.2 mM nitroblue tetrazolium salt [NB]; 100mM Tris (pH 9.5), 100 mM NaCl) was added to each slide. Staining was 4 h (72 h for *ARR7* probe) in a humid box in the dark. Slides were washed in water, de- and re-hydrated in ethanol series, 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. Based on qRT-PCR analysis, *ARR15* transcript was about 20-fold lower than *ARR7* transcript in transition-stage embryos (Fig. 2a). To visualise 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.