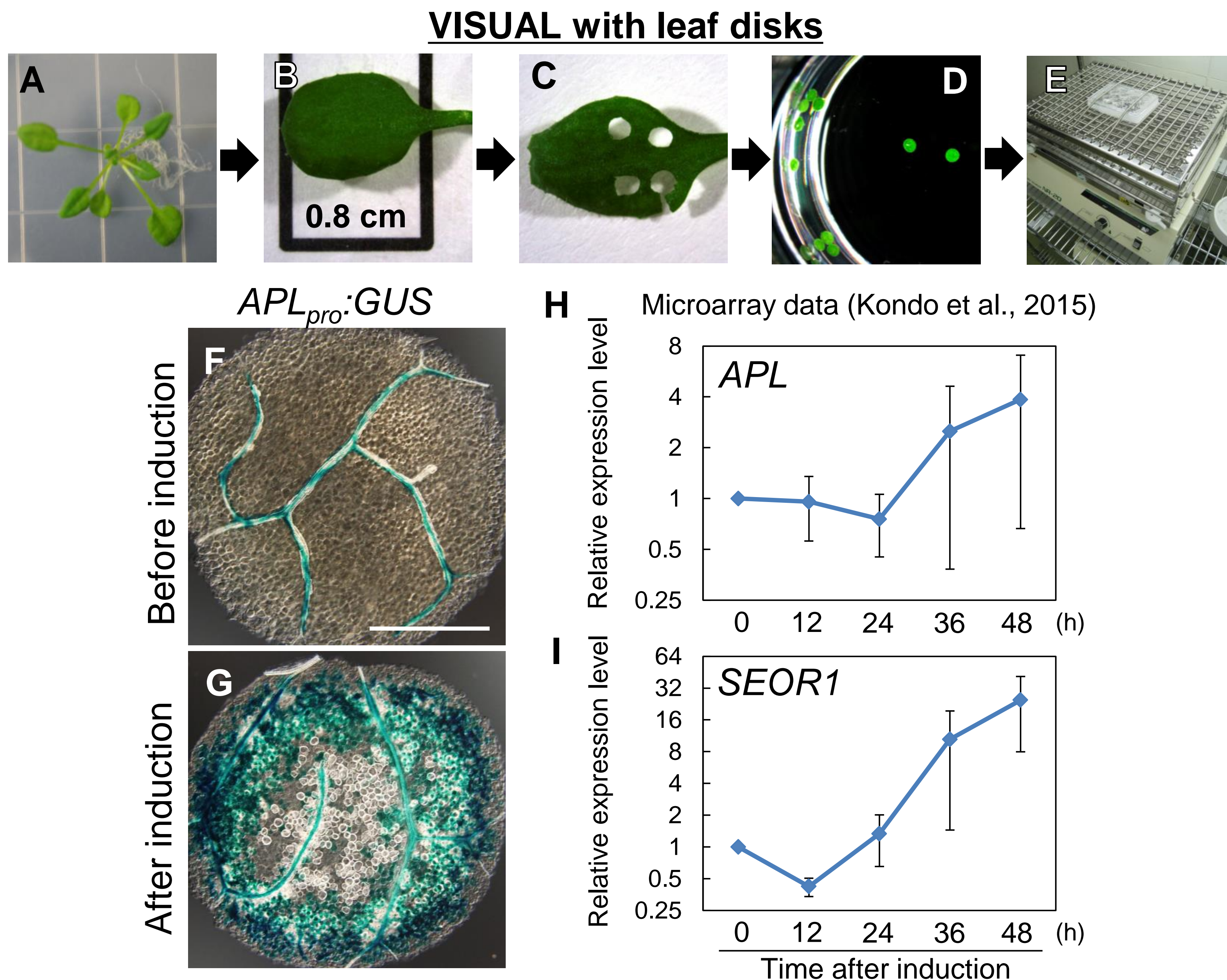
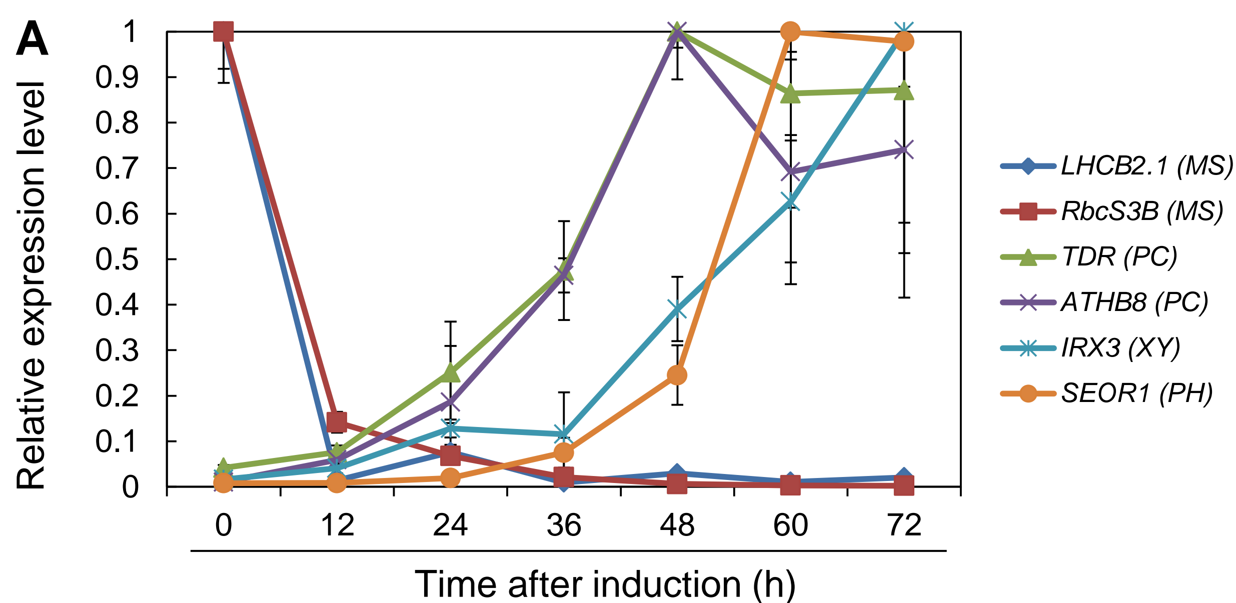
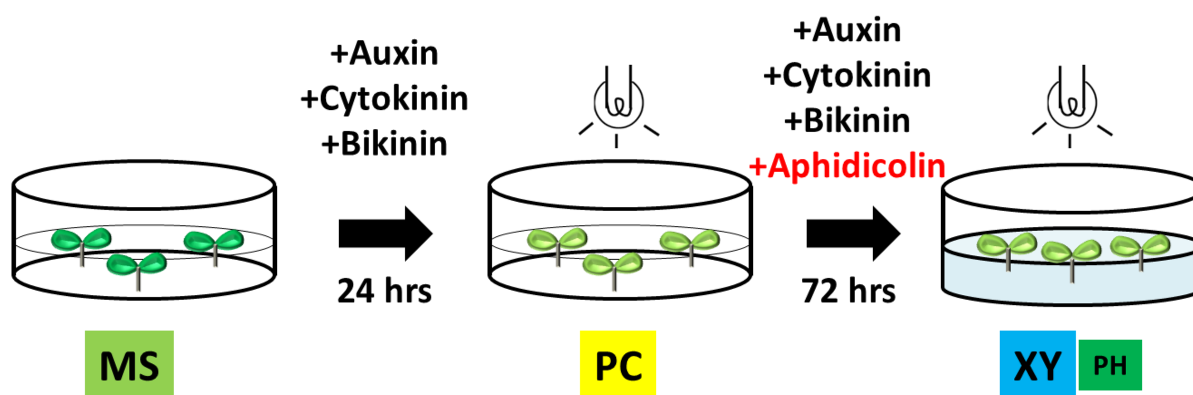
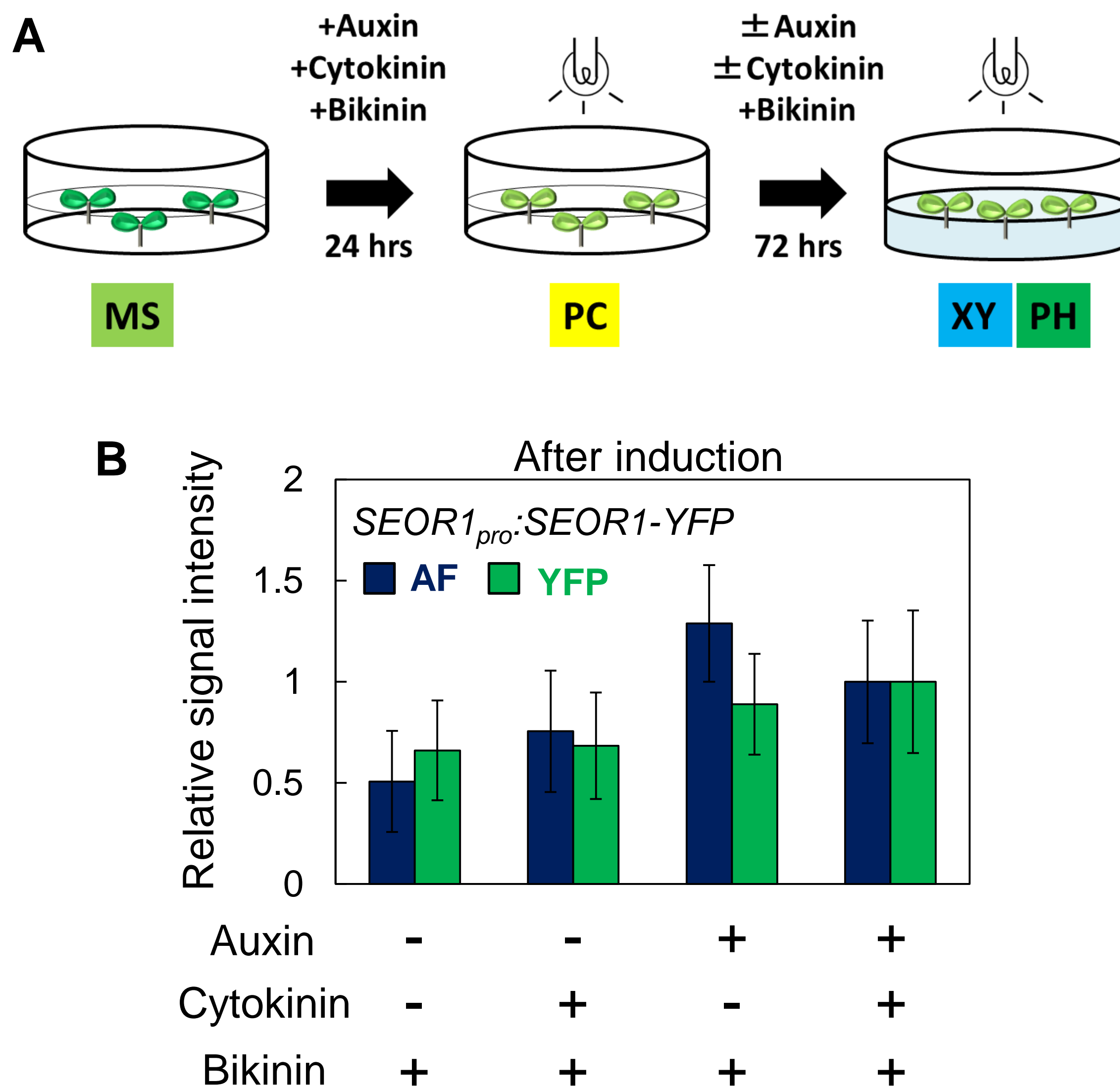


Supplemental Figure 1. Phloem Marker Expression in Leaf Disks Revealed by VISUAL
(A–E) Workflow of VISUAL with leaf disks. (A) *Arabidopsis* plants grown on MS agar plates for 3–4 weeks under continuous light. (B) Rosette leaf with proper leaf blade length (0.8–1.0 cm) for VISUAL. (C) Leaf disks made with a biopsy-punch from the basal part of the excised leaf. (D) Prepared leaf disks floated on liquid induction medium. (E) Cultivation of leaf disks under continuous light in a 12-well plate containing induction medium. (F and G) Expression of *APLpro:GUS* before (F) and after (G) induction. Scale bar indicates 500 µm. (H and I) Expression of phloem marker genes in VISUAL. Time-course changes in expression levels of *APL* (H) and *SEOR1* (I) were calculated from microarray data from a previous report (Kondo et al., 2015). Error bars indicate SD (n = 3; biological replicates).

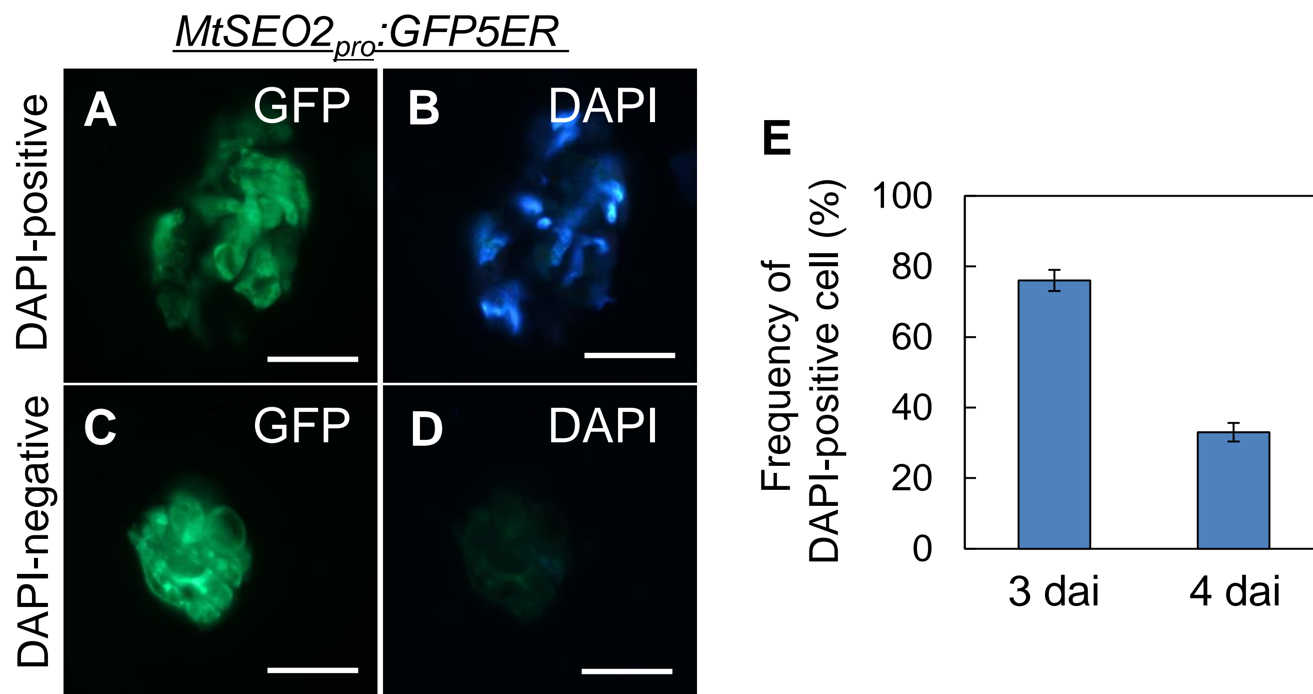




Supplemental Figure 2. Experimental Procedure for Aphidicolin Assay
(A) Expression profiles of several marker genes in cotyledons using VISUAL. Time-course changes in expression levels of genes related to mesophyll (*LHCB2.1* and *RbcS3B*), procambium (*TDR* and *ATHB8*), xylem (*IRX3*) and phloem (*SEOR1*) were calculated by RT-qPCR. Relative expression levels were calculated by comparison with the maximum expression level for each individual gene. Error bars indicate SD (n = 3; biological replicates). (B) Experimental procedure for aphidicolin application assay. First, aerial parts of seedlings were precultured in induction medium containing auxin, cytokinin and bikinin for 24 hours to induce reprogramming from mesophyll cells (MS) to procambial cells (PC). After preincubation, aphidicolin was added to the induction medium at various concentrations, and the cotyledons were further cultured for 72 hours to examine the differentiation into xylem cells (XY) and phloem cells (PH).



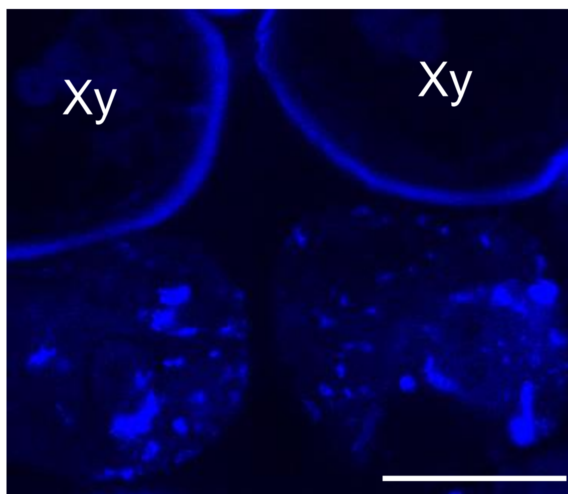
Supplemental Figure 3. Effects of Auxin and Cytokinin on Xylem and Phloem Cell Differentiation Revealed by VISUAL
(A) Experimental procedure for testing the effects of hormones on cell differentiation via VISUAL. Aerial parts of seedlings were pre-cultured in induction medium containing auxin, cytokinin and bikinin for 24 hours to induce procambial cell (PC) formation. Samples were then transferred to various types of media (± auxin, ± cytokinin, and bikinin) and cultured for 72 hours. (B) Quantification of fluorescence signal intensities from *SEOR1pro:SEOR1-YFP* cotyledons cultured in various types of media. Error bars indicate SD (n = 12; number of analyzed cotyledons).



Supplemental Figure 4. Enucleation during SE-like Cell Differentiation in VISUAL

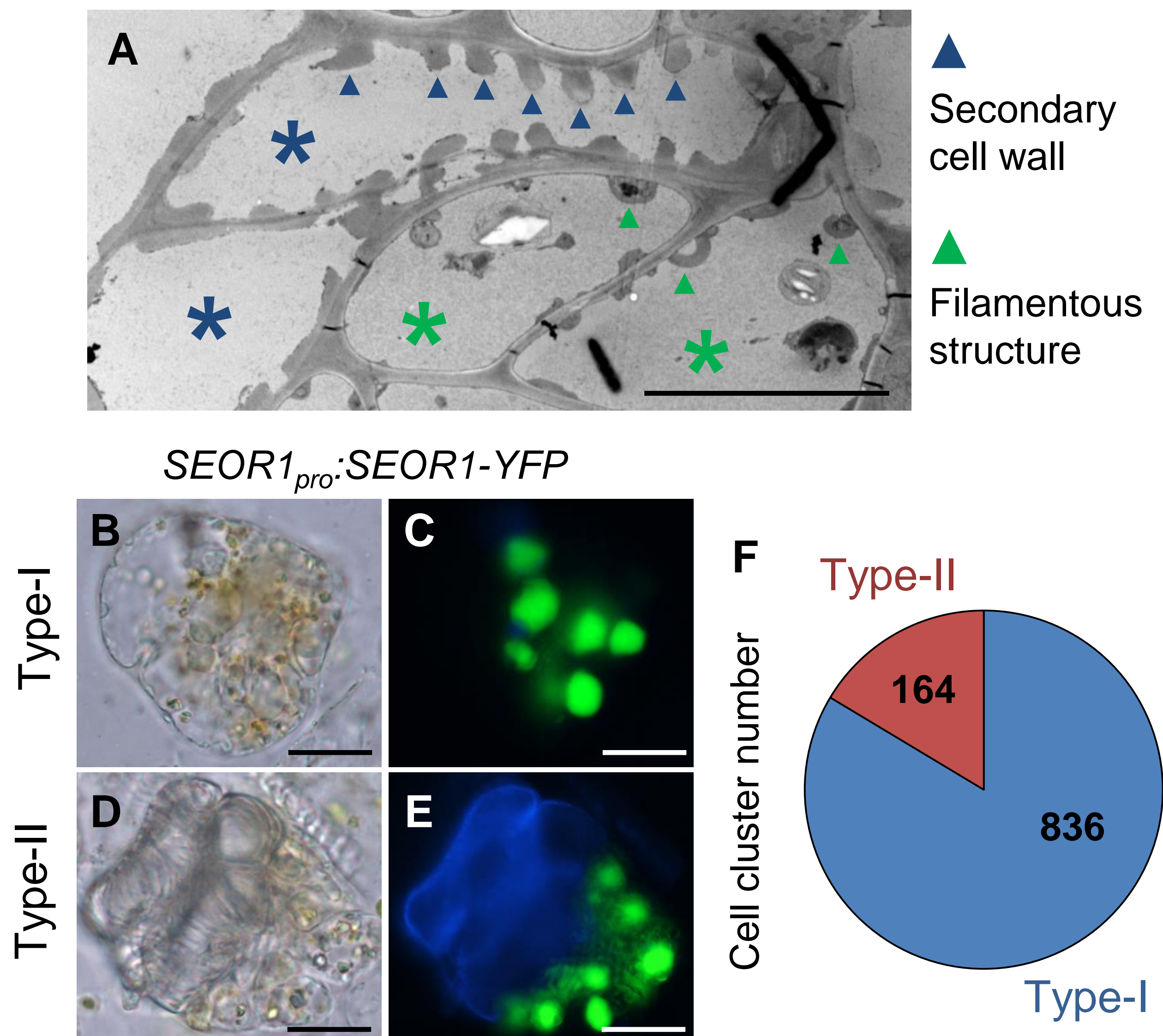
(A–D) DAPI staining in *MtSEO2pro:GFP5ER* cotyledons cultured with bikinin for 4 days. Both DAPI-positive cells (A and B) and DAPI-negative cells (C and D) were observed in isolated GFP-positive cells. Many nuclei were distorted at 4 dai compared with the nuclear structures at 3 dai (Figure 2G). (E) Frequencies of DAPI-positive cells in *MtSEO2pro:GFPER*-positive cells were calculated against 100 isolated cells from 3 or 4 dai samples. Error bars indicate SD ($n = 3$; biological replicates). Scale bars indicate 20 μm.

aniline blue staining
4 dai cotyledons



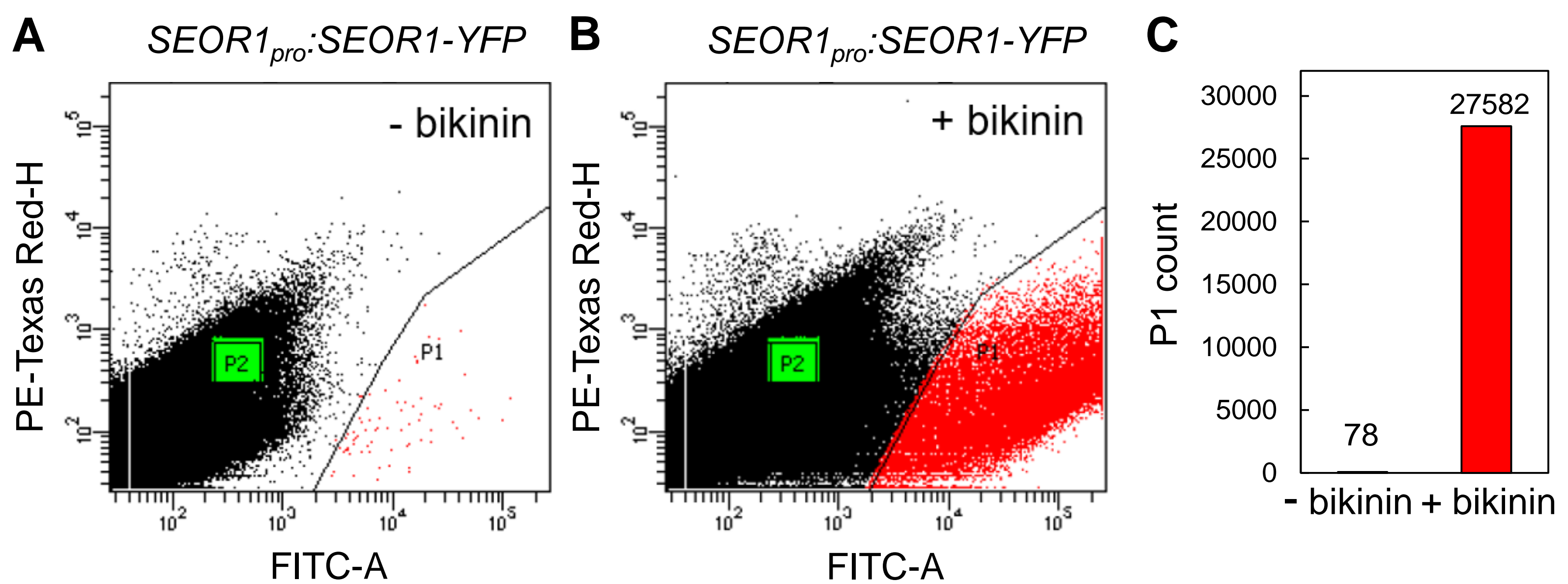
Supplemental Figure 5. A Confocal image of Differentiated Cells after Aniline Blue Staining in VISUAL

Aniline blue staining of differentiated cells in cotyledons cultured for 4 days with bikinin. The image was obtained using an FV1200 confocal microscope (Olympus) with 405 nm laser. Xy indicates xylem tracheary element. The other cells are phloem-like cells. Scale bar indicates 50 μm.



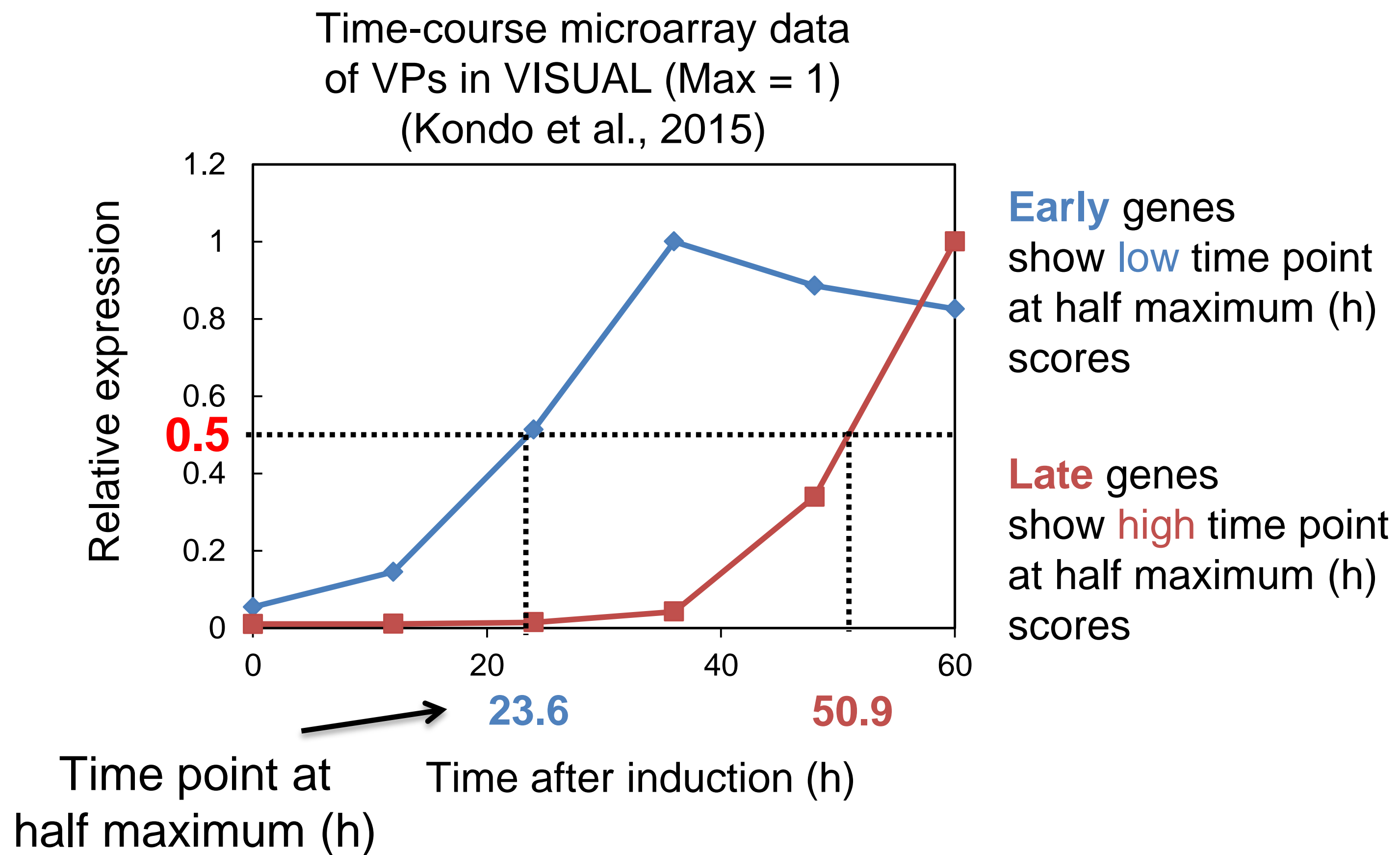
Supplemental Figure 6. TEs- and SEs-clusters in VISUAL

(A) High-magnification TEM image showing TE-like (blue asterisk) and SE-like (green asterisk) cells. TEs and SE-like cells were judged based on the presence of secondary cell walls (blue arrowheads) and filamentous structures (green arrowheads), respectively. (B–F) SE-clusters in VISUAL. Positive cells from 4 dai *SEOR1pro:SEOR1-YFP* cultured cotyledons were classified into two different types based on the presence of auto-fluorescence from TE secondary cell walls (Type I: without TE-signal, Type II: with TE-signal). Bright field images (B and D) and fluorescent merged images (C and E) for a Type-I and Type-II cell clusters are shown. (F) Frequencies of Type-I and Type-II clusters. Cell clusters number of Type-I and Type-II against total 1,000 cell clusters was counted. Scale bars indicate 10 μm for (A) and 20 μm for (B–E).

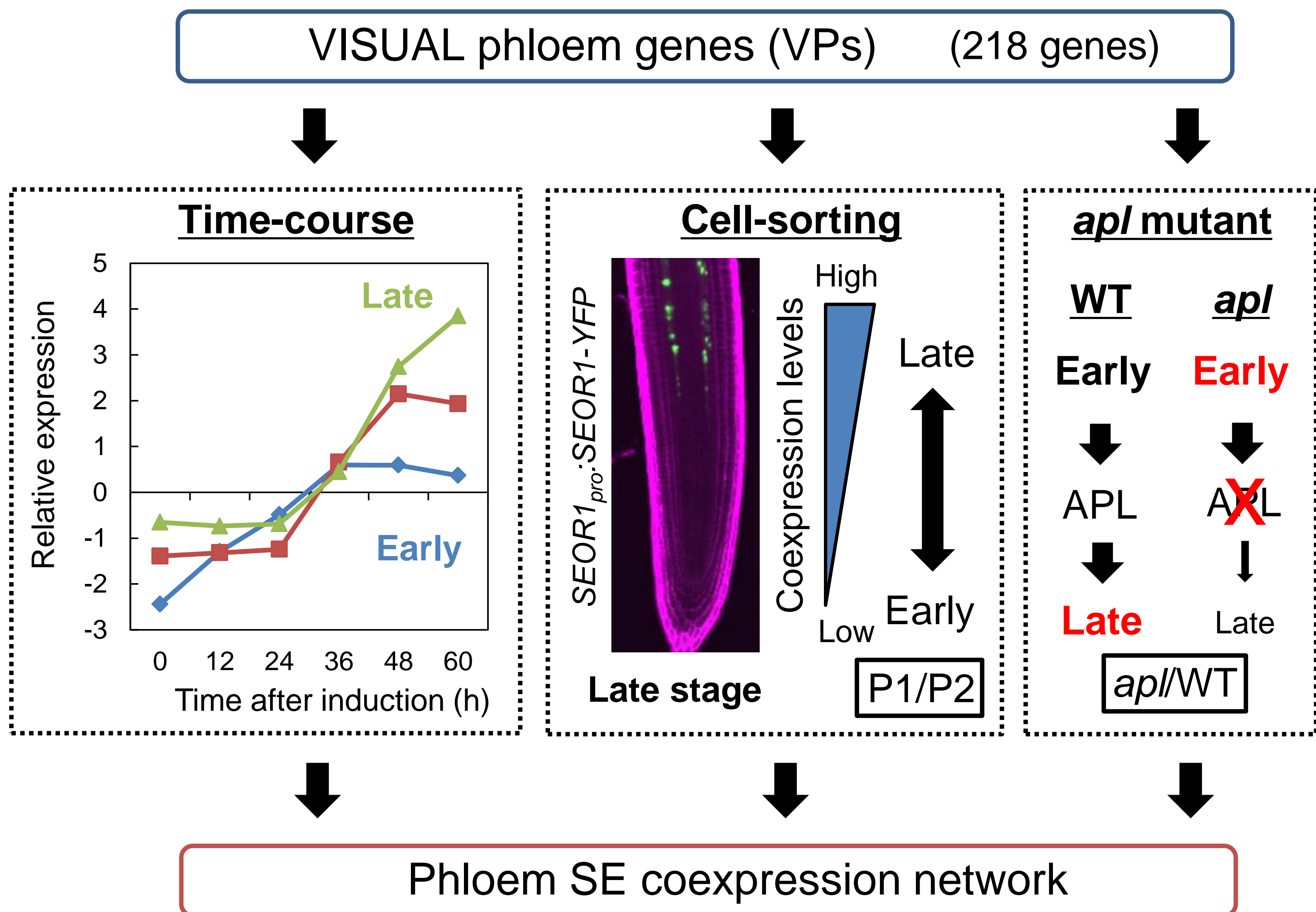


Supplemental Figure 7. Bikinin-Dependent Increase in the Number of YFP-Positive Cells

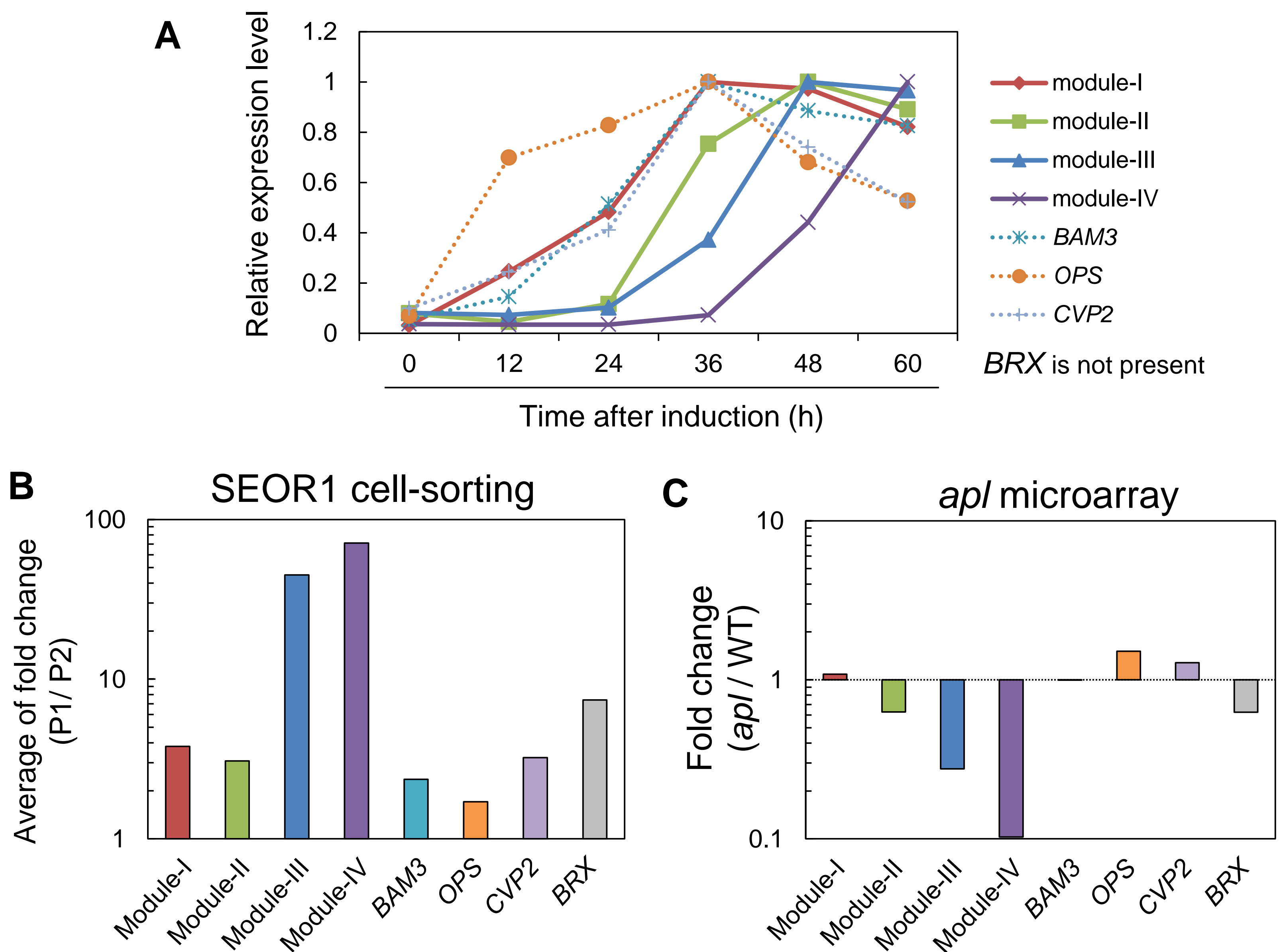
(A and B) Plots of fluorescent signal intensities for a total of 1,000,000 protoplasts from *SEOR1pro:SEOR1-YFP* cotyledons cultured without or with bikinin. (C) Number of YFP-positive cells (P1) in a total of 1,000,000 protoplasts from cotyledons cultured without or with bikinin.



Supplemental Figure 8. Definition of Time Point at Half Maximum
An example of the calculation of time point at half maximum scores based on time-course microarray data with VISUAL for VPs. Blue and red lines indicate the expression pattern for early and late phloem genes, respectively. Relative expression levels were calculated by comparison with the maximum expression level for each individual gene (max = 1). Time (h) when expression levels first reached 0.5 was calculated as time point at half maximum. In this study, time point at half maximum was used to judge whether gene expression is early or late in VISUAL.

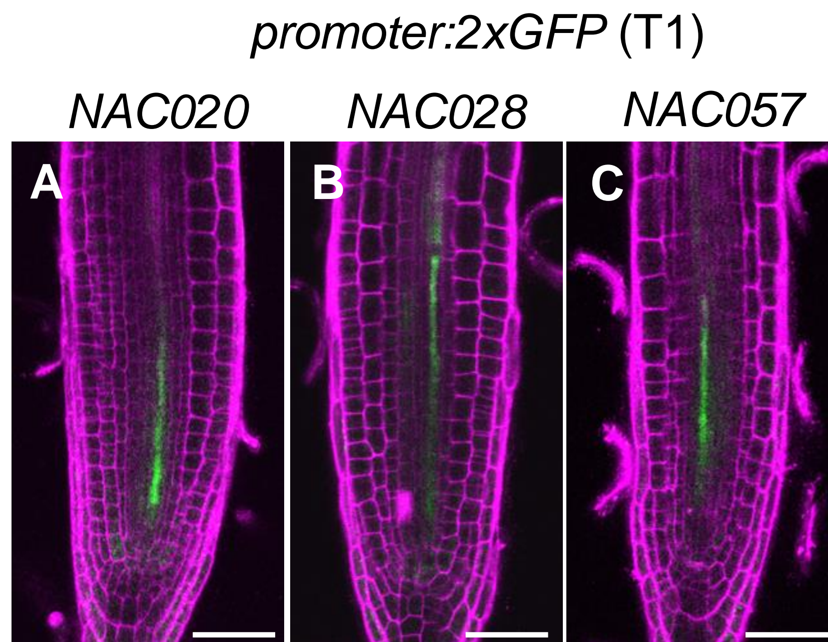


Supplemental Figure 9. Strategies for Constructing Phloem SE Coexpression Networks
For all VPs (218 genes), median normalized values were obtained from three sets of transcriptome data: time-course, WT *versus apl*, and SEOR1 cell-sorting data. Correlation of these values among VPs was examined with the WGCNA package for network construction.

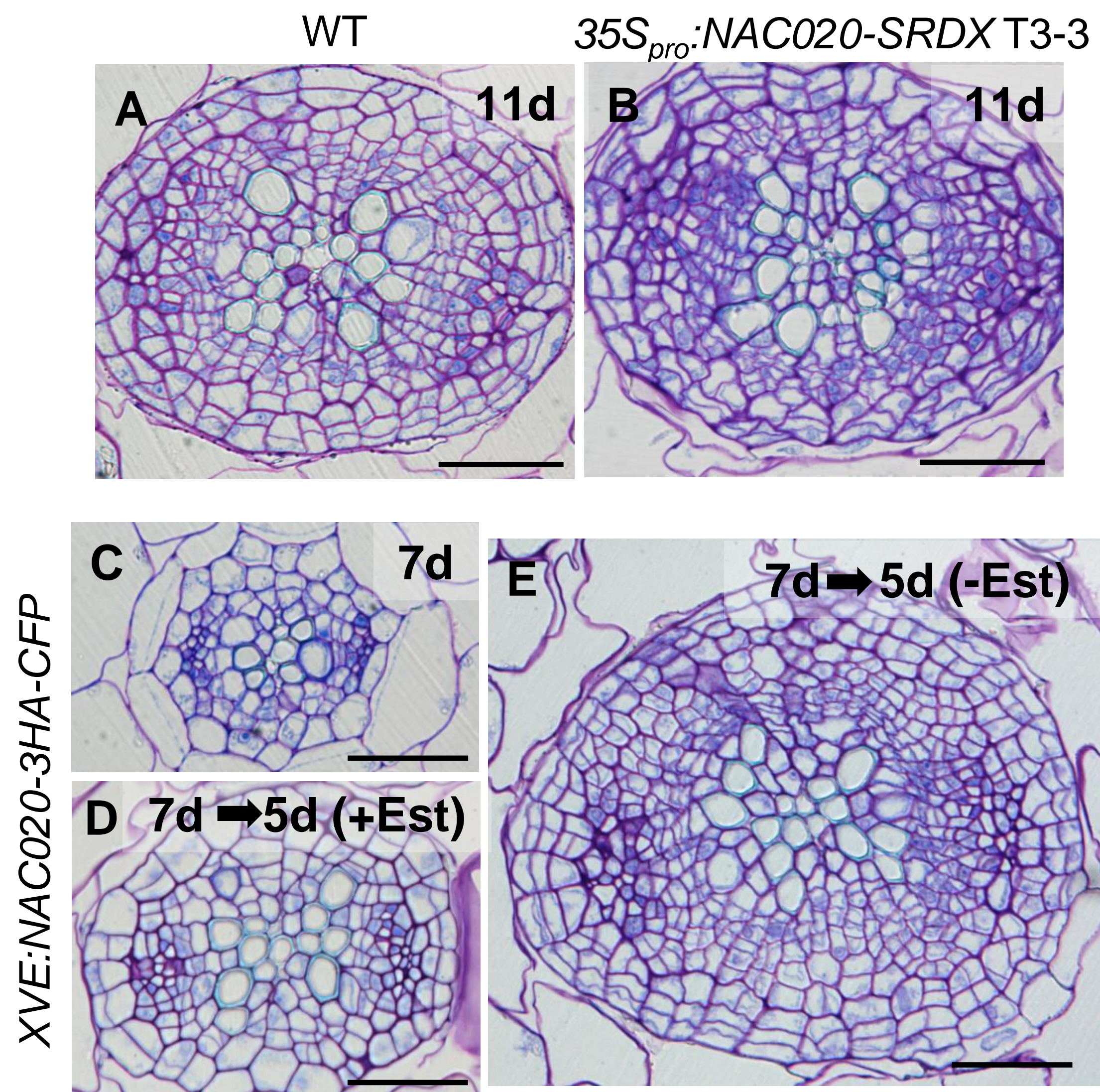


Supplemental Figure 10. Comparison between Known Early Phloem Regulators and Module Genes

(A) Comparison based on time-course microarray data in VISUAL. (B) Comparison based on SEOR1-YFP cell-sorting microarray data in VISUAL. (C) Comparison based on *apl* mutant microarray data in VISUAL.



Supplemental Figure 11. Expression Patterns of *NAC020*, *NAC028*, and *NAC057* in Roots
GFP expression patterns of $NAC020_{pro}$*:2xsGFP* (A), $NAC028_{pro}$*:2xsGFP* (B), and $NAC057_{pro}$*:2xsGFP* (C) lines with PI staining. Scale bars indicate 50 μm. Roots of Arabidopsis seedlings grown for 7 days were used.



Supplemental Figure 12. Cross Sections of Hypocotyl Vasculature in NAC020ox and NAC020-SRDX

(A and B) Technovit sections of hypocotyls of 11-day-old WT (A) and 11-day-old *35S$_{pro}$:NAC020-SRDX* (B). (C–E) Technovit sections of hypocotyls of 7-day-old *XVE:NAC020-3HA-CFP* (C), 7-day-old *XVE:NAC020-3HA-CFP* seedlings treated with (D), or without 10 μM estradiol (E) for 5 days. The samples were stained with Toluidine blue. Scale bars indicate 40 μm.

SUPPLEMENTAL METHODS

VISUAL

MATERIALS

REAGENTS

- Murashige and Skoog (MS) Basal Medium (Sigma, cat. no. M5519)
- Sucrose (Wako, cat. no. 196-00015)
- 2-morpholinoethanesulfonic acid monohydrate (MES; Dojindo, cat. no. 145224-94-8)
- D(+)-Glucose (Wako, cat. no. 049-31165)
- 2.5 g L^{-1} 2,4-dichlorophenoxyacetic acid stock (2,4-D, see REAGENT SETUP; Wako, cat. no. 044-18532)
- 0.5 g L^{-1} Kinetin stock (see REAGENT SETUP; Sigma, cat. no. K3378)
- 10 mM Bikinin stock (see REAGENT SETUP; ChemBridge)
- Bacto™ Agar (BD, cat. no. 214-010)
- Sodium Hypochlorite (Wako, cat. no. 197-02216)
- 10% (vol/vol) Triton-X100 (Nacalai Tesque, cat. no. 35501-15)
- Potassium hydroxide (Wako, cat. no. 168-03855)
- Acetic acid (Wako, cat. no. 017-00251)
- Ethanol (Wako, cat. no. 057-00451)
- Tri-chloro acetaldehyde monohydrate (Chloral hydrate; Wako, cat. no. 032-02165)
- Glycerol (Wako, cat. no. 075-00611)
- Dimethyl sulfoxide (DMSO; any)
- Purified, autoclaved Milli-Q

- Liquid growth medium (see REAGENT SETUP)
- MS agar plates (see REAGENT SETUP)
- Induction medium (see REAGENT SETUP)
- Fixative solution (see REAGENT SETUP)
- Clearing solution (see REAGENT SETUP)
- Sterilizing solution (see REAGENT SETUP)

EQUIPMENT

- Sterilized 1.5 mL sampling tubes (any)
- Sterilized 6-well plate (Sumilon, cat. no. MS-80060)
- Sterilized 12-well plate (Sumilon, cat. no. MS-80120)
- 95 mm x 95 mm Square grid plates (any)
- Petri dish (any)
- 0.22 µm filter (Merck Millipore, cat. no. SLGV004SL)
- 1 mm disposable biopsy punch (Kai Industries, cat. no. BP-10F)
- 1 µL Inoculating Loop (VWR, cat. no. 12000-806)
- Surgical forceps (any)
- Surgical tape (Micropore, cat. no. 1530-0)
- Autoclave (any)
- Clean bench (any)
- Continuous light chamber (22°C, neutral white fluorescent lamp; 45–55 µmol m^{-2} s^{-1}; any)
- Continuous light chamber (22°C, neutral white fluorescent lamp; 60–70 µmol m^{-2} s^{-1}; any)

- Rotary shaker (Taitec)
- Glass slides and cover slip (Matsunami, cat. no. S2441)
- Fluorescence microscope (BX53; Olympus)

REAGENT SETUP

2,4-D Dissolve in ethanol and dilute to 2.5 g L^{-1} using purified autoclaved Milli-Q. Sterilize using 0.22 µm filter. Aliquot 200 µL in sampling tubes and store at -20°C.

Kinetin Dissolve and dilute to 0.5 g L^{-1} using 0.1 M KOH and sterilize using 0.22 µm filter. Aliquot 200 µL in sampling tubes and store at -20°C.

Bikinin Dissolve and dilute to 10 mM using DMSO and sterilize using 0.22 µm filter. Aliquot 50 µL in sampling tubes and store at -20°C.

Liquid growth medium (for VISUAL with cotyledons) Dissolve 2.2 g L^{-1} MS Basal Medium, 10 g L^{-1} Sucrose and 0.5 g L^{-1} MES in Milli-Q and adjust the pH to 5.7 using KOH. Dispense in small glass bottles as appropriate and autoclave at 120°C for 20 min to sterilize. The prepared liquid growth medium can be stored at room temperature for a couple of weeks.

MS agar plates (for VISUAL with leaf disks) Measure 0.6% of Agar and pour into a conical flask. Add 2.2 g L^{-1} MS Basal Medium, 10 g L^{-1} Sucrose and 0.5 g L^{-1} MES solution (pH 5.7). Autoclave (120°C, 20 min) to sterilize and pour no less than 40 ml of the solution into each square grid plates (95 mm x 95 mm) inside a clean bench.

Induction medium (common) Prepare 2.2 g L^{-1} MS Basal Medium containing 50 g L^{-1} D(+)-Glucose in Milli-Q and adjust to pH 5.7 with KOH. Dispense in small glass bottles as appropriate and autoclave at 120°C for 20 min to sterilize. The prepared

induction medium can be stored at room temperature for a couple of weeks. Add 2,4-D, Kinetin and Bikinin (final concentration is 1.25 mg L^{-1} for 2,4-D, 0.25 mg L^{-1} for Kinetin and 10 μM for Bikinin) to the solution before culturing the seedlings. Repeated freezing and thawing of hormones and the chemical compound reduces the efficiency of differentiation and is strictly not recommended (see TROUBLESHOOTING).

Fixative solution Mix acetic acid and ethanol at a ratio of 1:3.

Clearing solution Mix chloral hydrate, glycerol and distilled water at a ratio of 8:1:2.

Sterilizing solution Add 1 mL of 10% Triton-X100 in 10 mL of Sodium hypochlorite solution and dilute to 100 mL with Milli-Q. The solution should be prepared fresh.

PROCEDURE

Sterilization of seeds

1) Add 1 mL of sterilizing solution to the 1.5 mL sampling tube containing Arabidopsis seeds. Gently shake to mix and then use a rotator to stir for 5 min.
2) Spin down and transfer the sample inside a clean bench. Allow to stand for 5 min.
3) Remove the supernatant using a P1000 pipette.
4) Add 1 mL of purified, autoclaved Milli-Q and mix well.
5) Remove the supernatant, as the seeds sink to the bottom of the tube.
6) Repeat steps 4 and 5 twice.
7) Add 1 mL of purified, autoclaved Milli-Q.

Stratification of seeds

8) Store the sterilized seeds at 4°C for 2–3 days to stratify.

Preparation of Arabidopsis leaves

9) The procedure and timing differ slightly between options A (cotyledons) and B (leaf disks). For the VISUAL method using cotyledons, plants are usually grown in 6-well-plate using liquid growth medium, whereas MS agar plates are used for leaf disks. Perform the following steps inside a clean bench.

A) **Cotyledons**

   i) Pour 10 mL of liquid growth medium into each well of a 6-well-plate

   ii) Sow 10–15 seeds per well using a P1000 pipette.

   iii) Seal the plate using surgical tape.

   iv) Incubate the plate at 22°C under continuous light (neutral white fluorescent lamp; 45–55 µmol m^{-2} s^{-1}) for 6 days with shaking at 110 rpm on the rotary shaker. The cotyledons unfold and very small true leaves appear after 6 days of incubation, which is the most appropriate time for the induction of vascular differentiation. Additional culture of 1 day is possible if the growth of seedlings is unfavorable (see TROUBLESHOOTING).

B) **Leaf disks**

   i) Plate 13 seeds per MS agar plate using a P200 pipette.

   ii) Dry the plate inside a clean bench for around 15 min.

   iii) Seal the plate with surgical tape.

   iv) Incubate for 3–4 weeks under continuous light (22°C, neutral white fluorescent lamp; 45–55 µmol m^{-2} s^{-1}).

Induction of vascular differentiation

10) The procedure and timing differ slightly between options A (cotyledons) and B

(leaf disks). For the VISUAL method using cotyledons, the central part of the hypocotyl is cut and roots are removed before dipping into induction medium, whereas leaf disks are punched from the basal part of 0.8–1.0 cm long leaves before transferring to the induction medium. Perform the following steps inside a clean bench.

A) **Cotyledons** (TIMING: 4 days)

i) Defrost the hormones and the chemical compound (2,4-D, Kinetin and Bikinin) at room temperature before the start of the experiment.

ii) Add 0.1 mL of 2,4-D stock (2.5 g L^{-1}), 0.1 mL of Kinetin stock (0.5 g L^{-1}) and 0.2 mL of Bikinin stock (10 mM) to 200 mL of sterilized Induction medium (see REAGENT SETUP: Induction medium).

iii) Add 2.5 mL of the prepared induction medium into each well of a 12-well-plate.

iv) Use the inner surface of the lid of the 6-well-plate to place the seedlings, and cut across the middle section of the hypocotyl using surgical forceps (Fig. 1B). Be careful not to cause physical damage or stress to the plants while using forceps. Gently unwind the entangled roots of the seedlings in the 6-well-plate to separate them.

v) Remove the roots and lower half of the hypocotyl and return the aerial part of the explants to the liquid growth medium to prevent desiccation (Fig. 1C).

vi) When all the samples are ready, gently rescue the explants using forceps and transfer about 4–6 explants to each well containing the induction medium. Make sure not to harm the leaves with forceps while transferring (Fig. 1D) (see TROUBLESHOOTING).

vii) Use surgical tape to seal the 12-well-plate.

viii) Incubate at 22°C under continuous light (neutral white fluorescent lamp; 60–70 μmol m^{-2} s^{-1}) for 4 days. The light intensity is stronger than for plant growth (Fig. 1E) (see TROUBLESHOOTING).

B) **Leaf disks** (TIMING: 3 days)

i) Defrost the hormones and the chemical compound (2,4-D, Kinetin and Bikinin) at room temperature before the start of the experiment.

ii) Add 0.1 mL of 2,4-D stock (2.5 g L^{-1}), 0.1 mL of Kinetin stock (0.5 g L^{-1}) and 0.2 mL of Bikinin stock (10 mM) to 200 mL of sterilized Induction medium (see REAGENT SETUP: Induction medium).

iii) Add 2.5 mL of the prepared induction medium into each well of a 12-well-plate.

iv) Cut Arabidopsis true leaves from 3–4-week-old plants across the petiole using forceps and select the ones with leaf blade length of 0.8–1.0 cm (Supplemental Figure 1B). We have obtained the most efficient and stable vascular differentiation with this leaf size (Kondo el al., 2015).

v) Wear double rubber gloves and place 3–4-week-old rosette leaves of Arabidopsis upside down onto an index finger (the adaxial side of the leaf faces to finger).

vi) Using a biopsy punch of 1 mm diameter on the gloved hand, carefully clip out leaf disks from the basal part of the rosette leaves (Supplemental Figure 1C). We have achieved faster differentiation rates with basal leaf disks compared to apical ones. (CAUTION: Double rubber gloves are indispensable to avoid skin injury from the biopsy punch.)

vii) Insert a P1000 pipette at the back of the biopsy punch and pump the piston to push out the disks into a Petri dish containing liquid growth medium. The leaf disks are highly prone to desiccation and cannot be left in open air for long.

viii) Transfer 10–15 of the collected leaf disks into each well containing the induction medium (Supplemental Figure 1E) (see TROUBLESHOOTING).

ix) Seal the 12-well-plate with surgical tape.

x) Incubate at 22°C under continuous light (neutral white fluorescent lamp; 60–70 μmol m^{-2} s^{-1}) for 3 days (Supplemental Figure 1E) (see TROUBLESHOOTING).

Observation of vasculature

11) Fix the samples in fixative solution overnight.

12) Transfer into clearing solution and leave for over 3 hours.

13) Mount on a glass slide and observe under a bright field microscope.

14) Switch to UV exposure to observe auto-fluorescence from xylem secondary cell walls for more visible images.

ANTICIPATED RESULTS

If the differentiation was successfully induced, the color of the cotyledons and leaf disks fades from green to yellow or white. This phenomenon serves as a good indicator to easily predict the efficiency of xylem cell differentiation without fixation. After the fixation of samples, the area of ectopically formed xylem cells can be measured with imaging tools such as ImageJ to determine/calculate the differentiation rates. Normally, differentiation rates of ectopic xylem cell differentiation in VISUAL are approximately

50–70% in terms of xylem area per whole leaf area. For differentiation rates lower than 50%, please refer to the troubleshooting advice. You can also check the reliability of the experiments by comparing the expression levels of *IRX3* and *SEOR1* between samples collected before (at 0 h) and after induction (at 72 h). Usually, successful differentiation upregulates expression levels over 50–100 fold.

TROUBLESHOOTING

Problem	Possible reason	Solution
Poor plant growth	Old seeds	Refresh the seeds and keep them under good conditions
	Old culture medium	Use fresh culture medium
Low differentiation rate	Old bikinin	Bikinin should be separately stored at -20℃ Bikinin should not be used repeatedly more than 2 times Use new stocks of bikinin or increase bikinin concentration
	Old hormones	Use new stocks of hormones
	Ler accession	Our results have shown that Col-0 accession but not Ler is suitable for xylem cell induction
	Weak light	Light is indispensable for xylem cell induction Check the light conditions
	Types of sugar	Induction medium does not contain "sucrose"
	Sample conditions	Age and size of leaves are quite important for differentiation Unhealthy leaves are also not suitable
	Sample density	High density of samples in each well may affect differentiation
	Sample desiccation (in leaf disks)	Keep the lid of the agar plate closed during sample preparation Complete sample preparation as quickly as possible
	Repeated use of biopsy-punch (in leaf disks)	A blunt blade of a biopsy-punch may cause tissue crushing at the cutting edge Change the biopsy-punch often (e.g., for every 30 disks)