The phloem transports photosynthetic assimilates and signalling molecules. It mainly consists of sieve elements (SEs), which act as “highways” for transport, and companion cells (CCs), which serve as “gates” to load/unload cargos. Though SEs and CCs function together, it remains unknown what determines the ratio of SE/CC in the phloem. Here we develop a new culture system for CC differentiation in Arabidopsis named VISUAL-CC, which almost mimics the process of the SE-CC complex formation. Comparative expression analysis in VISUAL-CC reveals that SE and CC differentiation tends to show negative correlation, while total phloem differentiation is unchanged. This varying SE/CC ratio is largely dependent on GSK3 kinase activity. Indeed, gsk3 hexaploid mutants possess many more SEs and fewer CCs, whereas gsk3 gain-of-function mutants partially increase the CC number. Taken together, GSK3 activity appears to function as a cell-fate switch in the phloem, thereby balancing the SE/CC ratio.
Multicellular organisms possess a variety of functional cells with a proper ratio for their life maintenance. In plants, the phloem tissue is composed of two major cell types: sieve elements (SEs) as conductive tubes and phloem companion cells (CCs) as helper cells for phloem transport. Phloem CCs function to support neighboring SEs through connected plasmodesmata. Although they function together with each other to ensure phloem transport, it has long been a deep mystery how the ratio of SE/CC is strictly controlled in the phloem. Recent technical advances enabled to identify various regulators that control SE differentiation\(^1\). In contrast to SEs, understanding of the molecular mechanism underlying CC differentiation remains a long-standing challenge.

Vascular cell induction culture system using *Arabidopsis* leaves (VISUAL) is a culture system that can artificially mimic plant vascular development\(^2\). In the VISUAL system, mesophyll cells are reprogrammed into vascular stem cells, and then differentiated into xylem vessel elements or phloem SEs within a couple of days. VISUAL enables the molecular genetic studies of vascular development, leading to the in-depth understanding of the regulatory network, especially for phloem SE differentiation. Even in VISUAL, differentiation into phloem CCs rarely occurs\(^3\), which makes it difficult to study CC development in detail.

In this study, we develop a new culture system for inducing CC-like cell differentiation named VISUAL-CC by modifying the conventional VISUAL method. Based on comprehensive gene expression analysis in VISUAL-CC, here we reveal that GLUCOSYL SYNTHESE KINASE 3 (GSK3) activity plays an important role in determining the SE/CC ratio. In vivo genetic analyses confirm the importance of GSK3 probably as a cell-fate switch in phloem development.

Results

**VISUAL-CC is a culture system for inducing CC-like cells.** The conventional VISUAL system can induce ectopic xylem (XY) or phloem SEs via the stage of vascular stem cell (Fig. 1a). Toward the understating of CC differentiation, we modified the VISUAL based on a luciferase-based screen with SUCROSE-PROTON SYMPORTER 2 (*SUC2*) pro:ELUC, a specific CC marker\(^4\) (Fig. 1b). In this screen process, vascular stem cells were induced by the conventional VISUAL method in advance, and subsequently were exposed to a variety of culture media. After a series of screens with different media, we could induce *pSUC2:ELUC* activity (Fig. 1c) and ectopic expression of the *pSUC2::YPFynls* marker in cotyledons within 4 days using CC medium (Fig. 1d–f). Hereafter, we refer to this culture system as “VISUAL-CC”. To further investigate the spatial pattern, a dual phloem marker line expressing *pSUC2::YPFynls* and SIEVE-ELEMENT-OCCULSION-RELATED 1 (*SEOR1*) pro:SEOR1-RFP\(^5\), a specific SE marker was established. Detailed observation of the dual phloem marker line by confocal microscopy after tissue-clearing treatment (ClearSee)\(^4\) revealed that CCs expressing *pSUC2::YPFynls* (green) are detected only in dividing cell clusters and are always limited to the cells adjacent to SEs expressing *pSEOR1::SEOR1-RFP* (magenta) (Fig. 1g). Thus, CC and SE markers appeared next to each other after several rounds of cell division (Fig. 1g). Observations using a field-emission scanning electron microscope (FE-SEM) or transmission electron microscopy (TEM) consistently visualized CC-like cells with dense cytoplasm adjacent to SEs with brighter cytoplasm (Fig. 1h). These cells showed minor vacuolation and developed the branched plasmodesmata typically seen in SE–CC complexes in vivo (Fig. 1h–j). In VISUAL, SMXL4 and SMXL5 are known as important regulators for early phloem SE development (Supplementary Fig. 1a)\(^6,7\). In VISUAL-CC, the double mutant *smxl4 smxl5* significantly suppressed CC-like cell differentiation (Supplementary Fig. 1b), suggesting that SE and CC differentiation partially shares a common developmental process from vascular stem cells. Taken together, these results suggest that VISUAL-CC can mimic the SE–CC complex formation.

**VISUAL-CC can induce known CC-related gene expression.** To validate the promoter-based assay, we compared *SUC2* mRNA accumulation with the promoter:LUC activity in the same sample (Fig. 2a, b). qRT-PCR analyses of VISUAL-CC samples and samples cultured in the conventional VISUAL medium (VISUAL, V) as negative controls revealed a strong correlation between promoter activity and mRNA level of *SUC2* (Fig. 2b; \(r = 0.97, P < 0.005\)) (Fig. 2b). We used these data for classification of VISUAL-CC samples into strong LUC activity (CC-strong, S) and moderate LUC activity (CC-moderate, M), according to their *SUC2* levels (Fig. 2a, b; Supplementary Fig. 2). Expression of SISTER OF ALTERED PHOLOM DEVELOPMENT (SAPL), another CC marker gene\(^8\), also showed a strong correlation with *SUC2* expression (Fig. 2c; \(r = 0.91, P < 0.005\)). A microarray analysis using the same samples was performed to obtain a comprehensive gene expression profile (Supplementary Fig. 3a). As expected, genes previously characterized as CC-related, including SULFATE TRANSPORTER 2;1 (*SULTR2;1*)\(^9\), SODIUM POTASSIUM ROOT DEFECTIVE 1 (*NaKR1*)\(^10\), C-TERMINALLY ENCODED PEP-PEPTIDE RECEPTOR (*CEPR1*)/XYLEM INTERMIXED WITH PHLOEM 1 (*XIP1*)\(^11,12\), and MYB-RELATED PROTEIN 1 (*MYR1*)\(^13\), showed similar expression patterns to *SUC2* and SAPL (Fig. 2d). Consistently, quantitative RT-PCR assay for these genes validated the microarray result (Supplementary Fig. 4). By utilizing the variation in expression observed between samples (S, M, and V), we identified 186 VISUAL-CC-inducible genes that satisfied the following patterns of expression levels: \(S > M > V\) and \(S/V > 4\) (Fig. 2e and Supplementary Fig. 2). According to the previous dataset of root cell-type-specific transcriptome\(^14\), these genes were mainly expressed in root CCs or phloem pole pericycles (PPPs, Fig. 2f). PPPs are also known to participate in phloem unloading from SEs in roots via intervening plasmodesmata\(^15,16\) (Fig. 2f). Here we grouped 67 genes as VISUAL-CC-related (VC) genes based on CC-preferential expression (Supplementary Table 1). Transporter genes were overrepresented among these VC genes, reflecting the functional aspect of phloem transport (Supplementary Fig. 3b, c).

SE and CC differentiation shows a negative correlation. We previously identified 137 VISUAL-XY-related (VX) genes and 218 VISUAL-SE-related (VS) genes using VISUAL microarray data\(^2\). Then, expression of VX and VS genes was examined in the VISUAL-CC transcriptome dataset. Although there was no regular pattern of VX gene expression, expression of VS genes was very low in the S samples in contrast to that of VC genes (Fig. 2g–i). Correlation analysis among these gene sets revealed that expression levels of VC genes negatively correlate with those of VS genes (Supplementary Fig. 5b; \(r = −0.91, P < 0.05\)) but not with those of VX genes (Supplementary Fig. 5a; \(r = −0.28, P > 0.05\)). To further assess this tendency, we calculated the quantitative expression levels of vascular marker genes in individual samples. Although there was a strong correlation between expression of SAPL (CC) and *SUC2* (CC) (Fig. 2c; \(r = 0.91, P < 0.005\)), no correlation was found between IRREGULAR XYLEM 3 (*IRX3*)\(^16\) (XY) and *SUC2* (CC) expression (Fig. 3a, \(r = −0.27, P > 0.05\)). By contrast, expression of *SEOR1* (SE) showed a significant negative correlation with that of *SUC2* (CC) (Fig. 3b, \(r = −0.76, P < 0.05\)). All these results suggest that CC and SE differentiation tend to show negative correlation.
Interestingly, expression levels of ALTERED PHLOEM DEVELOPMENT (APL) (SE + CC), which is expressed in both SEs and CCs, was almost constant among all the samples, indicating that the total amount of differentiating phloem cells is almost unchanged (Fig. 3c and Supplementary Fig. 6a). Taken together, these results suggest that VISUAL-CC induces different ratios of CC-like cells and SEs without changing the total number of phloem cells, while VISUAL only produced SEs. This implies that a key determinant of SE or CC cell fate is present in VISUAL-CC cultures.

SE/CC cell fates mostly depend on GSK3 activity. To identify the determinants of SE/CC cell fate, we investigated the impact of auxin and bikinin, as their concentrations differed between VISUAL and VISUAL-CC media (Fig. 1c). Exogenous auxin reduced pSUC2:ELUC expression in a dose-dependent manner (Supplementary Fig. 7a). Although SUC2 expression levels showed a similar decreased trend (Supplementary Fig. 7b), SEOR1 expression was not affected by auxin level (Supplementary Fig. 7c). Similarly, bikinin showed a repressive effect on pSUC2:ELUC expression levels (Fig. 3d). Unlike auxin, treatment with bikinin decreased expression of SUC2 in a dose-dependent manner while simultaneously increasing SEOR1 expression (Fig. 3e–g).

Bikinin is a specific inhibitor of plant GSK3s, and thus these results suggest that GSK3 activity plays a role in determining the SE/CC ratio. GSK3 activity correlates with the expression of BR biosynthetic genes, as a negative feedback regulation. Indeed, DWF4 (DWARF4)20, one of BR biosynthetic genes, was downregulated following bikinin treatment in a dose-dependent manner (Fig. 3i). In the VISUAL-CC transcriptome, expression of BR biosynthetic genes tended to be higher in the S samples and lower in the M samples (Supplementary Fig. 8), also suggesting the relationship between the GSK3 activity and the SE/CC ratio. Previous studies have reported 113 bikinin-suppressed genes19; these results suggest that GSK3 activity plays a role in determining the SE/CC ratio.
investigated the correlation between these genes and VC genes in VISUAL-CC transcriptome data. The bikinin-suppressed genes showed higher expression in the S samples (Fig. 2j) and their expression exhibited positive correlation with expression of VC genes (Supplementary Fig. 5c; \( r = 0.94, P < 0.01 \)). To investigate the relationship further, we quantitatively compared the expression patterns of \( SUC2 \) and GSK3-affected genes in the S and M samples. Expression of \( DWF4 \), a typical GSK3-induced gene (Fig. 3i), showed a strong positive correlation with \( SUC2 \) expression (Fig. 3j, \( r = 0.89, P < 0.005 \)). Similarly, other GSK3-induced genes such as \( CONSTITUTIVE PHOTOMORPHOGENIC DWARF \) (\( CPD \)) and \( BRASSINOSTEROID-6-OXIDASE 2 \) (\( BR6ox2 \)) showed significantly higher expression in the S samples than in the M samples\(^{21,22} \) (Supplementary Fig. 6b). By contrast, expression of \( TOUCH 4 \) (\( TCH4 \)), a typical GSK3-suppressed gene\(^{23} \), showed a significant negative correlation with \( SUC2 \) expression (Fig. 3k, \( r = -0.72, P < 0.05 \)). These results suggest that the ratio of induced SE and -CC is largely dependent on GSK3 activity in VISUAL-CC (Fig. 3l).

**Manipulation of GSK3 activity alters in vivo SE/CC ratio.** Then, we analyzed the role of GSK3s in in vivo secondary phloem development in Arabidopsis hypocotyls. In hypocotyls, SEs are characterized by vacant cytoplasm, whereas CCs are deeply stained with toluidine blue and they usually appear as pairs in a transverse section (Fig. 4a). Inhibition of GSK3 activity by bikinin treatment induced clusters of SEs and far fewer CCs (Fig. 4b). Bikinin treatment consistently reduced expression of \( pSUC2:YFPnls \) and resulted in clusters of \( pSEOR1:SEOR1-RFP \) signals in the dual phloem marker line (Fig. 4c, d), indicating that bikinin promotes SE formation and decreased CC number in vivo. Next, we confirmed the function of GSK3 proteins genetically using knockout mutants of members of the SKII subfamily (\( BIN2 \), \( BIL1 \), and \( BIL2 \)) and RNAi knockdown for SKI subfamily members (\( AtSK11 \), \( AtSK12 \), and \( AtSK13 \))\(^{24} \), because they are the main targets of bikinin\(^{17} \). The phloem tissue of the \( bin2 \) \( bil1 \) \( bil2 \) \( AtSK13RNAi \) quadruple mutant exhibited a slight but significant decrease in CC occupancy (40%) when compared with wild-type plants (44%) (Fig. 4e, f, h). The gsk hextuple mutant (quadruple + \( AtSK11 \),
AtSK12RNAi showed a reduction in CC occupancy (20%), resulting in more SEs and few CCs (Fig. 4g, h). Moreover, in the hextuple mutant, some of the PPP cells unexpectedly differentiated into ectopic SE-like cells (Fig. 4g). Previous studies have revealed that the vascular cells express SKII subgroup genes BIN2 and BIN2-LIKE2 (BIL2). In addition, expression of SKI subgroup genes pSK11:GUS and pSK12:GUS was found in the vasculature, including the phloem tissue (Fig. 4j, k). Similarly to the GUS expression analysis, SKI/II gene expression was kept high in VISUAL time-course and in VISUAL-CC transcriptome data (Supplementary Fig. 9), indicating that six GSK3 members are present during phloem development. Next, we investigated local GSK3 activity in the vasculature using pDWF4:GUS, which is an indicator of high GSK3 activity. Supporting with our idea, pDWF4:GUS expression was detected in the phloem CCs but not in SEs (Fig. 4l). Taken together, our results indicate that GSK3 activity is required for maintaining high CC occupancy in planta.

GSK3s function as signaling hubs to control xylem differentiation in the cambium. Here to focus on phloem development, bin2-1, a stable form of GSK3, was driven under promoters specific to each stage of phloem development. As we had previously demonstrated that the sequential genetic cascade in phloem SE differentiation is NAC020 (early), APL (middle), SEOR1 (late), we induced expression of bin2-1 under these different phloem promoters and investigated their phloem phenotype (Fig. 5a–f). Expression of bin2-1 driven by the APL and SEOR1 promoters did not affect phloem phenotypes, but pNAC020:bin2-1 slightly increased the ratio of CCs in the phloem (Fig. 5a–f). To objectively confirm the results with the CC marker, the number of pSUC2:YFPnls signal in WT and pNAC020:bin2-1 was quantified using a confocal microscope (Fig. 5g). YFP-positive cell number estimated from 3D-reconstruction images was significantly higher in the pNAC020:bin2-1 than in the WT (Fig. 5h–j), indicating that activation of GSK3 in the early phloem

**Fig. 3 GSK3s activity balances the SE/CC ratio in VISUAL-CC.** a Expression of SUC2 (CC) and IRX3 (XY) showing no correlation between levels in S and M samples. The Pearson correlation coefficient and P value are marked on the chart. b Negative correlation between expression of SUC2 (CC) and SEOR1 (SE). c Positive correlation between expression of SUC2 (CC) and APL (SE + CC). d Time course of pSUC2:ELUC signal intensities during VISUAL-CC with various bikinin concentrations. Averaged LUC values are shown (n = 6). e–i Expression of SUC2 (CC, e), SAPL (CC, f), SEOR1 (SE, g), CALS7 (SE, h), and DWF4 (GSK3-induced). j) Positive correlation between expression of SUC2 (CC) and DWF4 (GSK3-induced). k) Negative correlation between expression of SUC2 (CC) and TCH4 (GSK3-suppressed). l) Schematic model showing dose-dependent regulation of the SE/CC ratio by GSK3 activity.
leads to increment of CC cell number. Taken together, our results suggest that GSK3s appear to function as cell-fate switches for determining differentiation into phloem CCs or SEs, and that GSK3 activity will be important for ensuring the proper ratio between CCs and SEs (Fig. 5k).

**BR-BES1 signaling is not involved in SE/CC-fate regulation.** Finally, we examined the involvement of BR in CC differentiation, because GSK3s function as signal mediators in BR signaling. However, application of brassinolide (BL), an active BR, did not alter the SE/CC ratio in hypocotyls (Supplementary Fig. 10). Moreover, bes1 buz1 loss-of-function and bes1-D buz1-D gain-of-function mutants for BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAXOLE RESISTANT 1 (BZR1), which are well-known transition factors phosphorylated by GSK3s in BR signaling, exhibited a normal phloem development in terms of the SE/CC ratio (Supplementary Fig. 11). These results suggest the possibility that other signaling pathway(s) than BR participates in controlling the SE/CC ratio.

**Discussion**

Recent studies have revealed that CCs are not only of importance for phloem transport, but also act as a signal center integrating environmental information into the developmental program. We have established VISUAL-CC as a powerful tool for analyzing the functional and developmental processes of CCs. During secondary vascular development, it has been widely believed that SEs and CCs are derived from the same phloem precursors via asymmetric cell division. In VISUAL-CC, SEs and CCs were formed from vascular stem cells as neighboring complexes after several rounds of cell division, suggesting that VISUAL-CC may reflect the process of secondary phloem development. By taking advantages of VISUAL-CC, we identified GSK3s as key molecular switches to specify cell fate toward SE or CC. Indeed, gsk3 gain-of-function and loss-of-function mutants altered the ratio of SE/CC differentiation in the hypocotyl vasculature, and our expression analysis together with previous results consistently revealed that SKI/I/II subgroup members of GSK3s including BIN2 are expressed in the vascular tissues of hypocotyls. As SEs lose their nuclei during the differentiation process, support from adjacent CCs is essential for their function. Thus, maintenance of the SE/CC ratio by GSK3s will be an important mechanism ensuring survival under various environmental conditions. Although GSK3s act as a central regulator of SE/CC development, genetic experiments suggested the involvement of other signaling than BR mediated by GSK3s in this regulation. GSK3s have been implicated in regulation of phloem development through the interaction with OCTOPUS (OPS), which genetically functions together with BREVIS RADIX (BRX). Further studies on such interacting proteins and reverse genetic approaches combined with VISUAL-CC transcriptome data will be helpful for elucidating a new signaling cascade controlling the SE/CC ratio.
Fig. 5 Early phloem-specific activation of GSK3 activity increases the CC cell number in planta. a–d Toluoid blue-stained transverse sections for hypocotyls of 11-day-old Col (wild type, a), pNAC020:bin2-1 (b), pAPL:bin2-1 (c), and pSEOR1:bin2-1 (d) plants. e Genetic cascade in phloem development revealed by VISUAL. f Box-and-whisker plots of SE/CC ratios (%) in wild type (WT), pNAC020:bin2-1, pAPL:bin2-1, and pSEOR1:bin2-1 calculated from toluidine blue-stained sections (n = 14–16). g Schematic illustration for 3D confocal imaging of pSUC2:YFPnls marker expression in hypocotyls. h, i Maximum intensity projection of pSUC2:YFPnls marker expression in the WT (h) and pNAC020:bin2-1 (i) was shown from XY (upper) and XZ (lower) angles. j Quantification of pSUC2:YFPnls-positive cells from 3D-reconstruction images in the WT and pNAC020:bin2-1 (n = 11–12). Asterisks: significant differences determined using Student’s t test (** P < 0.005). k Schematic showing that GSK3 activity determines the SE/CC ratio. For box-and-whisker plots, median values were indicated by central lines. The first (Q1) and third (Q3) quartile were shown as a box. Lines show the range of Q1 – 1.5× interquartile and Q3 – 1.5× interquartile. Dots indicated distributions of each sample. Scale bars: 10 µm (a–d); 50 µm (h, i).

GSK3s also function in animals as molecular switches determining differentiation into alternate cell types, suggesting their common and important role as cell-fate switches. On the other hand, GSK3 activity regulates asymmetric cell division in the stomata lineage by interacting with polarly localized proteins, which are required for specifying stomatal cell fate. Further investigation with the context of asymmetric cell division will uncover the extent to which GSK3s serve as a common mechanism determining cell fate.

Methods

Plant materials. Arabidopsis plants used in this study is Col-0 accession, except for gsk3 high-order mutants (Ws background). To construct the CC-reporter lines, approximately 2.0 kb of the SUC2 promoter region was cloned and then fused with ELUC (Toyobo) or YFP containing a nuclear localization signal. A genomic fragment of SEOR1, approximately 4.8-kb long and containing 1.6 kb of the promoter, was fused with RFP to make pSEOR1:SEOR1-RFP; this was subsequently transformed into pSUC2:YFPnls to generate the double phloem marker line. To transform Arabidopsis plants, seeds were co- cultured with 200 µM o-luciferin (Wako) in white 24-well plates (PerkinElmer). The time course of luciferase (LUC) activity was measured automatically using a TriStar2 LB942 (Berthold) within a growth chamber (Nihonika).

Microscopic observation. For deep imaging with confocal microscopes, isolated tissue samples were fixed for 3 h under vacuum in a fixative solution (4% paraformaldehyde and 0.01% Triton X-100 in 1× phosphate-buffered saline (PBS)). Fixed samples were washed twice with 1× PBS and transferred to ClearSee solution (25% urea, 15% xyitol, and 10% sodium deoxycholate). ClearSee solution was replaced with fresh solution every 2 days for 3–4 weeks. Calcofluor staining was performed 1 week before microscopic observations by adding 0.1% (w/v) calcofluor white to the ClearSee solution. The samples were stained overnight and then washed with ClearSee solution without calcofluor. Once the samples were stained, washing was continued as described above. Cleared samples were observed using LSM880 (Zeiss) or FV1200 (Olympus) confocal microscopes with Z stack. For the quantification of YFP-positive cells, we counted the number of cells in a phloem bundle of approximately 420-µm length of hypocotyls based on reconstructed 3D images.

Electron microscopy. Sample preparation for electron microscopy observation was modified slightly from a previous study. Briefly, leaf disks induced by VISUAL-CC were fixed and embedded in resin. Thin sections (100 nm) were mounted on glass slides. Sections were stained with 0.4% uranyl acetate solution (UA) and a lead citrate solution (PB), and then coated with osmium tetroxide. Observations of slides were made using a FE-SEM (Hitachi SU 8220). Thinner (80 nm) sections were mounted on formvar-coated 1-slot copper grids, stained with 4% UA and Pb, and then observed using an 80-kV transmission electron microscope (JEOL JEM-1400 Flash).

qRT-PCR and microarray experiments. Total RNA was extracted from four cotyledons using RNeasy plant mini kit (Qiagen) after LUC measurement. After reverse transcription reaction, qRT-PCR was performed using LightCycler 480II (Roche) by a universal probe method. The expression value was normalized with...
an internal control UBQ14. Microarray experiments were conducted with the Arabidopsis Gene 1.0 ST Array (Affymetrix) and analyzed with Subio platform and R gplots package. Primers used in this study are listed in Supplementary Table 2.

**Cross-section.** Hypocotyls of 10- or 11-day-old seedlings were fixed with FAA (formalin:acetic acid:alcohol, 1:1:18) for 1 day. Fixed hypocotyls were subjected to preincubation, samples were embedded in a mixture of Technovit 7100 without Hardener II for 1 day. After the preincubation, samples were embedded in a mixture of Technovit 7100 + Hardener II (12:5:1) and incubated at 37 °C for more than 1 h to harden. Technovit samples were sliced into 2-µm sections using a LEICA RM2255 microtome and stained with 0.1% toluidine blue to enable distinguishing of SEs (white) under microscopy. We counted the CC/SE cell number per phloem pole on VISUAL-CC Arabidopsis Gene 1.0 ST Array (Affymetrix) and analyzed with Subio platform and ANOVA (Tukey–Kramer method).

**VISUAL-CC.** This protocol was a modified one from the previous VISUAL method. VISUAL-CC consists of two distinct steps: vascular stem cell formation and subsequent phloem differentiation. As the initial step, 6- or 7-day-old seedlings were cultured with the conventional VISUAL medium for 2 days in order to induce sufficient amount of (pro)cambial cells. After that, samples are transferred into VISUAL-CC medium for SE–CC complex differentiation.

**Materials for growth of plant samples before VISUAL induction.**

1. MS growth medium: It contains 2.2 g/L MS Basal Medium (Sigma), 10 g/L sucrose, and 0.5 g/L 2-morpholinooethanesulfonic acid monohydrate (MES) in Milli-Q water and the pH is adjusted to 5.7 with KOH. The solution is autoclaved at 120 °C for 20 min and can be stored at room temperature up to several weeks.

2. Sterilizing solution: Sodium hypochlorite solution is diluted in Milli-Q water in the ratio 1:9 (v/v) and 0.1% of Triton X-100 is added. This solution is prepared immediately before the sterilizing procedure.

3. Sterilized 6-well plate (Sumilon).

4. Autoclaved Milli-Q water.

5. Surgical tape.

6. Continuous light chamber (22 °C, 45–55 µmol/m²/s).

7. Rotary shaker (Taitec).

**Materials for VISUAL and VISUAL-CC.**

1. VISUAL base medium: It contains 2.2 g/L MS Basal Medium and 50 g/L D (+)-Glucose in Milli-Q water and the pH is adjusted to 5.7 with KOH. The solution is autoclaved at 120 °C for 20 min and can be stored at room temperature for several weeks.

2. VISUAL-CC base medium: It contains 2.2 g/L MS Basal Medium and 10 g/L D (+)-Glucose in Milli-Q water and the pH is adjusted to 5.7 with KOH. The solution is autoclaved at 120 °C for 20 min and can be stored at room temperature for several weeks. Note that Glucose concentration is different from that of VISUAL base medium.

3. 2,4-D stock: About 2.5 g/L 2,4-D stock dissolved in autoclaved Milli-Q water and sterilized through 0.22-µm filter units. It is stored in small amounts in sampling tubes at −20 °C.

4. Kinetin stock: About 0.5 g/L Kinetin stock dissolved in 0.1 M KOH and sterilized through 0.22-µm filter units. It is stored in small amounts in sampling tubes at −20 °C.

5. Bikinin stock: About 10 mM Bikinin stock dissolved in DMSO and sterilized through 0.22-µm filter units. It is stored in small amounts in sampling tubes at −20 °C.

6. Bikinin stock: About 10 mM Bikinin stock dissolved in DMSO and sterilized through 0.22-µm filter units. It is stored in small amounts in sampling tubes at −20 °C.

7. 2,4-D stock, kinetin stock, and bikinin stock are defrosted at room temperature before use. The tubes are transferred inside a clean bench and added to the VISUAL base medium to obtain a final concentration of 1.25 mg/L 2,4-D, 0.25 mg/L Kinetin, and 10 µM Bikinin. About 2.5 mL of the above medium is then added into each well of a 12-well plate.

8. A pair of sharp surgical forceps are used to cut the bottom half of Arabidopsis 6–7-day-old plants across the center of the hypocotyl and the roots are removed. About 4 of the Arabidopsis explants are then transferred carefully to each well containing the induction medium using forceps, and the 12-well plate is sealed with surgical tape. The explants are cultured for 2 days under continuous light (22 °C, 60–70 µmol/m²/s) with shaking at 110 rpm on a rotary shaker.

**Methods for VISUAL.**

1. 2,4-D stock, kinetin stock, and bikinin stock are defrosted at room temperature before use. The tubes are transferred inside a clean bench and added to the VISUAL-CC base medium to obtain a final concentration of 0.25 mg/L 2,4-D, 0.25 mg/L Kinetin, and 1 µM Bikinin. About 2.5 mL of the above medium is then added into each well of a 12-well plate. Note that auxin and bikinin concentration is decreased when compared with the VISUAL.

2. VISUAL-induced samples were transferred into the new CC medium and then cultured for 4 days under dark conditions (22 °C) with shaking at 110 rpm on a rotary shaker. Note that light severely affects the CC differentiation ratio.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Accession number of microarray data for VISUAL-CC is GSE141037. The Supplementary information includes the 67 VC genes list characterized in this study as Supplementary Table 1.

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Author contributions
Y.K. designed the experiment. T.T., S.O., M.N., Y.O., T.F., Masato S., Mayuko S., M.W., K.T., and Y.K. performed the experiments. H.F. and Y.H. shared materials and information. T.T., H.F., and Y.K. wrote the paper.

Competing interests
The authors declare no competing interests.

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