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Supplementary Information for

Transcriptional control of local auxin distribution by the CsDFB1-CsPHB module regulates floral organogenesis in cucumber

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This PDF file includes:

Other supplementary materials for this manuscript include the following:

Materials and Methods

Plant materials and growth conditions

Wild-type and transgenic cucumber (*Cucumis sativus* 'Xintaimici') plants were grown in a greenhouse under natural light conditions from the end of February to July or from the end of August to December in Beijing, China. For RNA-seq, shoot tips from cucumber plants at the cotyledon stage (S0) and one-leaf stage (S1) were sampled. For real time quantitative PCR (RT-qPCR) analysis, we sampled different organs: roots, stems, leaves, flowers, and ovary from 2-month-old plants.

Nicotiana benthamiana was used for transient transformation. Seeds were surface sterilized in 70% ethanol for 10 min, then rinsed three times with sterilized water before being sown onto Murashige and Skoog (MS) plates for germination. Five to seven days after germination, seedlings were transferred into pots containing peat soil and vermiculite (2:1 ratio by volume) and grown inside a growth chamber at 25°C with a relative humidity of 55% under a 16 h light/8 h dark photoperiod.

Sequence alignment, phylogenetic analyses, and gene cloning

The full-length protein sequences encoded by Cucurbitaceae and Arabidopsis cystatins genes were downloaded from the Cucurbit Genomics database (http://cucurbitgenomics.org/) and the Arabidopsis Information Resource database (http://arabidopsis.org/). Some of the cystatins from other species were found in the GenBank database (https://ncbi.nlm.nih.gov/). Sequence alignment was performed with the ClustalX2 and ESPript programs (http://espript.ibcp.fr/ESPript/ESPript/). The phylogenetic tree was constructed with MEGA (v7.0) by the maximum likelihood method and with the Whelan and Goldman (WAG) substitution model with a BIONJ starting tree. The MEME (version 4.12.0) program was applied with the following parameters to predict conserved motifs: maximum size: 60,000; maximum number of motifs: 10; minimum and maximum motif widths: 6 and 50, respectively. Accession numbers used for the phylogenic analysis are listed in Table S1.

All full-length cDNAs were amplified by PCR with the appropriate primers, based on information gathered at the Cucurbit Genomics database. The miRNA-resistant version of *CsPHB* was previously described (1, 2). Amplified fragments were cloned into the pMD18-T vector (Takara, Japan) and then confirmed by sequencing. The primer sequences used are listed in Table S2.

Generation of transgenic cucumber plants

For overexpression, the full-length coding sequences of *CsDFB1* and *CsPHB** were amplified with appropriate primers. After digestion with *KpnI* and *SacI*, the fragments were inserted into the pCAMBIA1300 expression vector, which places the PCR product under the control of the Cauliflower Mosaic Virus (CaMV) *35S* promoter. For RNA interference, we amplified a 167 bp *CsDFB1* cDNA fragment with appropriate primers containing different restriction sites and then inserted the resulting fragment into the *AscI*/*SwaI* sites (sense orientation) and *SpeI*/*BamHI* sites (antisense orientation) of the binary vector pFGC1008, placing the fragments under the control of the *35S* promoter. These constructs were transferred into Agrobacterium (*Agrobacterium tumefaciens*) strain EHA105 and then transformed into cucumber as previously described (3) with minor modifications. Specifically, cotyledonary nodes were vacuum-infiltrated with Agrobacterium solution and subsequently cultured on co-cultivation medium (MS medium containing 0.5 mg/L 6-BA, 1 mg/L ABA, and 100 μM acetosyringone). After co-cultivation in the dark for 2–3 days at 28℃, transformants were transferred to screening medium (MS medium containing 0.5 mg/L 6-BA, 1 mg/L ABA, 500 mg/L carbenicillin and 10–20 mg/L hygromycin) and incubated in a chamber at 28℃ under a 12 h light/12 h dark photoperiod. When the shoots reached 0.5–1 cm, they were transferred to rooting medium (MS medium containing 500 mg/L carbenicillin and 10–20 mg/L hygromycin) in jars. Regenerated plants formed roots within 2-3 weeks and were then transferred into pots containing peat soil and vermiculite (2:1 ratio by volume). Transgenic plants were transferred to the greenhouse from China Agricultural University in Beijing. More than twenty T_0 transgenic lines were generated and two RNAi/overexpression T_2 transgenic lines were selected each for further study. For genetic analysis, the *CsPHB**-OE#20 line (♀) and *CsDFB1*-OE#3 line (♂) were crossed to generate F_1 hybrid plants. The primer sequences used are listed in Table S2.

In situ hybridization

The shoot tip and ovary/fruit from 6-week-old cucumber plants were fixed in 3.7% FAA (3.7% formaldehyde, 5% acetic acid, and 50% ethanol) at 4℃ overnight. Embedding, hybridization, and detection were carried out according to the methods in (4). Sense and antisense probes were amplified with specific primers using SP6 and T7 polymerase, respectively. Anti-digoxigenin, conjugated to alkaline phosphatase (AP), was used to detect digoxigenin-labeled RNAs. Images were obtained with an Olympus BX53 microscope (Olympus, Japan). The primer sequences used are listed in Table S2.

Histochemical staining

To observe lignin deposition, hand-cut cross sections were decolorized using 75% ethanol. The sections were then stained with phloroglucinol-HCl (0.2% phloroglucinol in 6 N HCl) and examined under a Leica S8 APO microscope (Leica, Germany).

Floral buds were fixed and embedded in paraffin (Thermo Fisher Scientific, USA). We sectioned floral buds into 8 μm sections and stained with 0.25% toluidine blue. Slides were viewed with an Olympus BX53 microscope (Olympus, Japan).

Extraction and quantification of endogenous auxins

About 50 mg samples were harvested from the shoot tips of cucumber plants for measurement of auxin content. The extraction and quantification of endogenous auxin were performed using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) according to methods previously described (5). Three biological replicates were performed for each sample.

Immunolocalization of IAA

Shoot tips from cucumber were fixed in 3.7% FAA at 4℃ overnight. Procedures for dehydration, paraffin-embedding, sectioning, and immunolocalization were as previously described (6). A primary antibody against indole-3-acetic acid (Agrisera, Sweden) and an anti-rabbit secondary antibody conjugated to fluorescein isothiocyante (FITC) or AP (Sigma-Aldrich, USA) were used in this experiment. The negative controls were specimens not incubated with the primary anti-auxin antibody. Specimens were processed using the same parameters (dyeing time, exposure time and gain). The images were obtained with an Olympus BX53 microscope or an Olympus Confocal Laser Scanning Microscope (Olympus, Japan).

Phytohormone treatments

Cucumber seeds were germinated at 28°C in the dark and then grown inside a growth chamber at 25°C under a 12 h light/12 h dark photoperiod. The shoot tips of cucumber seedlings at the one-leaf stage were sprayed with 50 μM 1-naphthylacetic acid (NAA) or water (as control) every 3 days for 15 days. Photographs were then taken and the phenotypes analyzed after the flowers formed (about 15–20 days after the last treatment). The experiment was performed independently three times.

Yeast one-hybrid (Y1H) assays

The full-length coding sequence of *CsPHB* was cloned into the prey vector pB42AD.

Oligonucleotides corresponding to the CsPHB-binding sites and their mutant versions were synthesized and then ligated into the pLacZi2μ vector. These constructs or the corresponding empty vectors were co-transformed into yeast strain EGY48 and incubated at 30°C on synthetic dextrose plates lacking Ura and Trp, but containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) to observe color development. The primer sequences used are listed in Table S2.

Recombinant protein purification and EMSAs

The full-length coding sequence of *CsDFB1* was cloned into the GST fusion vector pGEX-2T; *CsPHB* was cloned into the MBP fusion vector pMAL-c2X. The constructs were then introduced into *Escherichia coli* strain BL21 (DE3). The GST-CsDFB1, MBP and MBP-CsPHB recombinant proteins were produced using 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16°C for 20 h. The recombinant proteins were affinity-purified using Glutathione Sepharose 4B (GE Healthcare, USA) or amylose magnetic beads (NEB, USA). Probes harboring *cis*-elements were synthesized and labeled with biotin at their 5′ end (Sangon Biotech, China). For probe competition, unlabeled probe was added to the reactions. EMSA was performed using a chemiluminescent EMSA kit (Thermo Fisher Scientific, USA). Probe sequences are listed in Table S2.

Transient expression and dual luciferase reporter assays

The full-length coding sequences of *CsPHB**, *CsDFB1* and *GFP* were cloned into the vector pGreenII 62-SK to generate effector constructs; the *CsYUC2* (–33 bp to –817 bp) and *CsPIN1* (–210 bp to –2092 bp) promoters were cloned into pGreenII 0800 to drive expression of the luciferase (*LUC*) reporter gene. Renilla (*REN*) luciferase under the control of the *35S* promoter was used as a positive control and was included in the same vector. The *GFP* effector was used as a negative control. The combinations of effector plasmids (*35S::GFP* or *35S::CsPHB*) and reporter plasmids (*pCsYUC2::LUC* or *pCsPIN1::LUC*) were transiently transformed into cucumber protoplasts as described (7). The samples were incubated for 36–48 h, then luciferase activity was determined using a Luciferase Assay System kit (Promega, USA). The promoter activity was calculated as the ratio of LUC to REN activity. The experiment was performed independently three times.

For the transient transformation in *N. benthamiana* leaves, all constructs were introduced into Agrobacterium strain GV3101 harboring the pSoup plasmid. Overnight Agrobacterium cultures containing these constructs were collected by centrifugation and resuspended to a final OD_{600} of 1.5 in infiltration buffer (10 mM

$MgCl_2$, 100 μM acetosyringone, 10 mM MES, pH 5.7). Equal volumes of Agrobacterium cell suspensions carrying the indicated constructs were mixed and used together with the P19 strain (OD_{600} = 0.3) for infiltration into *N. benthamiana* leaves. After 2 days, the abaxial side of *N. benthamiana* leaves were sprayed with 1 mM luciferin (Promega, USA) and luminescence was detected using an automatic chemiluminescence image analysis system with an exposure time of 10 min. The experiment was performed independently three times. The primer sequences used are listed in Table S2.

Bimolecular fluorescence complementation (BiFC) assays

The full-length coding sequences of *CsDFB1*, *CsPHB**, *CsYAB2*, *CsCRC*, and *CsSUP* without stop codons were cloned and inserted into the pSPYNE and pSPYCE vectors containing the coding sequence for the 5′ end or 3′ end of *Yellow Fluorescent Protein* (*YFP*) to generate in-frame fusion proteins. Transient expression in *N. benthamiana* leaves was performed as described above. After 48–72 h of infiltration, fluorescence was observed after excitation/emission at 488/507 nm (GFP) and 587/610 nm (mCherry) by confocal imaging (Olympus, Japan). The experiment was performed independently three times. The primer sequences used are listed in Table S2.

Firefly luciferase complementation imaging (LCI) assays

The full-length coding sequences of *CsDFB1* and *CsPHB** without stop codons were cloned and inserted into the *35S::nLUC* and *35S::cLUC* vectors containing the 5′ or 3′ sequences of the *LUC* gene to generate in-frame fusion proteins. Transient infiltration in *N. benthamiana* leaves and the detection of luciferase activity were performed as described above. The experiment was performed independently three times. The primer sequences used are listed in Table S2.

Pull-down assays

Recombinant proteins (MBP-CsPHB and MBP) and prey protein (GST-CsDFB1) were incubated in 1 mL of immunoprecipitation buffer (50 mM HEPES pH 7.5, 10% glycerol, 150 mM NaCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1× protease inhibitor cocktail tablet) for 30 min at 4°C with 20 μL MBP-beads. After centrifugation at 3000 *g* for 5 min, the supernatant was discarded. After being washed three times with 1 mL of IP buffer, the beads were boiled in 100 μL SDS loading buffer to elute bound proteins, which were separated on 10% SDS-PAGE gels. For immunoblot analysis, anti-GST antibody (M20007, Abmart, China) at 1:5,000 dilution, and anti-MBP antibody (E8032S, NEB, USA) at 1:10,000 dilution were used to detect the levels of GST-CsDFB1 or MBP-CsPHB, respectively. Chemiluminescent signals

were visualized using ECL reagents (Millipore, USA) according to the manufacturer's protocol. Images were scanned using a Tanon-4500 instrument (Tanon, China) according to the manufacturer's instructions.

Subcellular localization

To examine the subcellular localization of CsDFB1 and CsPHB, the full-length coding sequences of *CsDFB1* and *CsPHB** were amplified with the appropriate primers. After digestion with *SpeI* and *KpnI*, the fragment was inserted into the *pSuper::GFP* expression vector for transient transformation. The *Super* promoter is mannopine synthase promoter with three copies of the octopine synthase enhancer (8). Transient expression in cucumber protoplasts and *N. benthamiana* leaves was performed as described above. *CsDFB1-GFP*, *CsPHB*-GFP* or *GFP* constructs and the nucleus-localized marker *NF-YA4-mCherry* were co-infiltrated in *N. benthamiana* leaves. Cucumber protoplasts were incubated with 4′,6′-diamidino-2-phenylindole (DAPI) for 5 min to stain nuclei. Fluorescence was observed after excitation/emission at 488/507 nm (GFP), 587/610 nm (mCherry), and 358/461 nm (DAPI) by confocal imaging (Olympus, Japan). The experiment was performed independently three times. The primer sequences used are listed in Table S2.

Western blot analysis

N. benthamiana leaves were ground in liquid nitrogen and homogenized in SDS loading buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 5% β-mercaptoethanol). The samples were denatured at 98°C for 10 min and subjected to SDS-PAGE analysis. For immunoblotting, protein extracts were separated on 10% SDS-PAGE gels and then transferred to a polyvinylidene difluoride membrane (Millipore, USA) using transfer buffer (39 mM glycine, 48 mM Tris, 20% (v/v) methanol). The target proteins were detected with an anti-GFP antibody at a 1:5,000 dilution (M2004, Abmart, China). Chemiluminescent signals were visualized using ECL reagents (Millipore, USA) according to the manufacturer's protocol. Images were scanned using a Tanon-4500 instrument (Tanon, China) according to the manufacturer's instructions.

In vitro inhibitor activity assays

Recombinant protein (GST-CsDFB1) was used in the inhibitor activity assay against papain. The inhibitory activity was determined according to a previously described method (9). Briefly, 1 μL papain (Sigma-Aldrich, USA) was incubated with 30 μL of assay buffer (50 mM sodium phosphate, pH 7.0, 5 mM L-cysteine and 0.1 % (v/v) Triton X-100) and 80 μL of 2 % (w/v) azocasein (Sigma-Aldrich, USA) was diluted in

the sodium phosphate buffer for 2 h at 37°C. At the end of the reaction, 300 μL of 10% (w/v) trichloroacetic acid was added and the residual azocasein was removed by centrifugation at 14,000g for 5 min at 4°C. The supernatant (350 μL) was added to 300 μL of 1 N NaOH, and the absorbance was measured at 450 nm. For inhibitory assays, the GST-CsDFB1 protein were diluted to the appropriate concentrations in the 30 μL of assay mixture. The enzyme and inhibitor solutions were allowed to incubate for 30 min at 25°C before addition of the substrate. For the assay, 1% BSA and 50 μM E-64 were used as negative and positive control, respectively. The experiment was performed independently three times.

RNA extraction and real time quantitative PCR (RT-qPCR) analysis

Total RNA was isolated from specified cucumber tissues using the Total RNA Isolation System (Waryoung, China). Total RNA (1 μg) from each tissue was then reverse-transcribed with the FastQuant RT Kit (Tiangen, China). RT-qPCR analysis was performed using the SYBR Premix Ex Taq Mix (Takara, Japan) on an Applied Biosystems QuantStudio 6 real-time PCR system. Relative gene expression levels were calculated according to the comparative ΔΔCt method. *TUBULIN* was used as the internal reference gene. The experiment was performed independently three times. The primer sequences used for RT-qPCR are listed in Table S2.

RNA sequencing, data processing, and analysis

Shoot tips of cucumber plants were sampled for total RNA extraction as described above. Total RNA from each sample was used for RNA-seq at Biomarker Technologies (Beijing, China). Two biological replicates were performed for each sample. All procedures during cDNA library construction were performed using a standard Illumina sample preparation protocol. The RNA-seq libraries were sequenced on an Illumina Genome HiSeq 2500 platform and paired-end reads were generated.

Various bioinformatics approaches were used for annotation and metabolic pathway analysis of unigenes. The unigenes were aligned to the Cucurbit Genomics database (http://www.cucurbitgenomics.org/) and Swiss-Prot protein database (http://www.gpmaw.com/html/swiss-prot.html), for retrieving putative protein functional annotations. GO analysis of unigenes was performed using the GO database (http://www.geneontology.org/). FPKM (fragments per kilobase of transcript per million mapped reads) values were used to normalize gene expression levels (10). Differentially expressed genes were identified using the DESeq package in R.

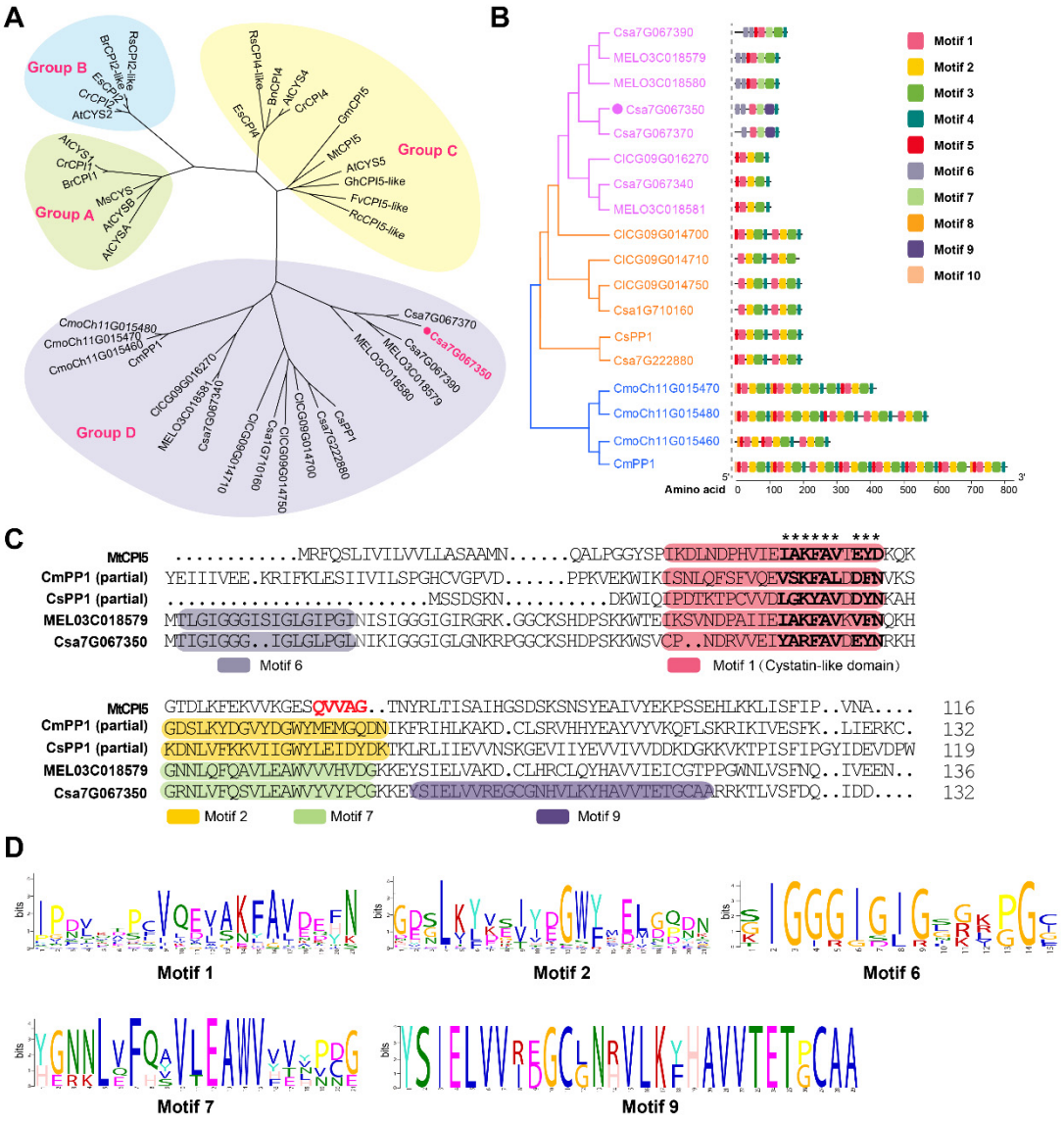

Fig. S1. Phylogenetic analysis of proteins from the plant cystatin superfamily. (*A*) The phylogenetic tree was constructed by the maximum likelihood method. The sequence information was obtained from the Cucurbit Genomics database, the Arabidopsis Information Resource database, and the GenBank database. Corresponding accession numbers are listed in Table S1. (*B*) Phylogenetic relationship (left) and architecture of conserved protein motifs (right) in proteins annotated as cystatin superfamily proteins. Conserved functional motifs are shown in different colors. The motifs, numbers 1–10, are displayed in different colored boxes. The length of the proteins can be estimated using the scale at the bottom. (*C*) Comparison of the predicted protein sequences derived from *Csa7G067350* and its homologs in *Medicago truncatula* ('MtCPI5'), *Cucurbita maxima* ('CmPP1'), *Cucumis sativus* ('CsPP1'), and *Cucumis melo* ('MELO3C018579'). The complete protein sequences were aligned in ClustalX2. Black asterisks indicate the conserved [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDQV]-[HYFQ]-N motif. (*D*) Protein sequence logos for motifs 1, 2, 6, 7, and 9.

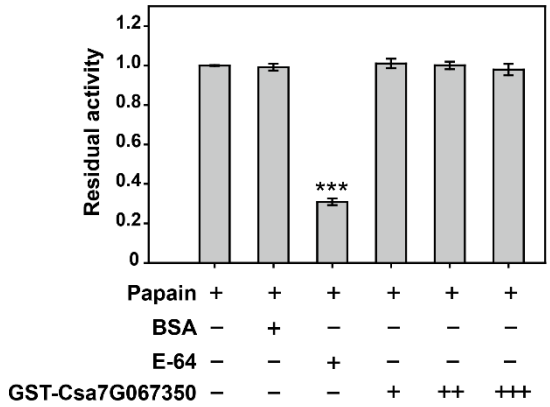

Fig. S2. Csa7G067350 does not inhibit papain *in vitro*. Residual papain activity was detected using azocasein as a substrate. Concentrations of 50 μM E-64 and 1% BSA were used as positive and negative controls, respectively. A gradient concentration of GST-Csa7G067350 was used (+, 1.0 μg; ++, 2.0 μg; +++, 3.0 μg). Error bars indicate SD of three biological replicates; two-tailed Student's *t*-test (***, $P < 0.001$). Abbreviations: BSA, bovine serum albumin; GST, glutathione S-transferase.

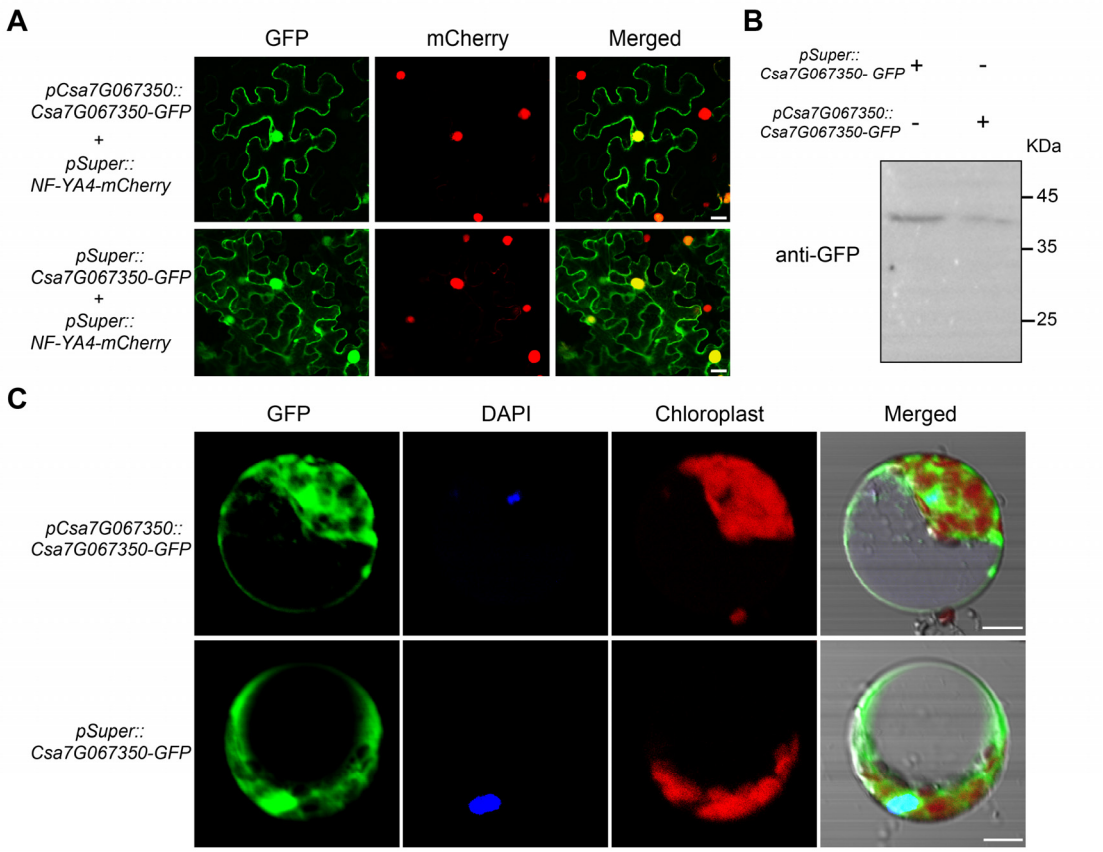

Fig. S3. Subcellular localization of Csa7G067350. (*A*) Subcellular localization of Csa7G067350-GFP fusion protein in *N. benthamiana* leaves. The *Csa7G067350-GFP* fusion construct was placed under the control of the *Csa7G067350* promoter and the *Super* promoter, respectively. The *Super* promoter consists of the mannopine synthase promoter with three copies of the octopine synthase enhancer. *Csa7G067350-GFP* was co-infiltrated with the mCherry-labelled nuclear marker *NF-YA4-mCherry*. (Scale bar, 20 μm.) (*B*) Immunoblot analysis of Csa7G067350-GFP accumulation in *N. benthamiana* leaves with an anti-GFP antibody. (*C*) Subcellular localization of Csa7G067350-GFP in cucumber protoplasts. (Scale bar, 10 μm.)

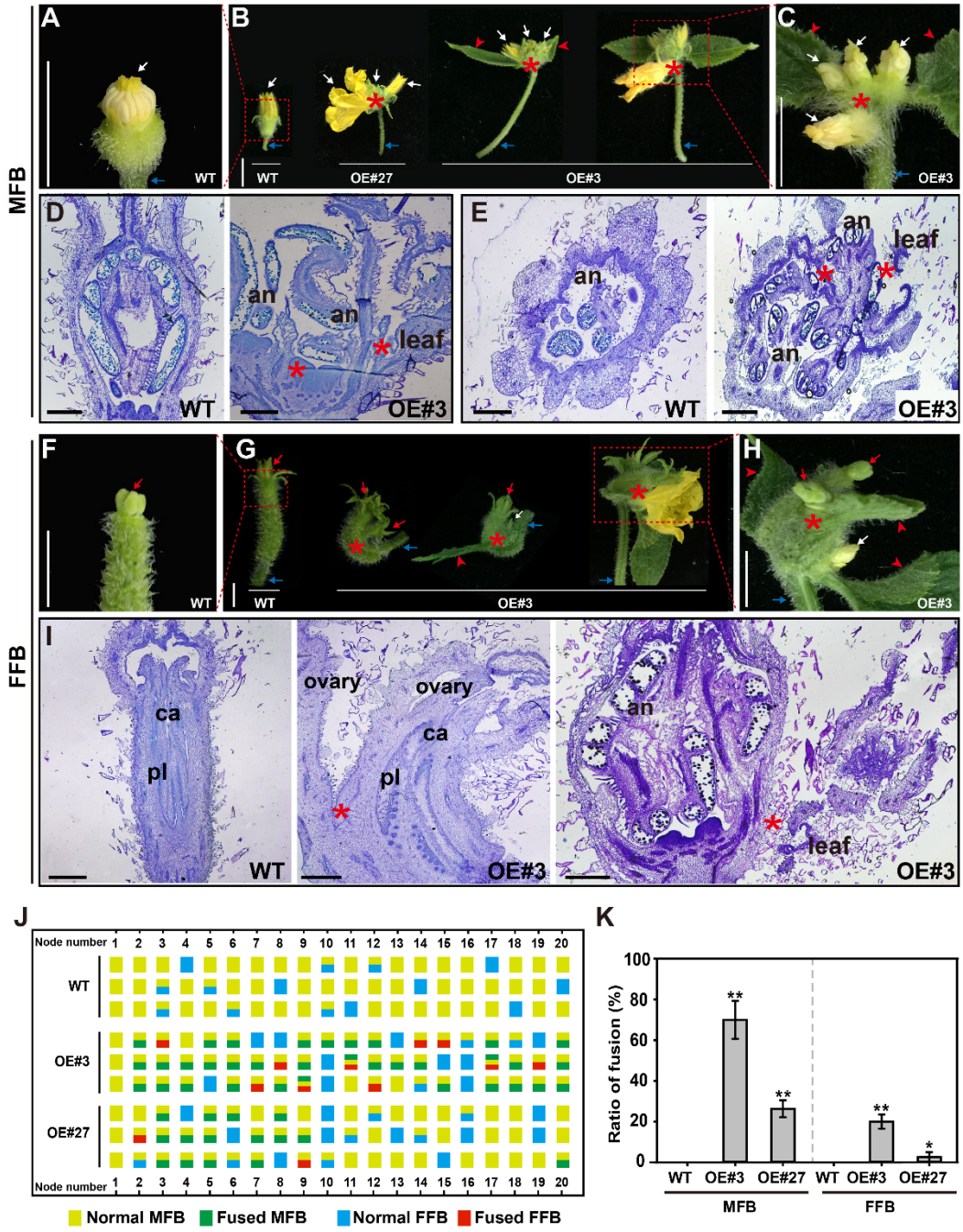

Fig. S4. Phenotypic analysis of organ fusion in *CsDFB1*-OE plants. (*A–I*) Representative organ fusion phenotype of MFB (*A–E*) and FFB (*F–I*) induced by overexpression of *CsDFB1*. (*A–C* and *F–H*) Morphology of MFB (*A–C*) and FFB (*F–H*) during early development from two transgenic lines (OE#3 and OE#27) and WT plants as control. (*A*, *C*, *F*, and *H*) Magnified views from the red dashed boxes in (*B* and *G*) without sepals and petals, respectively. Images show fused MFBs, or fused FFBs, or fused FFBs with MFB, as well as MFB/FFB fused with a leaf-like structure. White, red and blue arrows indicate anthers, stigma and pedicels/peduncles, respectively; red arrowheads indicate leaf-like structure; red asterisks indicate the organ fusion. (*D*, *E*, and *I*) Paraffin longitudinal- (*D* and *I*) and cross-sections (*E*) of MFB (*D* and *E*) and FFB (*I*). Images show the

fused tissues of anthers and leaf-like organs (*D* and *E*), as well as ovaries and leaf-like organs (*I*). (Scale bars, 1 cm in *A–C*, *F–H*; 500 μm in *D*, *E*, and *I*.) (*J* and *K*) Phenotypic analysis of organ fusion in two *CsDFB1*-OE lines (OE#3 and OE#27) and WT plants as control. (*J*) Diagram summarizing the position of normal and fused floral buds in two-month-old WT and *CsDFB1*-OE plants. Each row represents an individual plant. Yellow and blue areas represent normal MFB and FFB, respectively; green and red areas represent fused MFB and FFB, respectively. (*K*) Summary of the ratio of organ fusion in the WT and *CsDFB1*-OE plants. The ratio of deformation was calculated as the percentage of nodes with the fused phenotype. Data represent means ± SD (n = 20); two-tailed Student's *t*-test (**, $P < 0.01$; *, $P < 0.05$). Abbreviations: MFB, male floral bud; FFB, female floral bud; an, anther; ca, carpel; pl, placenta.

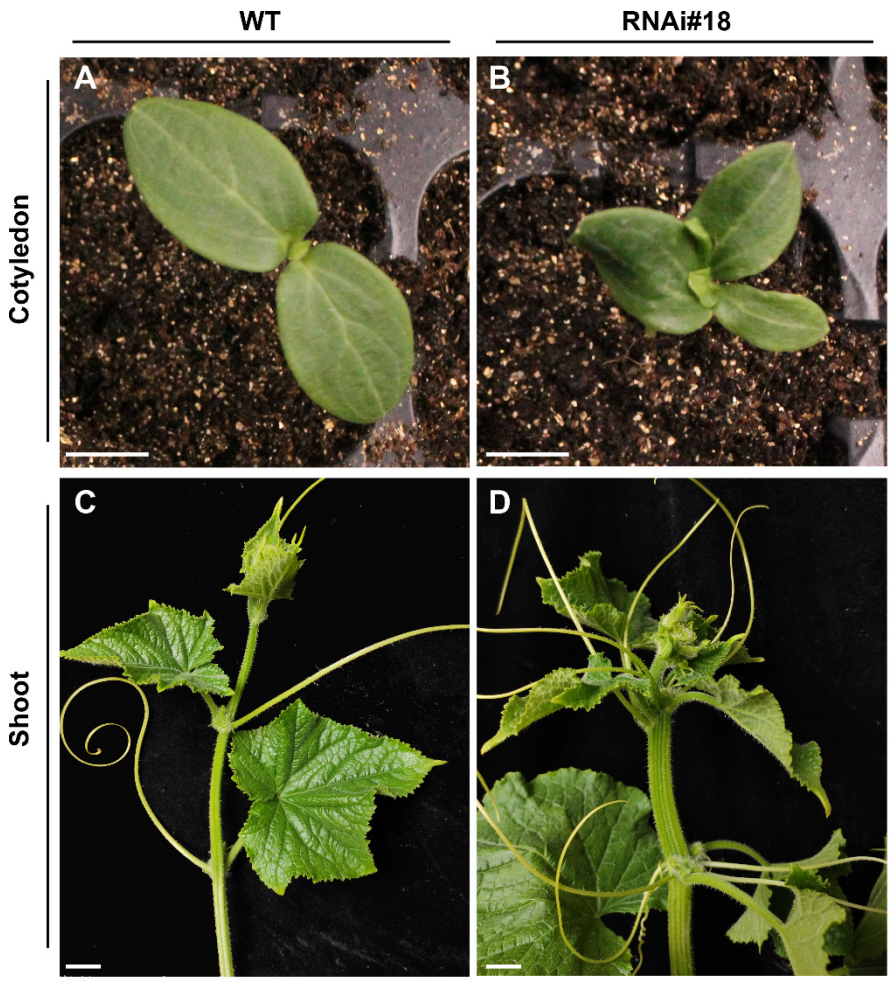

Fig. S5. Phenotype of cucumber *CsDFB1*-RNAi plants. (*A* and *B*) Representative images of cotyledons. (*C* and *D*) Representative images of shoots from 30-day-old plants. (Scale bar, 1 cm.)

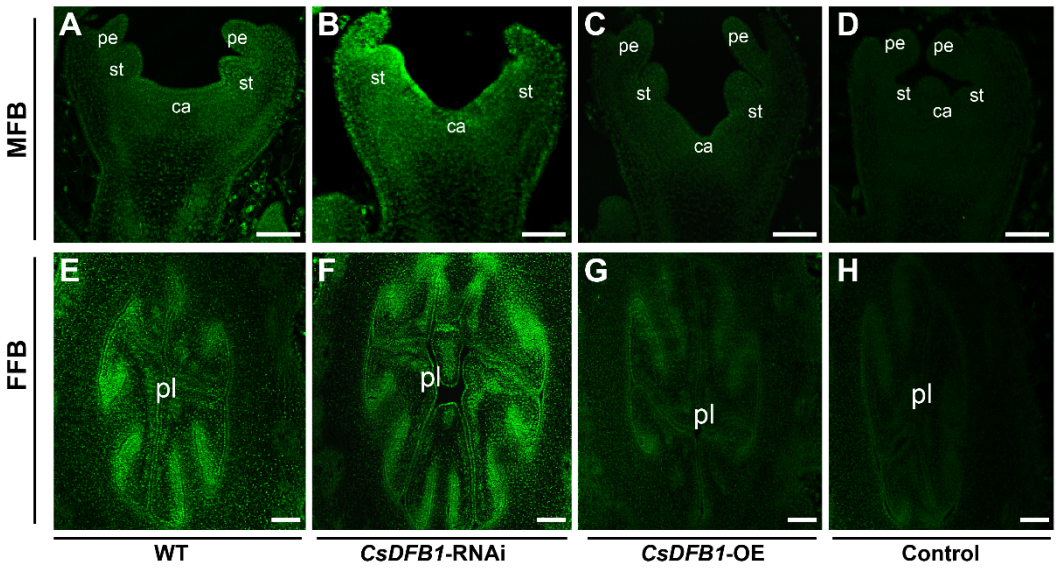

Fig. S6. Auxin distribution is altered in floral buds of *CsDFB1* transgenic plants. Immuno-localization of IAA in the MFB (*A–D*) and FFB (*E–H*) of WT (*A* and *E*), *CsDFB1*-RNAi (*B* and *F*) and *CsDFB1*-OE (*C* and *G*) plants using fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Specimens not incubated with the anti-auxin primary antibody served as the negative control (*D* and *H*). (Scale bar, 100 μm.) Abbreviations: MFB, male floral bud; FFB, female floral bud; pe, petal; st, stamen; ca, carpel; pl, placenta.

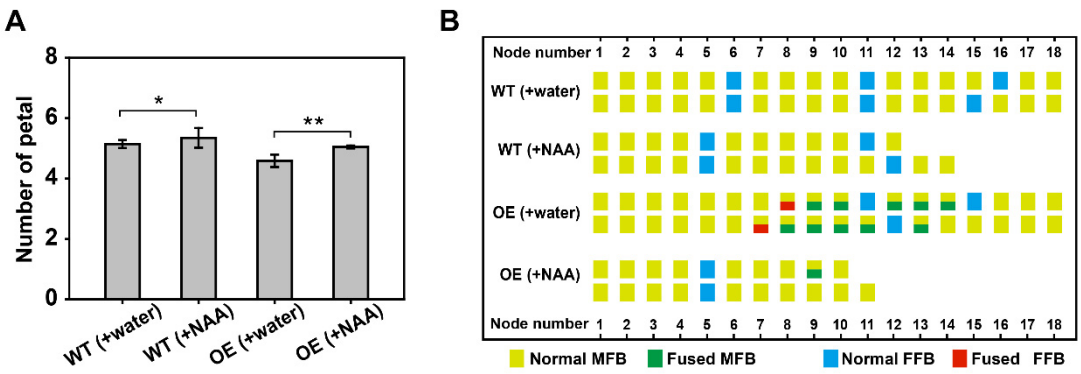

Fig. S7. Effects of exogenous auxin on cucumber floral buds. (*A*) Summary of petal number in male floral buds from the WT and transgenic plants with or without NAA treatment. Error bars indicate SD of 20 flowers from 3 individual plants; two-tailed Student's *t*-test (**, $P < 0.01$; *, $P < 0.05$). (*B*) Diagram representing the position of normal and fused floral buds in WT and *CsDFB1*-OE plants with or without NAA treatment. Each row represents an individual plant. Yellow and blue areas represent normal MFB and FFB, respectively; green and red areas represent fused MFB and FFB, respectively. Abbreviations: MFB, male floral bud; FFB, female floral bud; NAA, 1-naphthylacetic acid.

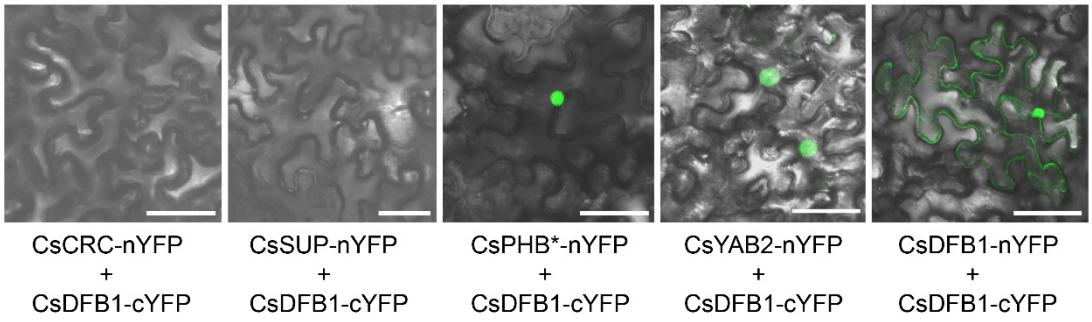

Fig. S8. CsDFB1 interacts with CsDFB1, CsPHB, and CsYAB2 proteins. Bimolecular fluorescence complementation assays show the interaction between CsDFB1 and other transcription factors related to auxin biosynthesis and transport. miRNA-resistant CsPHB is designated CsPHB*. (Scale bar, 50 μm.) Gene IDs: *DEFORMED FLORAL BUD1* (*CsDFB1*), Csa7G067350; *PHABULOSA* (*CsPHB*), Csa6G525430; *YABBY2* (*CsYAB2*), Csa6G426940; *CRABS CLAW* (*CsCRC*), Csa5G606780; *SUPERMAN* (*CsSUP*), Csa3G141870.

CsYUC2

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TGTCTAATAAAAACAACCCTTACAACTTCAATCCCCATAGATCAAAATCCAACTTTTCTTTCAAAACTA
AAAAA**GTATGCACAGAAAACCCGCCA**ATATACTAAACACATAACCCCTTTATTTCTT**CATG**

CsPIN1

CCTTCCATAAAACACCTTATTCACCTTTTCTTTAAGCATATATTTAAAAATTGGTTAATAGAAGCTTTA
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AAATCTTTAGTAAAGTGAATTGTAGTTAATGAATTAATTCAATTAAAAAGTTTAATTAATTAGTTCAAG
TTATTCAATGTTATTTTTATTTAGGTTATTCAGGTTATACCTCCTGATTCGTAAGTTGCCAATCCAACC
AACCCTAAAATATATGTTGGAGTGAGAATGTCCCAAACTCAACGTGCATTTTGAAACATTGAAATTCTA
CTTAAACGAATGGTGCTTATTTTACTTATTATTGTTATTTTATGTAGACAATATGCATGCTCAATTTTA
GGGTGGCATAAAAGGTTAATCAATGTTTTTTTATATTCTTTTTAAATCTTATTCATGTTCTTCTAACTT
GCTTTAAGATATAAATATGTCTAGAAATTGGTAAAGGATTTATTTGATAAATGTGTGGATATGTTTTTA
TGTTGATATAAAAGATTTATAAAAAATTAGATTATTTTAATTTGGTTTATAATAACATGTGTTTGTGGT
ATAAATTAGATTATTTTAGTTTGATAAAGAAATGATAAACATGGTAGAAACTAATAAAAAAAATGATGA
ATGTAAAATAATAAAAACGGTAGCAAATTTTATATTTTGAAATAGTGTTTACCGTAGCTAATTAAAGAT
TTTTAAATAGTATTTATTTTAACTAGAGGGGGTTATTATAGTCTTGCTTAATATCCTCACCTCAAACCA
TCTTTAACTATTATGACTAGGAATGTTCAAAAAATAGTTGAAAATAATCTAATTGATTGATAAATCGAA
TCGAAATCAAATGCATCCGTTTTGATTTGATGTGTGTTAAATTGAGATCGCTTTTTATGTGGTTTGGTT
CGGTTTAAGACATTAAACCGAATCTAAAACTAAATTGAACCACTTTATTTAAAAATAATTAAATATATA
AGTATGCCTATAACTTAGGGTAGAGGTAGAGCCATAGAGGAGAGGTAGACGAAACCCTTCGACAACGGC
AACGACATGACATGCAAACTTAGACGACCCGATGAAGATGAAGGCAAATCCTCCGACAACAACACGCAA
ATGCAGCACTTAGTACTTTACAATTTTCTTTTCCACTCTGATTACACATAAAGTTCTTCAGAACCCAAA
AATAAAAAATAAAAAAAATCAGCTTTTCCTTCTCCTTTTTCCCTTCCTTCTGTCCACCATTACAATGCC
T**CCATAAATACACCTCCCCACC**TCCCCTTTTCCTCTTACTTCTTCTTCTTTCTCCTCAATGGCTTAAAC
CCCATAAACAAAACACAGTGTACCCCCCCCAAAATTCCCAAC**ATG**

Fig. S9. Promoter analysis of *CsYUC2* and *CsPIN1* in cucumber. The translation initiation site (ATG) is indicated in red; the blue lines indicate the CsPHB binding site; yellow regions indicate the sequences used in yeast one hybrid and EMSA; and the black lines indicate the primers used in the dual luciferase reporter assay.

Table S1 List of accession numbers used in this study.

Gene Name / Gene ID	Species	Gene Name / Gene ID	Species
CsDFB1 / Csa7G067350	*Cucumis sativus*	CICG09G014700	*Citrullus lanatus*
CsPP1 / CsGy7G005820	*Cucumis sativus*	CICG09G014710	*Citrullus lanatus*
CsPHB / Csa6G525430	*Cucumis sativus*	CICG09G014750	*Citrullus lanatus*
CsAS1 / Csa3G264750	*Cucumis sativus*	CICG09G016270	*Citrullus lanatus*
CsAS2 / Csa2G070920	*Cucumis sativus*	*AtCYSA* / AT2G40880	*Arabidopsis thaliana*
CsKAN1 / Csa3G194380	*Cucumis sativus*	*AtCYSB* / AT3G12490	*Arabidopsis thaliana*
CsARF3 / Csa6G518210	*Cucumis sativus*	*AtCYS1* / AT5G12140	*Arabidopsis thaliana*
CsYUC2 / Csa1G242600	*Cucumis sativus*	*AtCYS2* / AT2G31980	*Arabidopsis thaliana*
CsYUC4 / Csa2G379350	*Cucumis sativus*	*AtCYS4* / AT4G16500	*Arabidopsis thaliana*
CsYUC6 / Csa2G375750	*Cucumis sativus*	*AtCYS5* / AT5G47550	*Arabidopsis thaliana*
CsPIN1 / *Csa4G430820*	*Cucumis sativus*	*BrCPI1* / XP_009121876.1	*Brassica rapa*
CsCRC / Csa5G606780	*Cucumis sativus*	*BrCPI2-like* / XP_009141203.1	*Brassica rapa*
CsSUP / Csa3G141870	*Cucumis sativus*	*BnCPI4* / XP_013655763.1	*Brassica napus*
CsYAB2 / Csa6G426940	*Cucumis sativus*	*CrCPI1* / XP_006288958.1	*Capsella rubella*
Csa1G710160	*Cucumis sativus*	*CrCPI2* / XP_006296241.1	*Capsella rubella*
Csa7G067340	*Cucumis sativus*	*CrCPI4* / XP_006284782.1	*Capsella rubella*
Csa7G067370	*Cucumis sativus*	*EsCPI2* / XP_006410333.1	*Eutrema salsugineum*
Csa7G067390	*Cucumis sativus*	*EsCPI4* / XP_006414340.1	*Eutrema salsugineum*
Csa7G222880	*Cucumis sativus*	*FvCPI5-like* / XP_004293371.1	*Fragaria vesca*
CmPP1 / AAC12676.1	*Cucurbita maxima*	*GhCPI5-like* / XP_016755444.1	*Gossypium hirsutum*
CmoCh11G015460	*Cucurbita moschata*	*GmCPI5* / XP_003541633.1	*Glycine max*
CmoCh11G015470	*Cucurbita moschata*	*MsCYS* / AAZ98791.1	*Medicago sativa*
CmoCh11G015480	*Cucurbita moschata*	*MtCPI5* / XP_013454866.1	*Medicago truncatula*
MELO3C018579	*Cucumis melo*	*RcCPI5-like* / PRQ58619.1	*Rosa chinensis*
MELO3C018580	*Cucumis melo*	*RsCPI2-like* / XP_018481394.1	*Raphanus sativus*
MELO3C018581	*Cucumis melo*	*RsCPI4-like* / XP_018467671.1	*Raphanus sativus*

Table S2. List of primers used in this study.

Primers for gene clone	
CsDFB1_for	5′-ATGACAATTGGTATTGGTGGAGG -3′
CsDFB1_rev	5′-TTAGTCGTCGATTTGGTCGAAAGA -3′
CsPHB _for	5′-ATGGCGTTAGTTATCCACAAAGA -3′
CsPHB* _rev	5′-CAACGATTCCAATAGAATCGGGGCCGGGTTTCATGCCAATC -3′
CsPHB* _for	5′-GATTGGCATGAAACCCGGCCCCGATTCTATTGGAATCGTTG -3′
CsPHB _rev	5′- TCAAACAAAGGACCAGTTTATGAAC -3′
Primers for cucumber transformation	
CsDFB1 _for (KpnI)	5′- CGGGGTACCATGACAATTGGTATTGGTGGAGG -3′
CsDFB1 _rev (SacI)	5′- CGAGCTCTTAGTCGTCGATTTGGTCGAAAGA -3′
CsDFB1_S_for (AscI)	5′- AGGCGCGCCTCAACTAAAGCTCTCACAAA -3′
CsDFB1_S_rev (SwaI)	5′- ATTTAAATATTGGGACATACACTCCA -3′
CsDFB1_A_for (SpeI)	5′- AGAGACTAGTTCAACTAAAGCTCTCACAAA -3′
CsDFB1_A_rev (BamHI)	5′- CGCGGATCCATTGGGACATACACTCCA -3′
CsPHB* _for (KpnI)	5′-CGGGGTACCATGGCGTTAGTTATCCACAAAGA -3′
CsPHB*_ rev (SacI)	5′-CGAGCTCTCAAACAAAGGACCAGTTTATGAAC -3′
Primers for subcelluar location	
CsDFB1 _GFP_for (SpeI)	5′-CGGACTAGTATGACAATTGGTATTGGTGGAGG -3′
CsDFB1 _GFP_rev (KpnI)	5′-CGGGGTACCGTCGTCGATTTGGTCGAAAGA -3′
pCsDFB1_for (HindIII)	5′-GGGCCCGGCGCGCCAAGCTTGGTCGGTATCCACTTAGTAAAGA-3′
pCsDFB1 _rev (BamHI)	5′-CCCTTGCTCACCATGGATCCGTCGTCGATTTGGTCGAAAG-3′
CsPHB* _GFP_for (SpeI)	5′-CGGACTAGTATGGCGTTAGTTATCCACAAAGA -3′
CsPHB*_ GFP_rev (KpnI)	5′-CGGGGTACCAACAAAGGACCAGTTTATGAAC -3′
Primers for qRT-PCR	
CsDFB1_Q_for	5′- AGGTTTGCAGTGGATGAGTAC -3′
CsDFB1_Q_rev	5′- GATTCCCACACCCTTCTCTTAC -3′
CsPHB_Q_for	5′- CGAATGTCCAAAGCCTAGTT -3′
CsPHB_Q_rev	5′- GATTCCTTACGCTGCTTCTC -3′
CsAS1_Q_for	5′- CCCTTCTCCGAGCTTACGTCA -3′
CsAS1_Q_rev	5′- GAGAGCCTTTCTTCAAGCCAGG -3′
CsAS2_Q_for	5′-CTTAGGGATTGGTAACCACGC -3′
CsAS2_Q_rev	5'-TCCGTCGTAGTTGCTACCG -3'
CsKAN1_Q_for	5′-AATCGGATGGTTCGGGAG -3′
CsKAN1_Q_rev	5′-ACATCCCACTCTGCTCTGC-3′
CsARF3_Q_for	5′- CAGCTCGGGCTATCACATC -3′
CsARF3_Q_rev	5'- CCCTTTGGTGCAGTTGCT-3'
CsYUC2_Q_for	5′-GCAAGGCAAAAGACTTCACGATCC -3′
CsYUC2_Q_rev	5′-GCGACCGCTAAGCCCG -3′
CsYUC4_Q_for	5′-GGGTTGTACACCGTCGGG -3′
CsYUC4_Q_rev	5'-ATCCGCCATTGCTCGGC -3'

CsYUC6_Q_for	5′-ACAGAGAAGATGAACGGCGG -3′
CsYUC6_Q_rev	5'-CGCCGCAGCTAATCCTGAG -3'
CsPIN1_Q_for	5'-TGTTTGGAGTTCGAGTGCG-3'
CsPIN1_Q_rev	5'-CGTTGAAATCTTTCGGGTTCATCCCA-3'
CsTUB_Q_for	5'-ACGCTGTTGGTGGTGGTAC-3'
CsTUB_Q_rev	5'-GAGAGGGGTAAACAGTGAATC -3'
Primers for BiFC and LCI	
CsDFB1_N/C _for (SpeI)	5′-CGGACTAGTATGACAATTGGTATTGGTGGAGG -3′
CsDFB1_N/C_rev (KpnI)	5′-CGGGGTACCGTCGTCGATTTGGTCGAAAGA -3′
CsSUP_YCE _for (SpeI)	5′-CGGACTAGTATGGAGAAGAACTGTTTACGCAC -3′
CsSUP_YCE _rev (KpnI)	5′-CGGGGTACCAGTTGAGTACCCTAATCGAAGCTCC -3′
CsCRC_YCE _for (SpeI)	5′-CGGACTAGTATGAGTAACTCCGAAGACAAATCTTCCA -3′
CsCRC_YCE _rev (KpnI)	5′-CGGGGTACCTTCATTGTTGTTGTGAGTACCAGAAACC -3′
CsYAB2_YCE _for (SpeI)	5′-CGCGGATCCATGTCACTGGACATGATGGC -3′
CsYAB2_YCE _rev (KpnI)	5′-CCGCTCGAGTTTTGTTTGCTTATTTCCATCC -3′
CsPHB*_YCE _for (SpeI)	5′-CGGACTAGTATGGCGTTAGTTATCCACAAAGA -3′
CsPHB*_YCE _rev (KpnI)	5′-CGGGGTACCTCAAACAAAGGACCAGTTTATGAAC -3′
CsDFB1_nLUC_for (KpnI)	5′-CGGGGGACGAGCTCGGTACCATGACAATTGGTATTGGTGGAGG -3′
CsDFB1_nLUC _rev (SpeI)	5′-ATCTGGTCGACTCCACTAGTGTCGTCGATTTGGTCGAAAGA -3′
CsPHB*_ cLUC _for (KpnI)	5′-ACGCGTCCCGGGGCGGTACCATGGCGTTAGTTATCCACAAAGA -3′
CsPHB*_ cLUC _rev (SpeI)	5′-TGCAGGTCGACTCCACTAGTTCAAACAAAGGACCAGTTTATGA-3′
Primers for *in situ* probes	
CsDFB1_SP6	5'-GATTTAGGTGACACTATAGaatGCTTCAACTAAAGCTCTCACAAA-3'
CsDFB1_T7	5'-tgTAATACGACTCACTATAGGGATTGGGACATACACTCCA -3'
CsPHB_SP6	5'-GATTTAGGTGACACTATAGaatGCTGAGCAAGTTGAGGCATTAG -3'
CsPHB_T7	5'-tgTAATACGACTCACTATAGGGCAAACAAAGGACCAGTTTATG -3'
CsYUC2_SP6	5'-GATTTAGGTGACACTATAGaatGCTACTACACCGGTTACGTTACTC-3'
CsYUC2_T7	5'-tgTAATACGACTCACTATAGGGCCTTGAACCAAAATTTGTGGAAT-3'
CsPIN1_SP6	5'-GATTTAGGTGACACTATAGaatGCTAGTTGAGAGTGGTGGTGAGG-3'
CsPIN1_T7	5'-tgTAATACGACTCACTATAGGGGAAATCTTTCGGGTTCATCCCAC-3'
Primers for Y1H, EMSA and dual luciferase reporter assays	
CsPHB_pJG_for	5'-GATTATGCCTCTCCCGAATTCATGGCGTTAGTTATCCACAAAGA -3'
CsPHB_pJG_rev	5'-GAAGTCCAAAGCTTCTCGAGTCAAACAAAGGACCAGTTTATGA-3'
CsYUC2_pLacZi_for (EcoRI)	5'-aattcTAGATCAACAATCATTATATTATATTTCATTAGATCAACAATCAT TATATTATATTTCATTAGATCAACAATCATTATATTATATTTCATc-3'
CsYUC2_pLacZi_rev (XhoI)	5'-tcgagATGAAATATAATATAATGATTGTTGATCTAATGAAATATAATATA ATGATTGTTGATCTAATGAAATATAATATAATGATTGTTGATCTAg-3'
mtCsYUC2_pLacZi_for (EcoRI)	5'-aattcTAGATCAGCGATAACCAGATTCTATTTCATTAGATCAGCGATA ACCAGATTCTATTTCATTAGATCAGCGATAACCAGATTCTATTTCATc-3'
mtCsYUC2_pLacZi_rev (XhoI)	5'-tcgagATGAAATAGAATCTGGTTATCGCTGATCTAATGAAATAGAATC TGGTTATCGCTGATCTAATGAAATAGAATCTGGTTATCGCTGATCTAg-3'

CsPIN1_pLacZi_for (EcoRI)	5'-aattcTTATTTAATTGTAATTATTGCTATGTTTTATTATTTAATTGTAATTATTGCTATGTTTTATTATTTAATTGTAATTATTGCTATGTTTTAc-3'
CsPIN1_pLacZi_rev (XhoI)	5'-tcgagTAAAACATAGCAATAATTACAATTAAATAATAAAACATAGCAATAATTACAATTAAATAAg-3'
mtCsPIN1_pLacZi_for (EcoRI)	5'-aattcTTATTTAGTGTTCATCATCGTTGTATTTTATTATTTAGTGTTCATCATCGTTGTATTTTATTATTTAGTGTTCATCATCGTTGTATTTTAc-3'
mtCsPIN1_pLacZi_rev (XhoI)	5'-tcgagTAAAATACAACGATGATGAACACTAAATAATAAAATACAACGATGATGAACACTAAATAATAAAATACAACGATGATGAACACTAAATAAg-3'
CsPHB_pMAL_for	5'-AGGGAAGGATTTCAGAATTCATGGCGTTAGTTATCCACAAAGA-3'
CsPHB_pMAL_rev	5'-AGTGCCAAGCTTGCCTGCAGTCAAACAAAGGACCAGTTTATGA-3'
CsYUC2_Biotin_for	5'-TAGATCAACAATCATTATATTATATTTCAT -3'
CsYUC2_Biotin_rev	5'-ATGAAATATAATATAATGATTGTTGATCTA -3'
mtCsYUC2_Biotin_for	5'-TAGATCAGCGATAACCAGATTCTATTTCAT -3'
mtCsYUC2_Biotin_rev	5'-ATGAAATAGAATCTGGTTATCGCTGATCTA -3'
CsPIN1_Biotin_for	5'-TTATTTAATTGTAATTATTGCTATGTTTTA -3
CsPIN1_Biotin_rev	5'-TAAAACATAGCAATAATTACAATTAAATAA -3
mtCsPIN1_Biotin_for	5'-TTATTTAGTGTTCATCATCGTTGTATTTTA -3
mtCsPIN1_Biotin_rev	5'-TTATTTAGTGTTCATCATCGTTGTATTTTA -3
CsPHB_SK_for (BamHI)	5'-GCTCTAGAACTAGTGGATCCATGGCGTTAGTTATCCACAAAGA-3'
CsPHB_SK_rev (HindIII)	5'-TCGACGGTATCGATAAGCTTTCAAACAAAGGACCAGTTTATGA-3'
GFP_SK_for (BamHI)	5'-GCTCTAGAACTAGTGGATCCATGGTGAGCAAGGGCG-3'
GFP_SK_rev (HindIII)	5'-TCGACGGTATCGATAAGCTTTTACTTGTACAGCTCGTCC-3'
pYUC2_0080_for (BamHI)	5'-TCGACGGTATCGATAAGCTTCATTCATATACTTAACAATACTCTA-3'
pYUC2_0080_rev (HindIII)	5'-GCTCTAGAACTAGTGGATCCTGGCGGGTTTTCTGTGCATAC-3'
pPIN1_0080_for (BamHI)	5'-TCGACGGTATCGATAAGCTT CCTTCCATAAAACACCTTAT-3'
pPIN1_0080_rev (HindIII)	5'-GCTCTAGAACTAGTGGATCC GGTGGGGAGGTGTATTTATG-3'

Dataset S1. List of genes that were differentially expressed between cotyledon stage (S0) and one-leaf stage (S1) shoot tips.

Dataset S2. List of genes that were differentially expressed in the RNAi lines relative to WT plants.

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