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

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Plant cytokinins are cytokine protein inhibitors that play key roles in defense responses. In this work, we describe an unexpected role for the cytokinin-like protein DEFERED FLORAL BUD1 (CsDFB1) as a transcriptional regulator of local auxin distribution in cucumber (Cucumis sativus L.). CsDFB1 was strongly expressed in the floral meristems, floral primordia, and vasculature. RNA interference (RNAi)-mediated silencing of CsDFB1 led to a significantly increased number of floral organs and vascular bundles, together with a pronounced accumulation of auxin. Conversely, accompanied by a decrease of auxin, overexpression of CsDFB1 resulted in a dramatic reduction in floral organ number and an obvious defect in vascular patterning, as well as organ fusion. CsDFB1 physically interacted with the cucumber ortholog of PHABULOSA (CsPHB), an HD-ZIP III transcription factor whose transcripts exhibit the same pattern as CsDFB1. Overexpression of CsPHB increased auxin accumulation in shoot tips and induced a floral phenotype similar to that of CsDFB1-RNAi lines. Furthermore, genetic and biochemical analyses revealed that CsDFB1 impairs CsPHB-mediated transcriptional regulation of the auxin biosynthetic gene YUCCA2 and the auxin efflux carrier PIN-FORMED1, and thus plays a pivotal role in auxin distribution. In summary, we propose that the CsDFB1-CsPHB module represents a regulatory pathway for local auxin distribution that governs floral organogenesis and vascular differentiation in cucumber.

auxin distribution | transcriptional regulation | CsDFB1 | CsPHB | floral organogenesis

The phytohormone auxin plays fundamental roles in various processes involved in plant growth and development, including early embryo patterning (1, 2), meristem development (3), vascular differentiation (4, 5), floral organogenesis and flower fertility (5–7), fruit development (8, 9), and root patterning (2, 10). Auxin is synthesized locally in shoots and roots and then transported in a polar manner to other sites (7). Therefore, local auxin biosynthesis and polar auxin transport are critical for the establishment and maintenance of auxin gradients and are involved in regulating multiple developmental processes in plants (5, 7, 11–15).

Indole-3-acetic acid (IAA), the most common natural auxin in plants, is mainly synthesized from a tryptophan (Trp)-dependent pathway in a two-step reaction: Trp is first converted into indole-3-pyruvate (IPyA) by the TRYPOTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1) family of transaminases, and then IPyA is converted into IAA by the YUCCA (YUC) family of flavin-containing monoxygenases (7, 16–19). In its protonated form, IAA can enter the cell through passive diffusion or through active uptake by influx carriers of the AUXIN1/LIKE-AXU1 (AUX1/LAX) family (20). Auxin efflux is mainly mediated by PIN-FORMED (PIN) efflux carriers that regulate auxin distribution in plant tissues (21). In the past two decades, the regulatory mechanisms behind auxin have been dissected in great detail (17, 22). Several critical transcription factors contribute to the regulation of local auxin distribution by modulating the expression levels and patterns of YUC and PIN genes. For example, the HD-ZIP III transcription factor REVOLUTA (REV) is the direct upstream activator of YUC5 and TAA1 transcription (23). In agreement with this result, proper REV function is required for polar auxin transport in the shoot (24). Likewise, the MADS-box transcription factor AGAMOUS (AG) works together with the YABBY-type transcription factor CRABS CLAW (CRC) to regulate YUC4 expression (25). Similarly, SUPERMAN (SUP) plays a role in boundary formation of floral organs by regulating the expression of YUC1, YUC4, PIN3, and PIN4 (26). The cucumber (Cucumis sativus L.) MADS-box transcription factor FRUITFULL1 (CsFUL1) regulates fruit length by repressing CsPIN1 and CsPIN7 transcription (8). Therefore, transcriptional regulation of YUCs and PINs plays an important role in plant organ development.

The plant cytokinin protein superfamily contains a distinctive cytokinin-like domain ([LV[1]-[AGT]-[RKE]-[FY]-[AS]-[VI]-[EDQV]-[HYF]-N] in its N terminus (27). Plant cytokinins were originally identified in rice seeds (28) and have since been

Significance

Auxin is a key phytohormone influencing multiple aspects of plant development, including meristem maintenance, primordia initiation, floral organogenesis, and vascular differentiation. Local auxin biosynthesis and polar auxin transport are essential to establish and maintain auxin gradients that ensure proper plant development. Here, we demonstrate that CsDFB1, a member of the plant cytokinin superfamily, which was previously implicated in defense responses, plays a critical role in regulating local auxin distribution and thus influences floral organogenesis in cucumber. Genetic and biochemical assays suggest that CsDFB1 affects local auxin distribution by acting as an attenuator that interacts with CsPHB and modulates CsPHB-mediated transcriptional control of CsYUC2 and CsPIN1. Our results shed light on the fine tuning of local auxin distribution in plants.
characterized in monocots and dicots, including maize (Zea mays) (29), barley (Hordeum vulgare) (30), Arabidopsis (Arabidopsis thaliana) (31, 32), tobacco (Nicotiana tabacum) (33), and soybean (Glycine max) (34). They may play roles in defense responses to biotic and abiotic stresses (29–31, 35, 36), programmed cell death (32, 33), and regulation of protein turnover during seed development and germination (37). For instance, Arabidopsis CYSTATIN1 (CYS1) was preferentially expressed in the vasculature of all organs and in response to abiotic stresses such as high temperature and wounding (31). The silencing of tobacco CYS by RNA interference (RNAi) induced precocious cell death in the basal cell of the embryo, resulting in embryonic arrest and seed abortion (33). In pumpkin (Cucurbita maxima Duch.), PHLOEM PROTEIN1 (CmPP1) contains four copies of the cystatin-like domain, and the CmPP1 mRNA was localized in phloem companion cells at early stages of vascular differentiation (35). To date, plant cystatins have been generally considered as defense proteins and regulators of protein turnover, with little information suggesting that they might have additional roles during plant development.

Here, we report on the unexpected finding that the cucumber cystatin-like protein DEFORMED FLORAL BUD1 (CsDFB1) functions as a transcriptional regulator to attenuate auxin accumulation, and thus is indispensable for proper floral organogenesis and vascular differentiation (35). To date, plant cystatins have been generally considered as defense proteins and regulators of protein turnover, with little information suggesting that they might have additional roles during plant development.

Next, we conducted in situ hybridization to validate the spatial expression pattern of Csa7G067350 in shoot tips and ovaries/fruits (Fig. 1 F–T). In agreement with the RNA-seq and RT-qPCR results above, we observed strong signals in floral meristems, floral primordia, and the vasculature (Fig. 1 F–J). Specifically, Csa7G067350 was expressed throughout the floral meristems at the early stage (Fig. 1F), later accumulating in petal, stamen, and carpel primordia within floral buds (Fig. 1 G–J). In addition, Csa7G067350 transcripts specifically accumulated in the phloem region of the main (MVB), peripheral (PeVB), carpel (CVB), and placenta (PIVB) vascular bundles in the ovary/fruit (Fig. 1 K–O). These results suggested that Csa7G067350 might function in floral organogenesis and vascular development.

Phylogenetic analysis indicated that the Csa7G067350 protein belongs to group D of the plant cystatins, which is a new group consisting of proteins from Cucurbitaceae (SI Appendix, Fig. S1 A and B) (35, 42). Csa7G067350 shared high similarity with PHLOEM PROTEIN1 (PP1), previously identified in pumpkin and cucumber (SI Appendix, Fig. S1) (35, 42). Interestingly, as for several other group D members such as cucumber PP1 (CsPP1) and soybean PP1 (CmPP1), the predicted protein sequence of Csa7G067350 lacked the cysteine protease inhibitor activity associated with cysteine proteinase inhibitor activity (SI Appendix, Fig. SIC) (30, 35, 36). Consistent with the absence of this conserved motif, in vitro inhibitory assays indicated that the protein encoded by Csa7G067350 did not inhibit the activity of the cysteine proteinase papain (SI Appendix, Fig. S2). Further analysis of conserved motifs established that Csa7G067350 contained only one copy of the [LV]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDOV]-[HYFO]-N motif, while CsPP1 presented two copies of this motif 1 and CmPP1 had eight copies (35). As compared to CsPP1 and CmPP1, Csa7G067350 contained conserved motifs 6, 7, and 9, but lacked motif 2 (SI Appendix, Fig. S1 B–D).

We then tested the subcellular localization of Csa7G067350 by transiently expressing Csa7G067350 fused to the Green Fluorescent Protein (GFP) gene and placed under the control of the Super promoter, in both cucumber protoplasts and Nicotiana benthamiana leaves. We detected green fluorescence in the cytoplasm and the nucleus (SI Appendix, Fig. S3), which is similar to the localization of the homologous barley (H. vulgare) proteins CYSTEINE PROTEASE INHIBITOR1 (HvCPI1) and HvCPI4 (30). We repeated this experiment with the endogenous Csa7G067350 promoter driving the GFP fusion construct and observed a similar pattern, validating the localization of the protein (SI Appendix, Fig. S3).

Csa7G067350 is HighlyExpressed in the Floral Meristems, Floral Primordia, and Vasculature in Cucumber. Floral organogenesis is an important developmental process that directly affects fruit yield and quality in cucumber. After a very short juvenile stage, the indeterminate growth habit of cucumber plants is accompanied by the parallel production of both vegetative and reproductive structures (38). We observed floral buds in the axil of leaves in seedlings at the two-leaf (S2) and three-leaf (S3) stages (Fig. 1 A, Left). An anatomical analysis of shoot tips showed that floral meristems initiated at the one-leaf stage (S1) and subsequently formed floral primordia at and after S2 (Fig. 1 A, Right). To generate a floral meristem-specific gene-expression atlas, we collected the shoot tips of seedlings at the S0 (cotyledons only) and S1 stages for transcriptome deep-sequencing (RNA-seq) analysis. As expected, transcripts for several known floral-specific genes such as AGAMOUS-LIKE6 (AGL6), SEPALATA2 (SEP2), CRABS CLAW (CRC), APETALAI (AP1), CONSTANS-LIKE3 (COL3), and COL5 were abundant in S1 shoot tips but barely detectable in S0 shoot tips (Fig. 1B and Dataset S1) (39, 40). Notably, we noticed that the Csa7G067350 gene, encoding an unknown protein, exhibited high expression in S1 shoot tips as well (Fig. 1B). RT-qPCR indicated that Csa7G067350 transcript levels gradually increased from the S0 to the S2 in shoot tips, and then decreased at the S3 (Fig. 1C). We then determined the expression levels of Csa7G067350 in various organs and tissues: Csa7G067350 expression was relatively high in reproductive organs, including male flowers, female flowers, and ovaries (Fig. 1D). Notably, the vascular bundles (VBs) in the ovary/fruits of cucumber are arranged in four systems, two collateral and two bicollateral (with internal and external phloem located on both sides of the xylem) (41). Accordingly, based on our previous transcriptome of fruit phloem during early development (41), Csa7G067350 was found to show a significantly higher expression in the phloem of those four VB systems when compared to the fruit flesh (Fig. 1E).

To investigate the molecular function of Csa7G067350 in cucumber, we generated transgenic cucumber lines with higher (overexpression) and lower (RNAi) levels of Csa7G067350. We obtained more than 40 primary (T0) transformants in total and selected two independent RNAi T2 lines (RNAi#18 and RNAi#33) and two overexpression T2 lines (OE#3 and OE#27) based on their Csa7G067350 expression levels for further study. RT-qPCR confirmed that Csa7G067350 transcript levels were dramatically reduced in the floral buds of RNAi lines, but increased in OE lines, as compared to wild-type (WT) plants (Fig. 2A).

Cucumber plants bear both male and female flower buds (designated MFB and FFB, respectively). Importantly, both MFBs (Fig. 2B–D) and FFBs (Fig. 2E–I) displayed a deformed phenotype relative to wild-type flowers in RNAi and OE transgenic lines, which inspired the Csa7G067350 gene name DEFORMED FLORAL BUD1 (CsDFB1). Silencing of CsDFB1 by RNAi produced more floral organs per flower compared to WT...
Fig. 1. Expression profile of Csa7G067350 in wild-type cucumber. (A) Morphology of cucumber plants from S0 to S3 (Left) and corresponding longitudinal paraffin sections of shoot tips (Right). White arrowheads indicate floral buds. (Scale bars, 1 cm at Left and 100 μm at Right.) (B) Heatmap representation of relative expression values of several differentially expressed genes between S0 and S1 shoot tips. (C and D) Expression analysis of Csa7G067350 by RT-qPCR in shoot tips from S0 to S3 (C) and different organs (D). Error bars indicate SD of three biological replicates from different plants. (E) Expression of Csa7G067350 by laser capture microdissection (LCM)-derived RNA-seq analysis in phloem systems of cucumber ovary/fruit during early development. For detailed experimental methods, please refer to Sui et al. (41). (F–T) In situ hybridization detection of Csa7G067350 transcripts in the shoot tip (F–J) and ovary/fruit (K–O and Q–T). (F) Floral meristem. (G) Floral bud at early stage. (H) Male floral bud. (I and J) Female floral bud. (K) Cross-section of the ovary/fruit. (L–O) Close-up views of different types of vascular bundles, indicated by the red dashed boxes in K. (P–T) Negative control for in situ hybridization, using Csa7G067350 sense probe in the shoot tip (P) and ovary/fruit (Q–T). Purple staining in F–J and red triangles in L–O indicate positive signal. (Scale bar, 50 μm.) Abbreviations: S0, cotyledon stage; S1, one-leaf stage; S2, two-leaf stage; S3, three-leaf stage; FPKM, fragments per kilobase of transcript per million mapped reads; R, root; S, stem; L, leaf; MF, male flower; FF, female flower; Ov, ovary; sam, shoot apical meristem; le, leaf or leaf primordia; fm, floral meristem; vb, vascular bundle; se, sepal primordia; pe, petal primordia; st, stamen primordia; ca, carpel primordia; pl, placenta; MVB, PeVB, CVB, and PlVB, main, peripheral, carpel, and placental vascular bundle, respectively; EP, external phloem; X, xylem; IP, internal phloem; and P, phloem.
plants (Fig. 2 B, C, and E–G), with an increase in organ number of about 20 to 80% for petals, stamens, and carpels (Fig. 2K). By contrast, OE lines had fewer floral organs than WT plants (Fig. 2 B, C, E, G, and K). Furthermore, overexpression of CsDFB1 could lead to the phenotype of organ fusion between whorls of MFB and FFB, including fused MFBs, fused FFBs, and even fused FFBs with MFB (SI Appendix, Fig. S4 A–I). This fusion defect was observed in the range of 25 to 70% in the MFBs of CsDFB1-OE lines, and 3 to 20% in the FFBs of the OE plants (SI Appendix, Fig. S4 J and K).
The determination and patterning of VBs in the floral buds of CsDFB1 transgenic lines was similarly disrupted compared to that in WT plants (Fig. 2D and G–I). The number of pedicel/peduncle VBs (Fig. 2D and I) and ovary/fruit MVBs (Fig. 2G and K) was increased in RNAi lines but decreased in OE lines. Preparation of hand-cut sections followed by phloroglucinol staining confirmed the disruption of vascular distribution in the transgenic lines, especially in OE plants (Fig. 2D and I). Moreover, in RNAi lines, we observed an abnormal pattern of increased differentiation of the phloem at the expense of the

Fig. 3. CsDFB1 transgenic plants have disrupted auxin distribution. (A) Significantly enriched GO pathways in CsDFB1-RNAi plants based on RNA-seq analysis. (B) RT-qPCR analysis of genes related to polarity or meristem development (e.g., CsAS1, CsAS2, CsARF3, and CsKAN1), auxin biosynthesis (e.g., CsYUC2/4/6), and auxin transport (e.g., CsPIN1) in shoot tips of CsDFB1 transgenic plants and WT plants. Error bars indicate SD of three biological replicates from different plants. Gene IDs: ASYMMETRIC LEAVES1 (CsAS1), Csa3G264750; ASYMMETRIC LEAVES2 (CsAS2), Csa2G070920; AUXIN RESPONSE FACTOR3 (CsARF3), Csa6G518210; KANADI1 (CsKAN1), Csa3G194380; YUCCA2 (CsYUC2), Csa1G242600; YUCCA4 (CsYUC4), Csa2G379350; YUCCA6 (CsYUC6), Csa2G375750; and PIN-FORMED1 (CsPIN1), Csa4G430820. (C) Immunolocalization of IAA in shoot tips of CsDFB1 transgenic plants and WT plants using fluorescein isothiocyanate (FITC)-conjugated (Upper) and alkaline phosphatase (AP)-conjugated secondary antibody (Lower). Green fluorescence and purple staining indicate the signals. (Scale bar, 200 μm.) (D) IAA level in the shoot tips of CsDFB1 transgenic plants and WT plants. Error bars indicate SD of three biological replicates from different plants. (E) Representative photograph of CsDFB1-OE plants and WT plants with or without treatment with 50 μM NAA. Blue numbers inside white boxes indicate the number of petals. (Scale bar, 1 cm.) Significance analysis was conducted with the two-tailed Student’s t test (**P < 0.01; *P < 0.05). Abbreviations: gfw−1, per gram fresh weight; fm, floral meristem; and NAA, 1-naphthylacetic acid.
xylem in the MVB region of ovaries/fruits; conversely, OE lines exhibited xylem tissue partially surrounding the phloem (Fig. 2H). Accordingly, after 30 d of development, the fruits produced by RNAi lines were shorter and thicker than those of WT plants, whereas fruits from OE lines took on a hook-like shape, sometimes even bent and two-fused shape (Fig. 2J). These results demonstrated that CsDFB1 may participate in floral organogenesis and vascular differentiation in cucumber.

CsDFB1 Regulates Local Auxin Distribution. To explore the function of CsDFB1 during floral organ development and vascular formation, we conducted an RNA-seq analysis to access the complement of differentially expressed genes (DEGs) between WT and CsDFB1-RNAi transgenic plants in shoot tips. We identified 320 down-regulated and 366 up-regulated genes in the RNAi lines relative to WT plants (Dataset S2), using a false discovery rate (FDR) of 0.05 and fold change (FC) of at least 1.5 as related genes, such as ASYMMETRIC LEAVES1 (AS1), AS2, AUXIN RESPONSE TRANSCRIPTION FACTOR3 (ARF3), and KANADI1 (KAN), was markedly altered in RNAi and OE lines, in opposite directions (Fig. 3B) (43, 44).

Most importantly, we also noted strong enrichment of the GO categories auxin metabolic process, response to auxin, auxin transport, and auxin-activated signaling pathway (Fig. 3A). As auxin plays a crucial role in both floral organ development and vascular differentiation (5), we hypothesized that CsDFB1 might be involved in auxin homeostasis. Indeed, several YUCCA4 auxin biosynthetic genes (CsYUC2, CsYUC4, and CsYUC6) were expressed at levels two- to fourfold higher in the RNAi lines relative to WT, with slightly lower levels in the OE lines (Fig. 3B). Interestingly, CsDFB1-RNAi lines often developed three cotyledons and formed fasciated shoots, phenotypes that were reminiscent of the auxin transport-deficient transposon insertional mutant pin1::En134 (23, 48). Thus, we thus turned to auxin efflux carrier genes in cucumber: we discovered that the expression of the cucumber PIN1 ortholog CsPIN1 was reduced in the RNAi lines and increased in the OE plants (Fig. 3B).

Consistent with these results, immunolocalization of IAA on shoot tips demonstrated a strong auxin accumulation in the floral meristems (Fig. 3C) and floral primordia (SI Appendix, Fig. S6) of RNAi lines relative to WT plants. Conversely, OE shoot tips exhibited a weaker IAA signal. These results were corroborated by ultra-performance liquid chromatography (UPLC) followed by tandem mass spectrometry (MS/MS) measurements of IAA levels in shoot tips (Fig. 3D). Moreover, exogenous application of the synthetic auxin 1-naphthaleneacetic acid (NAA) increased the number of petals in WT plants and restored normal floral organogenesis (Fig. 3D) coincided with those of CSYUC2 and CSPIN1 transcripts (Fig. 5I) coincided with those of CSYUC2 and CSPIN1 promoters (Fig. 5H). To validate this interaction in vivo, we next performed luciferase (LUC) transactivation assays in cucumber protoplasts. LUC activity derived from the CsYUC2 promoter was significantly enhanced upon cotransformation with CSYUC2; by contrast, LUC activity from the CSYUC1 promoter was repressed upon coexpression with CSYUC2 (Fig. 5H). We also established that the accumulation patterns of CSYUC2 and CSYUC1 transcripts (Fig. 5I) coincided with those of CSYUC2 and CSYUC1 promoters (Fig. 5H).

Next, we attempted to decipher how CsDFB1 influences CSYUC2 and CSPIN1 transcription in cucumber. Sequence analysis identified typical binding sites for HD-ZIP III transcription factor [GTAAT(G/C)ATTAC] in the CsYUC2 promoter was significantly enhanced upon cotransformation with CsPIN1*; by contrast, LUC activity from the CsYUC2 promoter was repressed upon coexpression with CsPIN1 (Fig. 5H). We also established that the accumulation patterns of CSYUC2 and CSYUC1 transcripts (Fig. 5I) coincided with those of CSYUC2 and CSYUC1 promoters (Fig. 5H).
Accordingly, we transiently coinfiltrated *N. benthamiana* leaves with the LUC reporters and the CsPHB and CsDFB1 effectors. CsDFB1 impaired the CsPHB-dependent activation of CsYUC2 and the repression of CsPIN1 (Fig. 6A) by preventing CsPHB binding to its cognate DNA-binding site (Fig. 6B). We also generated *CsPHB*-OE (♀) × *CsDFB1-OE (♂) hybrid plants. Notably, overexpression of *CsDFB1* rescued the floral organ and vascular bundle defects in *CsPHB*-OE
lines, as well as the abnormal expression of CsYUC2 and CsPIN1 (Fig. 6 C and D). Finally, we tested the accumulation pattern of CsPIN1 transcripts in WT, CsDFB1-RNAi, and CsDFB1-OE transgenic plants (Fig. 6E). Compared to WT plants, CsPIN1 transcript levels were greatly reduced in the shoot apical meristems, floral meristems, and VBs of CsDFB1-RNAi plants, but higher in CsDFB1-OE plants, especially in the vasculature (Fig. 6E).

Fig. 5. CsPHB regulates floral development and vascular patterning by controlling CsYUC2 and CsPIN1 expression. (A) RT-qPCR analysis of CsPHB expression in the floral buds of WT and CsPHB overexpression lines (CsPHB*-OE#6 and CsPHB*-OE#20). Error bars indicate SD of three biological replicates from different plants. (B) Summary of floral organ and MVB numbers in WT and CsPHB overexpression plants. Error bars indicate SD of 20 flowers from around 10 individual plants. (C) Phenotype of male flower and ovary/fruit from WT and the CsPHB*-OE20 line. Blue and white numbers indicate the number of petals and carpels, respectively; red arrows show the positions of MVBs. (Scale bar, 1 cm.) (D) Hand sections of ovary MVB stained with toluidine blue. (Scale bar, 100 μm.) (E) IAA levels in shoot tips of CsPHB*-OE20 and WT plants. Error bars indicate SD of three biological replicates. (F) RT-qPCR analysis of CsYUC2 and CsPIN1 expression in shoot tips of CsPHB*-OE plants and WT plants. Error bars indicate SD of three biological replicates. (G) Yeast one-hybrid assays testing the binding of CsPHB to the CsYUC2 and CsPIN1 promoters. (H) EMSAs showing that CsPHB binds to the CsYUC2 and CsPIN1 promoters. Competition for binding was performed using 10x and 100x unlabeled probes; mutant probe (mu1 and mu2), and MBP was used as negative controls. (I) Transcriptional activity of CsPHB* on the CsYUC2 (Left) and CsPIN1 (Right) promoters in cucumber protoplasts. Data are normalized to samples expressing GFP. Error bars indicate SD of three biological replicates. (J) In situ hybridization detection of CsYUC2 and CsPIN1 transcripts in shoot tips of wild-type cucumber. (Scale bar, 100 μm.)
Taken together, our findings reveal that CsDFB1 functions as a repressor of CsPHB to attenuate CsPHB-mediated activation of CsYUC2 and repression of CsPIN1 expression, and thus participates in controlling local auxin distribution and plant development in cucumber (Fig. 6). Local auxin biosynthesis and polar auxin transport are essential to establishing local auxin gradients throughout plant development. Local auxin biosynthesis is controlled by various environmental (high temperature, shading, and sugars) as well as endogenous signals (phytohormones and developmental processes). Transcriptional regulation of YUC genes, encoding the rate-limiting step of auxin biosynthesis, is crucial to controlling local auxin biosynthesis and thus is indispensable for proper plant development in Arabidopsis. The transcription factor PLETHORA (PLT) controls spiral phyllotaxis by ensuring sufficient production of auxin at the shoot apex, probably by regulating YUC1 and YUC4 genes.

**Discussion**

Local auxin biosynthesis and polar auxin transport are essential to establishing local auxin gradients throughout plant development (7, 10). Local auxin biosynthesis is controlled by various environmental (high temperature, shading, and sugars) as well as endogenous signals (phytohormones and developmental processes) (25, 26, 49–53). Transcriptional regulation of YUC genes, encoding the rate-limiting step of auxin biosynthesis (5), is crucial to controlling local auxin biosynthesis and thus is indispensable for proper plant development. In Arabidopsis, the transcription factor PLETHORA (PLT) controls spiral phyllotaxis by ensuring sufficient production of auxin at the shoot apex, probably by regulating YUC1 and YUC4 genes.
expression (15). The master regulator of flower determinacy AGAMOUS (AG) and the YABBY-type transcription factor CRABS CLAW (CRC) also regulate auxin biosynthesis by physically binding to the YUC4 promoter (25). However, auxin levels are not by themselves sufficient to initiate auxin-dependent biological processes; establishing an accurate auxin gradient is also essential. PIN proteins are major auxin efflux carriers that are required to establish auxin gradients and are associated with various important developmental events (10). Although many studies have focused on PIN regulation at the posttranscriptional level, transcriptional regulation of PIN is also vital for plant development. For instance, the MADS-box transcription factor AGL14/AXANTAL2 (XAL2) regulates auxin transport and auxin gradient in roots by acting on PIN transcription in Arabidopsis (54). Similarly, cucumber CsFUL1 functions as a transcriptional repressor that inhibits CsPIN1 and CsPIN7 expression and hence regulates fruit length (8).

Here, we discovered a transcriptional regulatory module, consisting of CsDFB1 and CsPHB, acting in local auxin biosynthesis and transport in cucumber. CsDFB1 encodes a cystatin-like protein from the plant cystatin superfamily (also known as the phytocystatins), which are generally considered to be involved in defense responses and regulation of protein turnover (28–37). Unexpectedly, silencing or overexpression of CsDFB1 resulted in defects in floral organogenesis (leading to malformed flowers) and vascular differentiation, which are classical features of plant development governed by auxin (4–6). Auxin content was higher in CsDFB1-OE lines but lower in CsDFB1-RNAi lines. More importantly, the floral defects of CsDFB1-OE lines were rescued by the application of exogenous auxin, further supporting the idea that CsDFB1 participates in modulating local auxin distribution.

CsDFB1 physically interacted with CsPHB, an HD-ZIP III transcription factor with critical roles in meristem formation, polarity establishment, and vascular differentiation (46, 55–60). In Arabidopsis, the gain-of-function mutation phb-1d produces rod-like leaves because of a transformation from abaxial to adaxial cell fate (60). The loss of function of PHB and the related factor REVOLUTA (REV) results in a reduction in flower meristem activity and floral organ number (57, 59). In cucumber, gain-of-function mutations in CsPHB result in curly leaves and increased floral organ number (46). Several HD-ZIP III transcription factors are also involved in regulating auxin homeostasis. In poplar (Populus trichocarpa), PtrHUB7 interacts with the Aux/IAA9-ARF5 module as a key regulator to control secondary xylem and phloem differentiation (55, 56). REV directly binds to the YUC5 and TAA1 promoters and induces their expression in Arabidopsis (23). In this study, overexpression of CsPHB* caused an increased number of floral organs and abnormal vascular differentiation concomitant with auxin accumulation. We demonstrated that CsPHB is a direct regulator that acts upstream of CsYUC2 and CsPIN1 but with opposite outcomes, as CsPHB induced CsYUC2 expression but repressed CsPIN1 expression. Accordingly, auxin content was elevated in CsPHB*-OE plants. Notably, in situ hybridization showed that CsDFB1 shared an expression pattern with CsPHB both spatially and temporally, indicating that CsDFB1 and CsPHB may indeed cooperate to fine tune the dynamics of local auxin homeostasis. In addition, CsDFB1 appeared to act as an attenuator of CsPHB function in the context of the activation of CsYUC2 expression and the repression of CsPIN1 expression. Adding CsDFB1 to the EMSAs reduced the binding of CsPHB to the two promoters. Moreover, overexpression of CsDFB1 rescued the abnormal expression of CsYUC2 and CsPIN1 in CsPHB*-OE plants. Thus, changes in auxin dynamics, based on the modulation of local auxin biosynthesis and polar transport, may explain how CsDFB1 affects floral and vascular development in cucumber.

Interestingly, in Arabidopsis sup mutants, the up-regulation of YUC1 and YUC4 and the down-regulation of PIN3 and PIN4 expression result in elevated auxin levels at the boundary between whorls 3 and 4, leading to increased number of reproductive organs (26). By contrast, the numbers of petals, stamens, and carpels all increased in CsDFB1-RNAi lines and CsPHB*-OE lines, indicating that the CsDFB1-CsPHB module influences auxin biosynthesis and auxin transport at whorls 2, 3, and 4. We found that the CsDFB1-CsPHB module functions redundantly with or in parallel to SUP in controlling the development of whorls 3 and 4 needs to be clarified.

Besides CsPHB, CsDFB1 also interacted with the YABBY-type transcription factor CsYAB2 as well. HD-ZIP III and YABBY-type transcription factors have opposite effects on polarity establishment (58, 61). Recently, the YABBY-type transcription factor CRC was reported to directly bind to the YUC4 promoter and activate its expression in flower primordia in Arabidopsis (25). It would be interesting to explore whether and how CsDFB1 may influence CRC-mediated auxin biosynthesis in cucumber.

CsDFB1 was expressed in the phloem, like its homologs AtCYS1 in Arabidopsis (31) and CmPP1 in pumpkin (35), suggesting that CsDFB1 might have similar functions to these cystatin family members in vascular tissues. However, CsDFB1 did not inhibit the activity of the cysteine proteinase papain in vitro. A previous study has reported that the expression of rice cystatins is suppressed by auxin treatment during callus differentiation (62). More interestingly, an aspartic protease in rice (OsAsp1) was recently shown to interact with a zinc finger transcription factor OsTIF1 to dismiss OsTIF1-mediated transcriptional inhibition of auxin biosynthesis gene OsTAA1 and thus increase IAA content (63). It will therefore be worth examining whether CsDFB1 function in auxin homeostasis evolved from an ancestor that originally possessed cystatin function.

Collectively, our findings revealed a regulatory pathway of local auxin distribution during floral organogenesis and vascular differentiation in cucumber. Whether this pathway is conserved in other species, and how it interacts with other known regulatory pathways, will be fruitful avenues of future inquiry.

Materials and Methods
A detailed description of plant materials, plant growth conditions, bioinformatic sequence analysis, vector construction and plant transformation, gene expression analysis (in situ hybridization, RT-qPCR, and RNA-seq), histochimical staining, quantification of endogenous auxins, immunolocalization of IAA, phytohormone treatments, subcellular localization, Western blot analysis, the protein–protein interaction assays (BiFC, LCI, and pull-down), the DNA–protein interaction assays (YTH, EMSA, and dual-LUC), and any associated references are available in SI Appendix, Materials and Methods. Accession numbers used for the phylogenetic analysis are listed in SI Appendix, Table S1. Primers used in this study are listed in SI Appendix, Table S2.

Data Availability. All study data are included in the article and/or supporting information.

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