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RESEARCH ARTICLE

Gene regulatory network controlling carpel number variation in cucumber

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ABSTRACT

The *WUSCHEL-CLAVATA3* pathway genes play an essential role in shoot apical meristem maintenance and floral organ development, and under intense selection during crop domestication. The carpel number is an important fruit trait that affects fruit shape, size and internal quality in cucumber, but the molecular mechanism remains elusive. Here, we found that *CsCLV3* expression was negatively correlated with carpel number in cucumber cultivars. *CsCLV3-RNAi* led to increased number of petals and carpels, whereas overexpression of *CsWUS* resulted in more sepals, petals and carpels, suggesting that *CsCLV3* and *CsWUS* function as a negative and a positive regulator for carpel number variation, respectively. Biochemical analyses indicated that CsWUS directly bound to the promoter of *CsCLV3* and activated its expression. Overexpression of *CsFUL1^{A}*, a *FRUITFULL-like* MADS-box gene, resulted in more petals and carpels. CsFUL1^{A} can directly bind to the *CsWUS* promoter to stimulate its expression. Furthermore, we found that auxin participated in carpel number variation in cucumber through interaction of CsARF14 with CsWUS. Therefore, we have identified a gene regulatory pathway involving *CsCLV3*, *CsWUS*, *CsFUL1^{A}* and *CsARF14* in determining carpel number variation in an important vegetable crop – cucumber.

KEY WORDS: Cucumber, CsCLV3, CsWUS, Carpel number, CsFUL1^{A}, Auxin

INTRODUCTION

Fruits and seeds are the predominant sources of food in our diets. To meet the increasing food demand, crop plants have undergone intense human selection for larger fruits and more seeds (Doebley et al., 2006; Kuittinen and Aguade, 2000). Flowers are the reproductive structures that give rise to fruits and seeds in higher plants. In the model plant *Arabidopsis thaliana*, the flower comprises four floral organs, sepals, petals, stamens and carpels, arranged in circular whorls from outer to inner most (Smyth et al., 1990). Cucumber (*Cucumis sativus* L.) is a world-wide cultivated vegetable crop in the Cucurbitaceae family bearing unisexual flowers. The cucumber fruit develops from the female flower in the leaf axil that typically consists of five sepals, five petals, three fused carpels and five suppressed stamens (Bai et al., 2004), and can be consumed freshly or processed into pickles at 8-18 days after anthesis (Weng et al., 2015). The carpel number (CN) is an important fruit trait that affects fruit shape, fruit size and internal quality in cucumber. In natural cucumber populations, carpel number (CN) can vary from two to seven (Li et al., 2016). An increase in carpel number (CN) is usually concomitant with enlargement of fruit diameter, and therefore results in changes in fruit shape, size and/or flavor. For example, most cultivated cucumbers bear cylindrical fruits that generally have three carpels (CN=3), whereas many cultivars in the Xishuangbanna region bear spherical fruits with CN=5 (Li et al., 2016). The CN of fruits is determined early in the floral meristem development, in which *WUSCHEL (WUS)-CLAVATA3* (*CLV3*) pathway genes play essential roles and have been under selection during crop domestication (Somssich et al., 2016). For example, the ancestor of tomato had only two locules (carpels) and the fruit was small, but modern cultivars have eight or more locules, owing to natural mutations of *WUS-CLV* signaling genes (Somssich et al., 2016). Similarly, in maize, disturbance of *WUS-CLV* pathways genes resulted in more kernel rows and higher yields (Bommert et al., 2013b; Je et al., 2016). However, the function of *WUS-CLV* pathway in carpel number variation remain largely unknown in cucumber.

The role of *WUS-CLV* pathway in shoot apical meristem (SAM) maintenance and floral organ development is well studied in *Arabidopsis*. CLAVATA3 (CLV3) is the founding member of the CLAVATA3/EMBRYO SURROUNDING REGION (ESR)-related (CLE) peptide family (Cock and McCormick, 2001). The proteins encoded by *CLV3* is a secreted peptide ligand that is required for stem cell maintenance in the SAM (Rojo et al., 2002). Overexpression of *CLV3* results in premature termination of the SAM, whereas loss of function of *CLV3* leads to enlarged SAM with over-accumulation of stem cells and production of more floral organs (Brand et al., 2000; Mayer et al., 1998; Miwa et al., 2009). *CLV3* is specifically expressed in the stem cells in the central zone of the SAM and thus serves as a stem cell marker in *Arabidopsis* (Müller et al., 2006). *WUSCHEL* (*WUS*) encodes a homeodomain transcription factor with a homeodomain, a WUS-box motif and an ERF-associated amphiphilic repression (EAR) motif (van der Graaff et al., 2009). *WUS* is expressed in the organizing center (OC) underlying the central zone to promote stem cell activity by stimulating *CLV3* expression in a non-cell-autonomous manner (Lenhard and Laux, 2003). Overexpression of *WUS* led to ectopic floral buds (Xu et al., 2005), whereas knockout of *WUS* caused stem

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cell depletion and arrest of organ initiation from the SAM (Laux et al., 1996). Inhibition of *WUS* expression at the floral meristem termination stage is required for gynoecium development and consequently affects the carpel number (CN) (Sun et al., 2009). As a feedback, *WUS* expression is restricted to the OC by the CLV signal transduction pathway involving CLV1, CLV2 and CLV3 (Brand et al., 2000; Schoof et al., 2000). *CLV1* encodes a leucine-rich repeat (LRR) receptor kinase with a transmembrane domain that acts as a key receptor to perceive and bind to CLV3 peptide (Clark et al., 1997; Ogawa et al., 2008). CLV2, a LRR receptor-like protein without a kinase domain (Jeong et al., 1999), interacts with another receptor-like kinase, CORYNE (CRN), to transmit CLV3 signal in parallel to CLV1 (Bleckmann et al., 2010; Guo et al., 2010; Müller et al., 2008; Zhu et al., 2010). The products of three *BARELY ANY MERISTEM* (*BAM*) genes, which are related to CLV1, appear to be indirect receptors of CLV3 in *Arabidopsis* (DeYoung et al., 2006). RECEPTOR LIKE PROTEIN KINASE2 (RPK2) acts as another LRR receptor of CLV3 signaling and functions redundantly with *CLV1*, *CLV2*/*CRN* and BAM pathways (Kinoshita et al., 2010; Replogle et al., 2013). Thus, the WUS-CLV regulatory circuitry forms a self-correcting mechanism that balances cell division and cell differentiation to maintain proper SAM size during continuous organogenesis.

In addition, the functions of *WUS* and *CLV3* have also been characterized in crops (Fletcher, 2018). In rice, the *WUS* ortholog *MONOCULM 3/TILLERS ABSENT 1/STERILE AND REDUCED TILLERING 1* (*MOC3/TAB1/SRT1*) is absent in the SAM but found in the premeristem zone that subsequently develop into axillary meristem (Shao et al., 2019). *OsWUS* knockouts failed to form tillers, and gave rise to disrupted inflorescence and spikelets (Lu et al., 2015; Tanaka et al., 2015). Mutation in the *FLORAL ORGAN NUMBER* (*FON2* or *FON4*), the *CLV3* ortholog in rice (Chu et al., 2006; Suzaki et al., 2006), leads to an enlarged SAM, inflorescence meristem and floral meristem (FM), and to an increased number of floral organs. In maize, WUS has two orthologs that display different expression patterns: *ZmWUS1* is expressed in the center of SAM, whereas *ZmWUS2* is expressed in leaf primordia (Nardmann and Werr, 2006). During tomato domestication, a regulatory mutation in *SlCLV3*, named as the *fasciated* (*fas*) locus, played an important role in the increased fruit size in modern cultivars (Rodríguez-Leal et al., 2017; Xu et al., 2015). The *SlCLV3* expression is mainly detected in the central cells of the SAM but is absent from the L1 layer. Knockdown of *SlCLV3* leads to increase in floral organ number and enlargement of the SAM size (Xu et al., 2015). Additionally, gain-of-function mutation of *SlWUS* underlying the *locule number* (*lc*) locus gave rise to fruits with more locules (Chu et al., 2019; Muños et al., 2011; Nardmann and Werr, 2006; Rodríguez-Leal et al., 2017).

Several regulators have been identified as participating in the SAM maintenance and organ development through mediating the WUS-CLV pathway. The bHLH transcription factor gene *HECATE1* (*HEC1*) is directly repressed by *WUS*, which is a negative regulator of *CLV3* expression; low level of *HEC1* is required for stem cell integrity in *Arabidopsis* (Schuster et al., 2014). HAIRY MERISTEM (HAM), a GRAS-domain transcription factor physically interacts with WUS to confine *CLV3* expression in the stem cells in the SAM (Zhou et al., 2015, 2018). *HANABA TARUNU* (*HAN*) encodes a GATA-3-like protein that confines *WUS* expression to the OC cells (Zhang et al., 2013; Zhao et al., 2004). The maize *COMPACT PLANT2* (*CT2*) gene, which encodes a putative α-subunit (Gα) of a heterotrimeric GTP binding protein, functions in the perception of CLV3 peptide through its direct interaction with the CLV2 ortholog FASCIATED EAR 2 (FEA2) (Bommert et al., 2013a). In tomato, mutations in arabinosyltransferase enzymes, including *REDUCED RESIDUAL ARABINOSE* (*RRA*) and *FASCIATED INFLORESCENCE* (*FIN*), caused upregulation of *CLV3* and *WUS* (Xu et al., 2015). In addition, several phytohormones participate in SAM activity via the WUS-CLV pathway. WUS negatively regulates cytokinin signaling by directly repressing response regulators *ARR5*, *ARR6*, *ARR7* and *ARR15* (Hwang and Sheen, 2001; Leibfried et al., 2005). Similarly, auxin accumulation is essential for lateral organ initiation from the peripheral zone of the SAM (Gaillochet et al., 2015). The auxin response factor (ARF) MONOPTEROS (MP) functions in the crosstalk of auxin and cytokinin pathways by repressing the expression of *ARR7* and *ARR15* (Zhao et al., 2010).

To reveal the molecular mechanism underling carpel number variation in cucumber, we performed functional analyses of *WUS* and *CLV3* through genetic transformation. We found that *CsWUS* and *CsCLV3* act as a positive and a negative regulator for carpel number variation in cucumber, respectively. Furthermore, we identified a FRUITFUL-like MADS-box protein (*CsFUL1*) that regulates floral organ development through directly promoting *CsWUS* transcription. Auxin participates in carpel number variation in cucumber through physical interaction with CsARF14 and CsWUS.

RESULTS

Sequence feature and phylogenetic analysis of CsCLV3 and CsWUS

Previous studies showed that orthologs of *CLV3* (*FAS*) and *WUS* (*LC*) genes are the key regulators responsible for locule number increase during tomato domestication (Muños et al., 2011; Rodríguez-Leal et al., 2017; Xu et al., 2015). Map-based cloning indicated that *CsCLV3* is the candidate gene underlying carpel number variation in cucumber (Li et al., 2016). To characterize the biological functions of *CsCLV3* and *CsWUS*, we cloned the coding sequence from cucumber inbred line R1461. CsCLV3 encodes a small peptide comprising a solo exon of 156 bp and CsWUS encodes a homeodomain protein consisting of three exons and two introns (Fig. 1A). CLV3 is a member of the *CLE* gene family, with a conserved 14 amino acid domain termed the CLE box (Cock and McCormick, 2001). Protein alignment of CLV3 homologs indicated that cucurbit CLV3 proteins, including cucumber (CsCLV3) and melon (CmCLV3), are shorter than those in *Arabidopsis* (AtCLV3) and tomato (SlCLV3) (Fig. 1B). There are 16 CLE family members in cucumber and 18 CLE genes in melon were identified by searching the Cucurbit Genomics Database. Phylogenetic analysis of CLE proteins showed that *CLV3* has only one homolog *CsCLV3* in cucumber (Fig. S1). WUS belongs to the WUS clade of WOX family, which typically has four conserved domains: homeodomain, acidic domain, WUS-box and EAR-motif (van der Graaff et al., 2009). Sequence alignment showed that the homeodomain, WUS-box and EAR-motif of CsWUS are highly conserved, whereas the acidic domain is quite divergent (Fig. 1B).

Expression pattern of *CsCLV3* and *CsWUS* in cucumber

Previous studies indicated that *CLV3* was specifically expressed in stem cells of the shoot apical meristem (SAM) and floral meristem (FM) in *Arabidopsis* and rice (Chu et al., 2006; Fiers et al., 2005). To investigate the expression pattern of *CsCLV3* in cucumber, we performed *in situ* hybridization in SAM, FM and flowers at different developmental stages. Interestingly, transcripts of *CsCLV3* were specifically enriched in the central region of SAM

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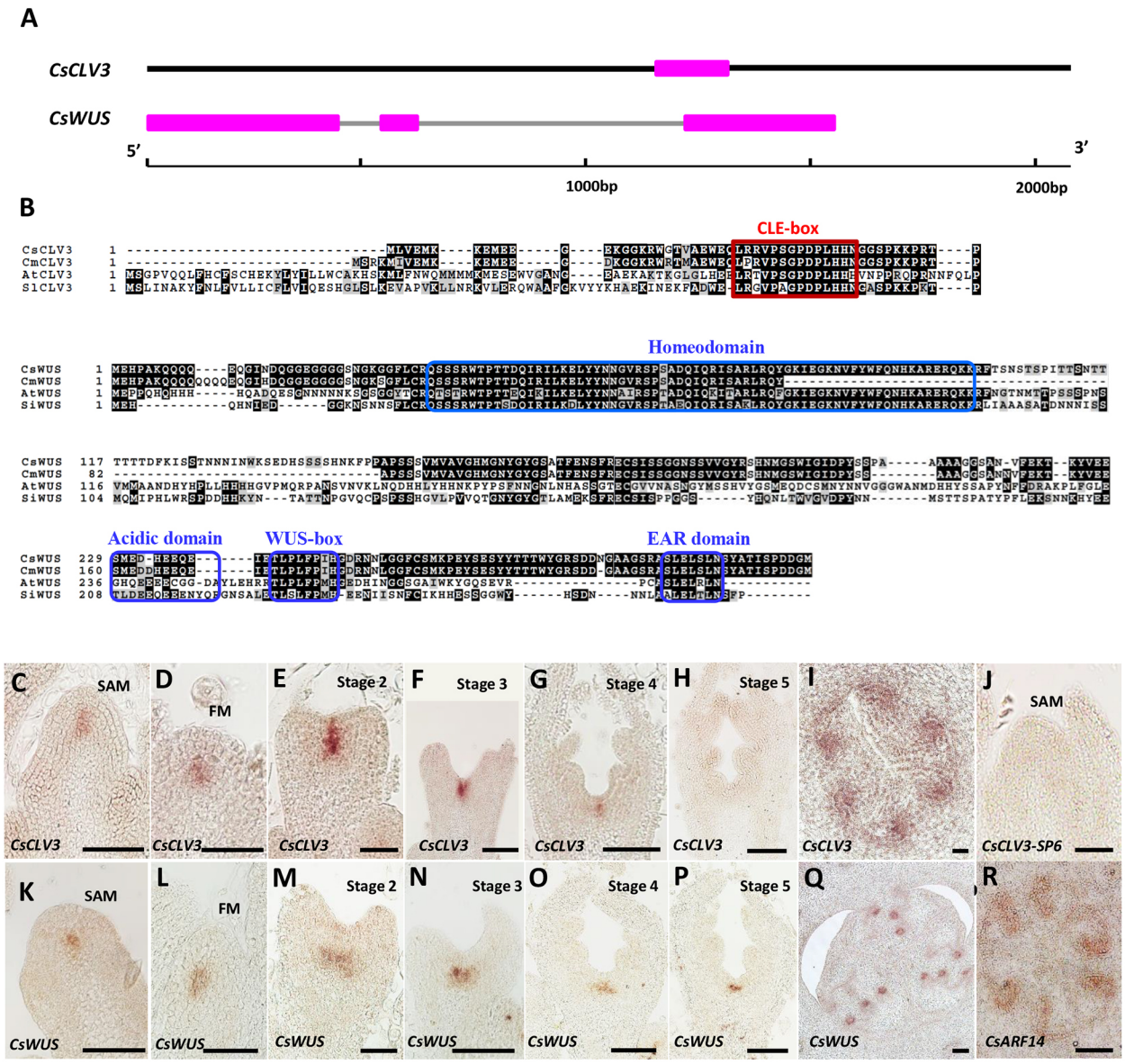

Fig. 1. Sequence features and expression analyses of *CsCLV3* and *CsWUS* in cucumber. (A) *CsCLV3* encodes a small peptide with only one exon (pink box). Black lines indicate the 1156 bp promoter region and 776 bp downstream sequence. *CsWUS* encodes a homeodomain transcription factor with three exons and two introns. Pink boxes indicate the exons and gray lines indicate the introns. (B) Protein sequence alignment of CsCLV3 and CsWUS in cucumber, melon, *Arabidopsis* and tomato. The red frame indicates the conserved CLE box in CsCLV3. The blue boxes show the conserved homeodomain, acidic domain, WUS-box and EAR-motif in CsWUS. (C-R) *In situ* hybridization analysis of *CsCLV3* (C-J), *CsWUS* (K-Q) and *CsARF14* (R) in cucumber. (C,D) *CsCLV3* is expressed in the middle of the central zone of the shoot apical meristem (SAM) (C) and floral meristem (FM) (D). (E-H) *CsCLV3* signal was detected throughout the central zone in the developing floral primordia at stage 2, and gradually became weaker from stage 3 to stage 5. (I) High levels of expression of *CsCLV3* were found in developing ovules in a cucumber female flower. (J) Negative control of *CsCLV3* hybridized with the sense probe in the SAM. (K-Q) *CsWUS* signal was detected in the putative stem cell organizing center of the SAM (K), FM (L) and floral buds (M-P), as well as in the nucellus of ovules (Q). (R) *CsARF14* was expressed in the placenta and ovule primordium in the ovary. Scale bars: 100 µm.

and FM, but were absent in stem cells (Fig. 1C,D). As the flower primordia develop, *CsCLV3* transcripts extended upward to the stem cells at the apex of FM from stage 2-3 (Fig. 1E,F) (Bai et al., 2004). The *CsCLV3* signal became weaker at stage 4 and disappeared by stage 5 (Fig. 1G,H). In cross-sections of cucumber young fruit, strong *CsCLV3* signals were found in developing ovules (Fig. 1I). As a control, no signal was detected upon hybridization with the sense *CsCLV3* probe (Fig. 1J). The expression pattern of *CsCLV3* appears to be similar to that of *WUS* in the SAM and FM (Brand et al., 2000; Yadav et al., 2011). For comparison, *in situ* hybridization was performed for *CsWUS*. Our data showed that *CsWUS* transcripts were specifically accumulated in the central region underneath the central zone in the SAM and FM (Fig. 1K,L), a region overlapping with that of *CsCLV3* (Fig. 1C,D). In the flower buds, *CsWUS* signal was maintained in a group of cells beneath the stem cells (Fig. 1M-P). In ovary, *CsWUS* was expressed in ovules in cucumber (Fig. 1Q).

CsCLV3 negatively regulates floral organ number in cucumber

Commercial cucumbers generally have three carpels (CN=3), but CN can vary from two to five in different cultivars (Fig. 2A). To

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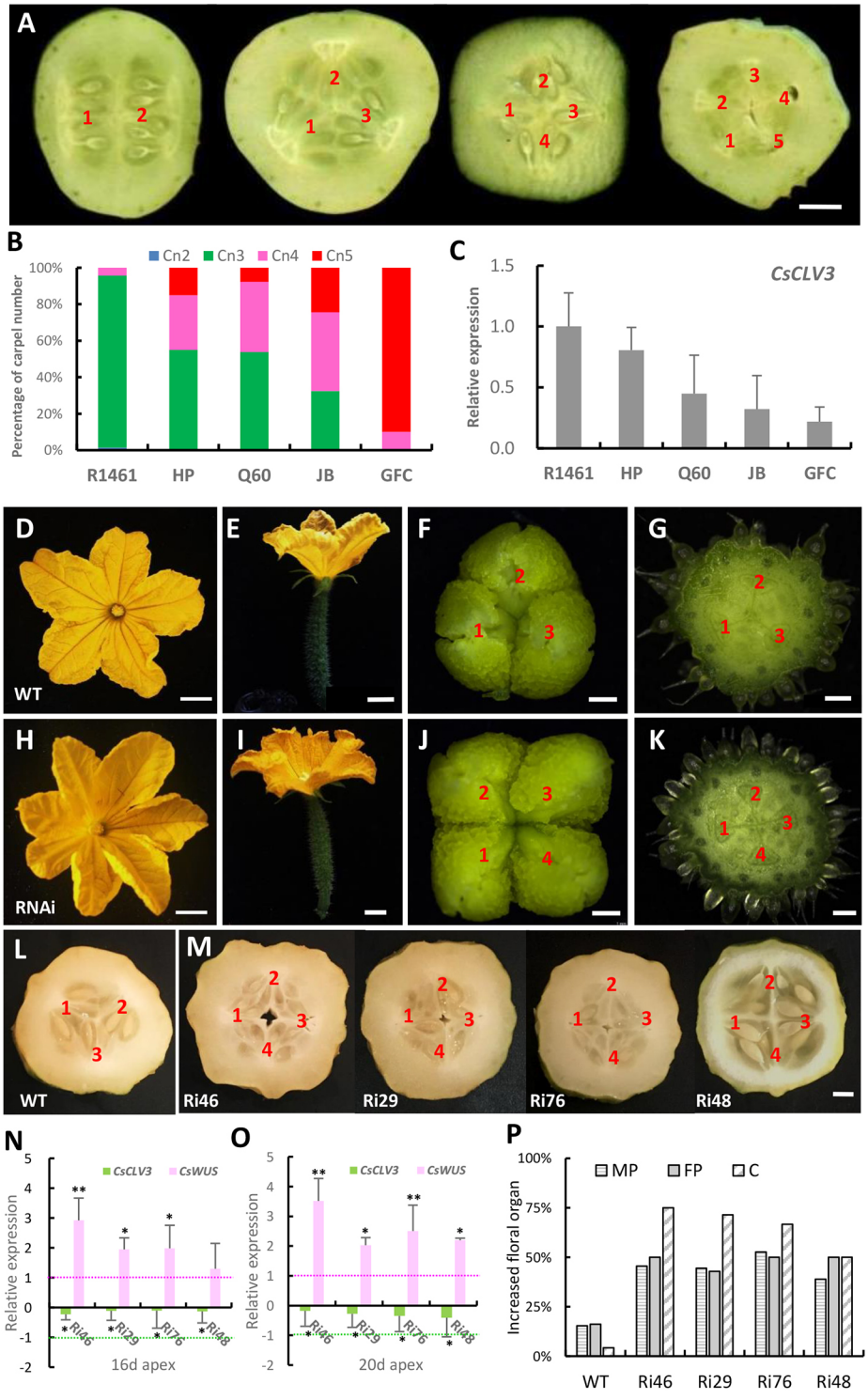

Fig. 2. *CsCLV3* negatively regulates floral organ number in cucumber. (A) Cross-sections of cucumber cultivars with two to five carpels. (B) Five cucumber cultivars with different CN variation. (C) *CsCLV3* expression in different cucumber cultivars. (D-M) Knockdown of *CsCLV3* by RNAi led to more floral organs in cucumber flowers. (D,H) Male flowers. (E,I) Female flowers. (F,J) Stigmas. (G,K) Cross-sections of young fruits. (L,M) Cross-sections of mature fruits in empty vector control (L) and four *CsCLV3*-RNAi transgenic lines (M). (N,O) Expression analyses by qRT-PCR of *CsCLV3* and *CsWUS* in the 16-day and 20-day apex of *CsCLV3*-RNAi transgenic lines. The green and pink dotted lines indicate the expression of *CsCLV3* and *CsWUS* in wild-type plants, respectively. (P) The frequency of increased floral organ number in wild-type and *CsCLV3*-RNAi transgenic lines. MP, male flower petal number; FP, female flower petal number; C, carpel number. Scale bars: 1 cm in A,D,E,H,I,L,M; 1 mm in F,G,J,K. Red numbers indicate carpel. Data are mean±s.e.m. in N,O. **P*<0.05; ***P*<0.01. Three biological replicates and three technique replicates were performed for each qRT-PCR analyses.

dissect the biological function of *CsCLV3* in carpel number variation, we performed qRT-PCR in 16-day-old seedling apices of five cucumber cultivars with different CN frequency (Fig. 2B,C). The expression of *CsCLV3* was found to be negatively correlated with CN variation (Fig. 2C). In the GFC line with mostly CN=5, the expression of *CsCLV3* decreased to 21% when compared with that in the R1461 line with predominantly CN=3 (Fig. 2C).

To further verify the negative role of *CsCLV3* in CN variation, we obtained 12 transgenic cucumber lines using cauliflower mosaic virus 35S (35S) promoter followed with a double-stranded RNA interference (RNAi) construct containing the whole-length coding sequence of *CsCLV3* (*CsCLV3-RNAi*), and chose four representative lines for further characterization. In the control plants, both male and female flowers have five petals (Fig. 2D,E), and there are three stigmas and three carpels in the female flower (Fig. 2F,G). In the *CsCLV3-RNAi* lines, the number of floral organs was greatly increased, including more petals, stigmas and carpels (Fig. 2H-M). Expression analysis indicated that transcripts accumulation of *CsCLV3* decreased to 13-32% of that in wild type, and *CsWUS* expression significantly upregulated two to threefold in the 16-day

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and 20-day apex of *CsCLV3-RNAi* plants (Fig. 2N,O), consistent with the negative feedback of CLV3 on WUS in *Arabidopsis* (Brand et al., 2002; Müller et al., 2006; Schoof et al., 2000). Correspondingly, phenotypic severity positively correlates with *CsCLV3* knockdown (Fig. 2P). In the most severe line Ri46, over 45% male flowers have seven petals and 75% female flowers have four carpels (Fig. 2P). Therefore, *CsCLV3* acts as an important repressor for floral organ specification in cucumber, especially for petal and carpel development.

CsWUS positively regulates carpel number variation in cucumber

Previous study showed that gain-of-function of *LC* (*WUS*) resulted in a high locule-number phenotype in tomato (Chu et al., 2019). To explore the biological function of *CsWUS* in cucumber, we generated overexpression lines of *CsWUS* driven by the 35S promoter. A total of five transgenic lines were obtained and three lines (OX-3, OX-5 and OX-9) were chosen for phenotypic observation. When compared with the vector control (WT), *CsWUS*-OX transgenic plants bore flowers with more floral organs, including increased number of sepals, petals and carpels (Fig. 3A-F). Statistical analysis indicated that the frequency of increased numbers of floral organs was significantly higher in the *CsWUS-OX* lines (Fig. 3G). These data suggest that *CsWUS* acts as a positive regulator of floral organ number in cucumber. Expression analysis showed that transcript accumulation of *CsWUS* and *CsCLV3* were significantly elevated in *CsWUS*-OX transgenic plants (Fig. 3H,I), consistent with the positive regulation of *CLV3*

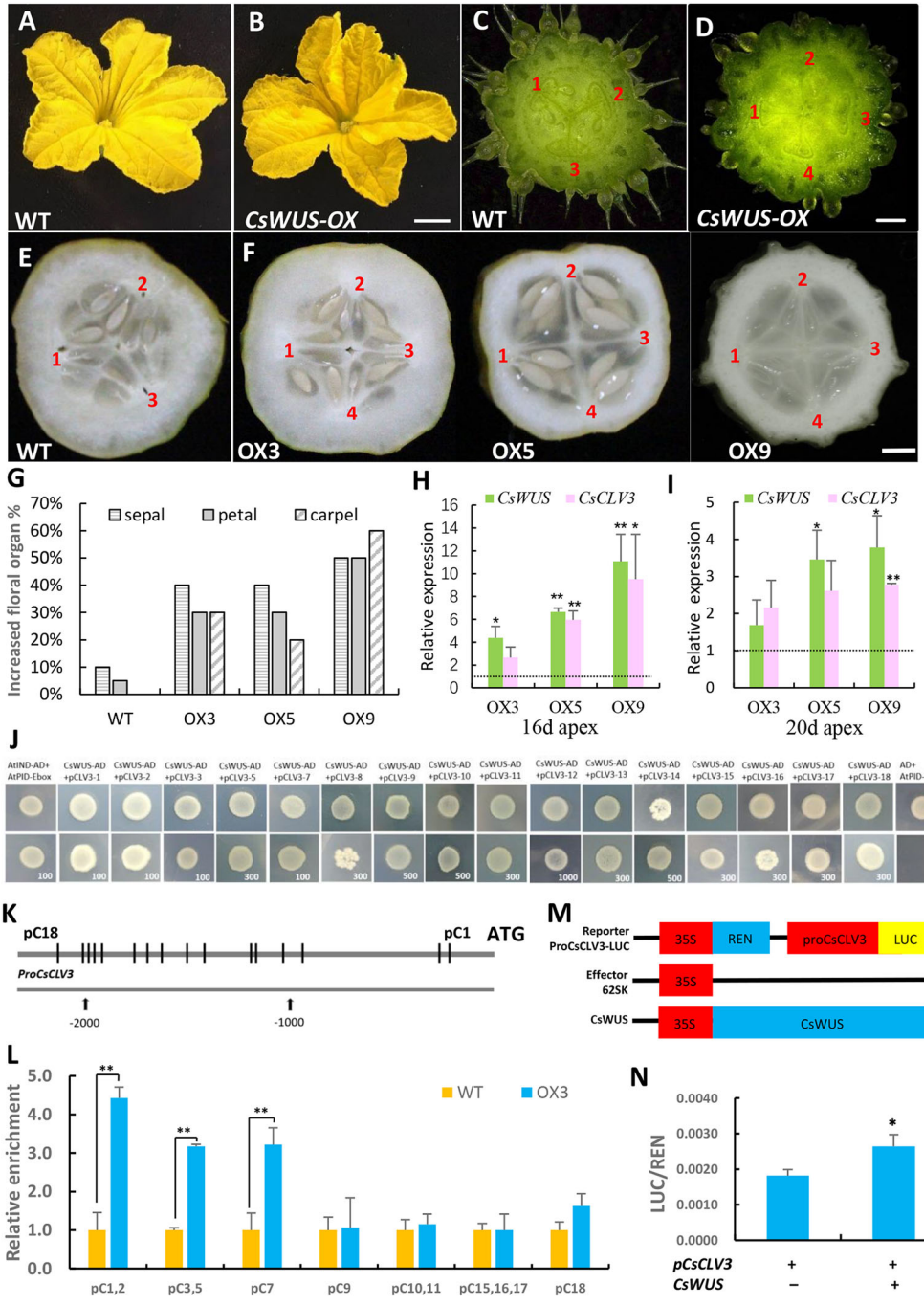

Fig. 3. *CsWUS* positively regulates floral organ number in cucumber. (A,B) Top view of flowers in empty vector control (WT) and *CsWUS*-OX transgenic plant. (C,D) Cross-sections of young fruits in wild-type and *CsWUS*-OX transgenic plants. (E,F) Cross-sections of mature fruits in wild type and three *CsWUS*-OX transgenic lines. (G) The frequency of increased floral organ number in wild type and *CsWUS*-OX transgenic lines. (H,I) Expression analyses by qRT-PCR of *CsCLV3* and *CsWUS* in the 16-day and 20-day apex of *CsWUS*-OX transgenic lines. The expression of *CsCLV3* and *CsWUS* in wild-type plants were set as 1 (dotted lines). (J) Y1H assay showed that CsWUS directly binds to the promoter fragments of *CsCLV3* containing the TAAT-box motif. The interaction between AtIND-AD and AtPID-Ebox was used as a positive control (Zhao et al., 2019). The empty AD and AtPID-Ebox was used as a negative control. The numbers in the bottom right corners indicate the basal concentration of AbA (ng/ml). (K) Schematic diagram of the promoter fragments of *CsCLV3* containing the TAAT-box motif used for Y1H assay and ChIP-qPCR. (L) ChIP-PCR showing the *in vivo* binding of CsWUS to *CsCLV3* promoter fragments. (M) Schematic diagram of the reporter and effector constructs used for the LUC/REN assay. (N) LUC activity measured after transient expression of *ProCsCLV3:LUC* and 35S:CsWUS in tobacco leaves. Scale bars: 1 cm. Red numbers indicate carpels. Data are mean±s.e.m. **P*<0.05; ***P*<0.01. Three biological replicates and three technique replicates were performed for each qRT-PCR analysis.

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expression by WUS in *Arabidopsis* (Ikeda et al., 2009; Müller et al., 2006; Yadav and Reddy, 2012).

Considering *CsCLV3* and *CsWUS* display overlapping expression patterns in the SAM and FM in cucumber, we performed protein-DNA interaction assays to examine whether interaction exists between them. In *Arabidopsis*, WUS binds to the *cis* element (TAAT box) to regulate *CLV3* expression (Perales et al., 2016). A total of 20 conserved TAAT boxes were found in the ∼2500 bp upstream of *CsCLV3* sequence. Y1H assay showed that CsWUS can bind to 16 fragments containing the TAAT-box motif in *CsCLV3* promoter (Fig. 3J,K). To verify the binding of CsWUS to *CsCLV3* promoter *in vivo*, chromatin immunoprecipitation (ChIP) combined with qPCR analysis (ChIP-qPCR) was performed using anti-myc antibodies in CsWUS-myc transgenic cucumber. The results showed that the three fragments (pC1,2, pC3,5 and pC7) had significant enrichment in the immunoprecipitated DNA of CsWUS when compared with wild type (Fig. 3L), indicating the direct binding of CsWUS to the *CsCLV3* promoter. A LUC transaction assay also showed that LUC activity was significantly enhanced upon co-transformation of 35S:CsWUS with *ProCsCLV3:LUC* in tobacco leaves (Fig. 3M,N), verifying the direct interaction between CsWUS and *CsCLV3*.

CsFUL1 participates in floral organ development through directly promoting *CsWUS* activity in cucumber

In our previous study, a FRUITFUL-like MADS-box transcription factor $CsFUL1^A$, but not $CsFUL1^C$, was found to be the functional allele for fruit length repression in cucumber (Zhao et al., 2019). Overexpression of $CsFUL1^A$ ($CsFUL1^A$-OX) led to flowers with increased floral organs, including more petals, stamens and carpels

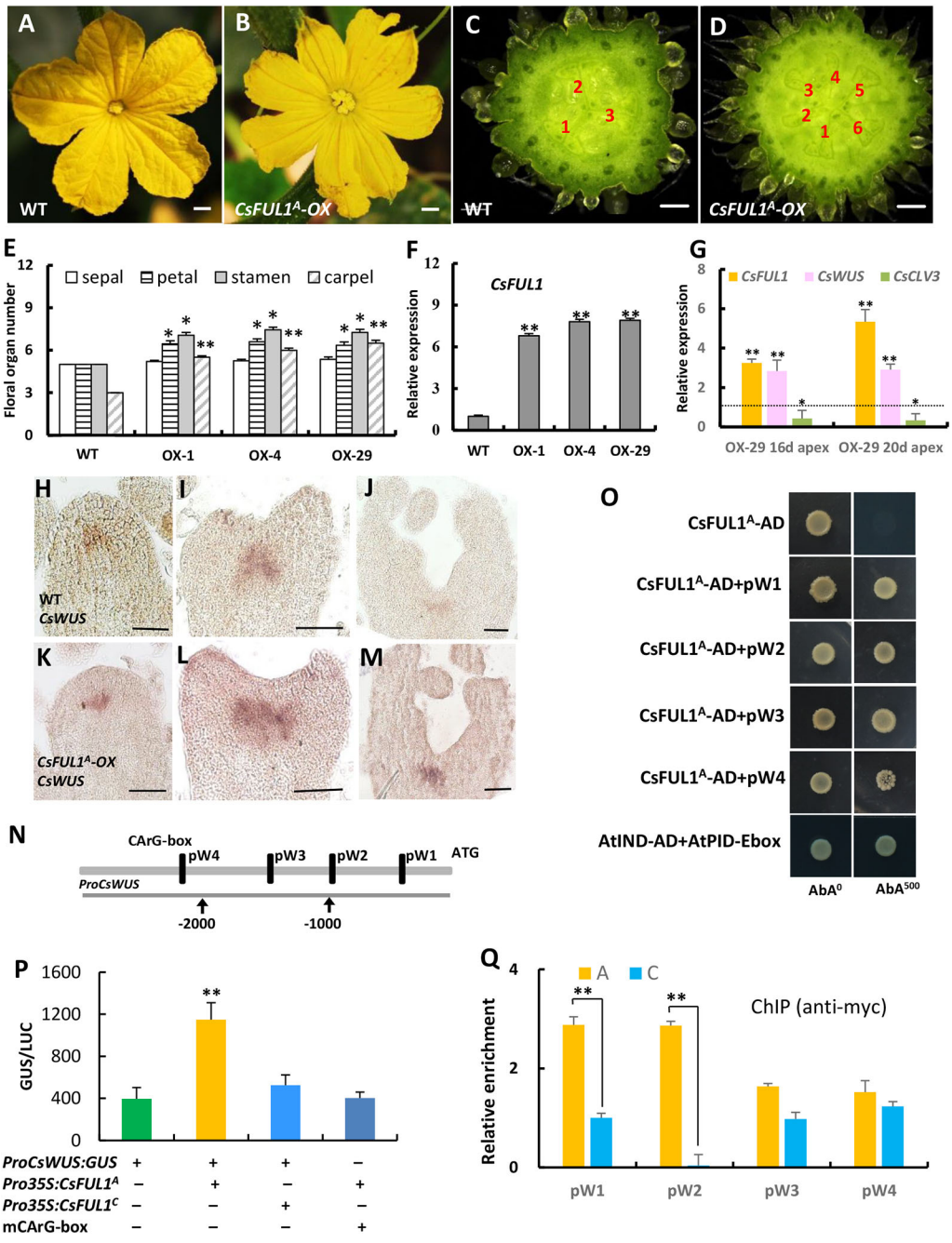

Fig. 4. $CsFUL1^A$ contributes to more floral organ numbers by directly stimulating *CsWUS* expression in cucumber. (A,B) Top view of male flowers in wild type (A) and the *35S:*$CsFUL1^A$ line (B). (C,D) Cross-section of ovaries at anthesis between wild type (C) and the *35S:*$CsFUL1^A$ line (D). (E) Statistical analysis of the numbers of sepals, petals and stamens in male flowers, and the number of carpels in female flowers in wild-type and *35S:*$CsFUL1^A$ lines. *P<0.05 and **P<0.01 compared with wild type. (F) qRT-PCR analysis of *CsFUL1* in young fruits of wild type and the *35S:*$CsFUL1^A$ transgenic lines OX-1, OX-4 and OX-29. (G) Expression analysis of *CsFUL1*, *CsWUS* and *CsCLV3* in 16-day and 20-day apices of wild-type and OX-29 transgenic plants by qRT-PCR. The values in wild-type plants were set as 1 (dotted line). (H-M) mRNA *in situ* hybridization of *CsWUS* in SAM and developing flowers of wild-type (H-J) and OX-29 transgenic plants (K-M). (N) Schematic diagram of the promoter fragments of *CsWUS* containing the CArG-box motif used for Y1H assay and ChIP-qPCR. (O) Y1H assay showing that $CsFUL1^A$ directly binds to the promoter fragments of *CsWUS* containing the CArG-box motif. Basal concentrations of AbA were 0 (left) and 500 (right) ng/ml. (P) GUS activity measurement in tobacco leaves after transient expression of *ProCsWUS:GUS* with Pro35S:$CsFUL1^A$ or Pro35S:$CsFUL1^C$, and *ProCsWUS*$^{mCArG\text{-}box}$*:GUS* with Pro35S:$CsFUL1^A$. Data are mean± s.e.m. (n=4; **P<0.01 compared with *ProCsWUS:GUS* control). (Q) ChIP-PCR showing the *in vivo* binding of $CsFUL1^A$ and $CsFUL1^C$ to *CsWUS* promoter fragments. Scale bars: 5 mm in A-D; 50 µm in H-M. **P<0.01 compared with $CsFUL1^C$-myc DNA enrichment.

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(Fig. 4A-D). The severe *CsFUL1*^{A} overexpression line (OX-29) can generate flowers with eight petals and six carpels. Quantification analysis indicated that the degree of floral organ increases positively correlates with *CsFUL1*^{A} expression levels (Fig. 4E,F). For example, the average carpel number was 3.0 in the wild-type plant, whereas it changed to an average of 6.5 in the severe line OX-29 (Fig. 4E,F). To explore whether *CsFUL1*^{A} regulates floral organ development through the classical WUS-CLV pathway, we examined the expression of *CsWUS* and *CsCLV3* by qRT-PCR in the apex of *CsFUL1*^{A}-OX plants (Fig. 4G). *CsWUS* transcription was significantly induced, whereas the expression of *CsCLV3* was decreased in the *CsFUL1*-OX plants (Fig. 4G). Next, *in situ* hybridization of *CsWUS* was performed in the shoot apex of wild-type and *CsFUL1*^{A}-OX plants (Fig. 4H-J). The signal of *CsWUS* was largely enhanced and expanded in the developing flowers of *CsFUL1*^{A}-OX (Fig. 4H-M).

To dissect the mechanism of *CsFUL1* regulating the WUS-CLV pathway, putative binding sites (CArG-boxes) of CsFUL1 were identified in promoters of *CsCLV3* and *CsWUS* (Smaczniak et al., 2012). Protein-DNA interactions between CsFUL1 and WUS-CLV pathway genes were examined with Y1H assays (Fig. 4N,O). Our data showed that CsFUL1^{A} can bind to four pW1-pW4 fragments containing the CArG-box motif in *CsWUS* promoter (Fig. 4O). Next, we performed a GUS transactivation assay to investigate the interaction of CsFUL1 and *CsWUS* in tobacco leaves. Our data indicated that the GUS activity was dramatically increased upon co-transformation of Pro35S:CsFUL1^{A} and *ProCsWUS:GUS*, whereas no significant changes were detected upon co-transformation of Pro35S:CsFUL1^{C} and *ProCsWUS:GUS* or upon co-transformation of Pro35S:CsFUL1^{A} with a CArG-box mutated *CsWUS* promoter (*ProCsWUS*$^{mCArG\text{-}box}$) (Fig. 4P), suggesting that CsFUL1^{A} may stimulate the expression of *CsWUS* in cucumber. To explore the *in vivo* binding of CsFUL1 to *CsWUS* promoter, ChIP-qPCR was performed using anti-myc antibodies in CsFUL1^{A}-myc and CsFUL1^{C}-myc transgenic plants. Our data indicated that the pW1 and pW2 fragments displayed significant enrichment in the immunoprecipitated DNA of CsFUL1^{A} when compared with CsFUL1^{C} (Fig. 4Q), suggesting the direct binding of CsFUL1^{A} to the *CsWUS* promoter to stimulate *CsWUS* expression in regulation of floral organ development in cucumber.

Auxin signaling is involved in cucumber carpel number regulation

Previous studies have identified that *CsCLV3* controls carpel number variation of cucumber using mapping populations derived from WI2757 (CN=3) and True Lemon (CN=5). There are 11 single nucleotide polymorphism (SNPs) between WI2757 and True Lemon, in which one SNP was confirmed to be crucial for carpel number variation (Li et al., 2016). We identified a spontaneous mutant Gui Fei Cui (GFC) from South China type cucumber 32X, in which the CN changed from 3 in 32X to 5 in GFC, despite the number of other floral organs, such as sepal, petal and stamen remain unchanged (Fig. 5A,B). Sequence cloning of *CsCLV3* in 32X and GFC indicated that they share the same 10 SNPs in WI2757 and True Lemon, including the most important SNP in the coding region associated with the carpel number variation (red box in Fig. S2). Additionally, there are two deletions (2 bp and 1 bp, respectively) in the promoter region and two deletions (3 bp and 4 bp, respectively) in the downstream genomic regions of CsCLV3, suggesting that CsCLV3 is responsible for the carpel number increase in the GFC mutant. As expected, *CsCLV3* expression was significantly reduced in GFC when compared with 32X, especially in the fruit at anthesis (Fig. 5C), which is consistent with the negative role of *CsCLV3* in regulation of carpel number variation in cucumber (Fig. 2). We examined *CsWUS* expression and found that it was upregulated in GFC when compared with 32X (Fig. 5D), supporting the notion of *CsCLV3* negative feedback on the transcription of *CsWUS* in cucumber.

To identify the downstream targets and gene pathways mediated by CsCLV3, we performed transcriptomic analysis by RNA-seq using fruit samples at anthesis from 32X and GFC (Fig. S3). When compared with GFC, 627 and 160 genes were up- and downregulated, respectively, in 32X (Table S1 and S2). Gene Ontology (GO) term enrichment analysis showed that sequence-specific DNA binding transcription factors were significantly enriched in upregulated genes in 32X (Fig. S3A), in which, genes in the AP2/ERF, C2H2, MYB, HB, bHLH, C2C2 family proteins occupied the majority (Fig. S3B,C). Previous studies have shown that AP2/ERF, MYB and bHLH family members play important roles in regulating plant growth and development through modulating multiple phytohormone signals, such as auxin, cytokinin and abscisic acid (Gu et al., 2017; Qi et al., 2015). We found many hormone-related genes were upregulated in 32X, such as auxin-responsive factors, cytokinin oxidase, response regulators, ethylene response factors and jasmonate-zim-domain proteins (Table S2).

To explore the role of hormones in CsCLV3-mediated carpel number variation, we measured the contents of auxin (IAA: 3-Indole acetic acid), cytokinin (ZR: trans-zeatin riboside), abscisic acid (ABA), gibberellins (GA3), jasmonic acid (JA) and brassinosteroid (BR) in fruits at anthesis in 32X (CN=3) and GFC (CN=5). Our data showed that the IAA and ABA levels were greatly reduced in the GFC when compared with 32X, and no significant differences were observed for ZR, GA3, JA and BR (Fig. 5E; Fig. S4). To visualize the distribution of IAA in cucumber, we performed IAA immunolocalization in transverse sections of 32X and GFC ovaries. IAA signals were found in the septum, vascular bundles and ovules of 32X. In GFC, the IAA signals were reduced (Fig. 5F), consistent with the decreased auxin accumulation (Fig. 5E). To investigate whether the increase of carpel number in GFC is related to the auxin signaling pathway, we examined the protein-protein interactions using Y2H and BiFC for CsARF1, CsARF3, CsARF4, CsARF5, CsARF12, CsARF13, CsARF14, CsARF17, CsPIN1 with CsCLV3 and CsWUS, respectively. Our data showed that only CsARF14 can bind to CsWUS in both assays (Fig. 5G,H). We examined *CsARF14* expression by qRT-PCR and found that transcript accumulation of *CsARF14* was elevated in GFC and *CsWUS*-OX plants (Fig. 5I,J), displaying a positive correlation with carpel numbers. Next, *in situ* analysis indicated that transcripts of *CsARF14* are enriched in developing ovaries (Fig. 1R), a pattern that overlaps *CsCLV3* and *CsWUS*, suggesting that auxin participates in carpel number variation through the CsARF14-mediated WUS-CLV pathway in cucumber.

DISCUSSION

CsCLV3 and *CsWUS* regulates carpel number variation in cucumber

The carpel number (CN) is an important fruit trait affecting fruit shape, fruit size and internal quality in horticultural crops. In cucumber, five-carpel fruits generally are rounder than three-carpel fruits (Weng et al., 2015). The roles of CLV3 in specifying the meristem size and flower organ numbers have been well documented (Soyars et al., 2016). In *Arabidopsis*, mutation of *CLV3* resulted in enlarged meristem size and increased number of all

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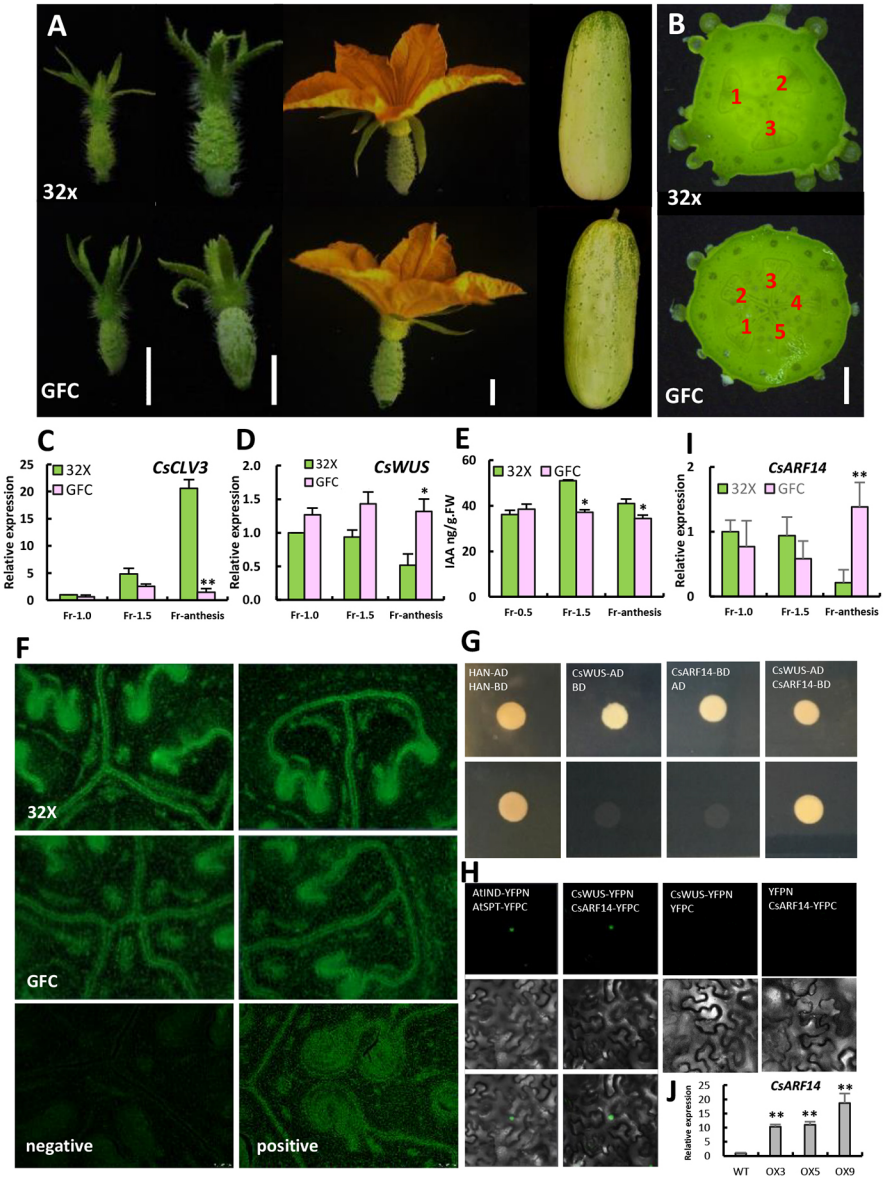

Fig. 5. The CsCLV3-CsWUS pathway regulates carpel number through the auxin response in cucumber. (A) Images of fruits at different developmental stages in inbred line 32X (Cn=3) and its mutant Gui Fei Cui (GFC) (CN=5). (B) Cross-section of fruits in 32X and GFC. (C,D) Expression analysis of *CsCLV3* and *CsWUS* in young fruits of 32X and GFC by qRT-PCR. Fr-1.0 and Fr-1.5 represent ovaries at 1.0 cm length and 1.5 cm length, respectively. Fr-anthesis indicates young fruit at anthesis. Data are mean±s.e.m. **P*<0.05 and ***P*<0.01 compared with 32X. (E) IAA (3-indoleacetic acid) content in young fruits at different developmental stages in 32X and GFC. **P*<0.05 and ***P*<0.01 compared to 32X. (F) IAA immunolocalization in cross-sections of cucumber ovaries at 1.5 cm length. The negative and positive control images of ovary in wild type were taken under the same exposure time and settings (Liu et al., 2018). (G,H) Protein interaction between CsWUS and CsARF14 was detected by Y2H (G) and BiFC assays (H). (I,J) Expression analysis of *CsARF14* in young fruits of GFC (I) and *CsWUS-OX* (J) plants by qRT-PCR.

four types of floral organs (Fletcher et al., 1999; Müller et al., 2006; Schoof et al., 2000). In rice, loss of function of *FON2* caused an increase in floral organ number (Suzaki et al., 2006). In maize, a *Mutator* insertion in *ZmFCP1* displayed fasciated ear phenotype (Je et al., 2016). In tomato, knockdown of *SlCLV3* showed branched and fasciated flowers and fruits with much more locules inside (Xu et al., 2015). *BrCLV3* was identified as conferring the multilocular trait in *Brassica rapa* (Fan et al., 2014). In *Lotus japonicus*, knockdown of *LjCLV3* resulted in meristem enlargement and increased ratio of flower number per peduncle (Okamoto et al., 2011). Here, we found that expression of *CsCLV3* is negatively correlated with carpel number variation in different cucumber cultivars, and downregulation of *CsCLV3* by RNAi led to increased numbers of petals and carpels (Fig. 2), suggesting that *CsCLV3* functions as a repressor for floral organ number in cucumber (Fig. 6). Furthermore, phenotypic variations exist among different alleles of *CsCLV3*. In the near isogenic lines GFC (CN=5) and 32X (CN=3), there is no difference in the number of floral organs except carpel number (Fig. 5; Fig. S2). Most semi-wild Xishuangbanna cucumbers bear fruits with CN=5 and male flowers with increased petal number (Li et al., 2016). Moreover, unlike most species, such as *Arabidopsis*, maize and tomato, no significant phenotypic changes were observed in the SAM of *CsCLV3* mutant alleles or RNAi lines, despite enlarged FM being observed in the True Lemon versus WI2757 (Li et al., 2016). This is probably due to the unique growth characteristics of cucumber: unisexual flowers are produced from the leaf axils and the SAM is responsible only for continuous leaf generation (Zhao et al., 2018). More severe alleles are needed to explore the function of *CsCLV3* in SAM maintenance in cucumber.

In *Arabidopsis*, WUS activates *AGAMOUS* (*AG*) to initiate reproductive development; in turn, AG represses *WUS* expression to terminate meristem activity. (Bollier et al., 2018; Lenhard et al., 2001). A *WUS* gain-of-function mutant displays ectopic floral buds phenotype in *Arabidopsis* (Xu et al., 2005), and increased locule number in tomato (Muños et al., 2011). Knockout of *ROSULATA* (*ROA*), a *WUS* ortholog in *Antirrhinum majus*, results in a meristem maintenance defect that is reminiscent of the *wus* phenotype in *Arabidopsis* (Kieffer et al., 2006). In rice, knockout of *OsWUS* produces a tiller-like structure, and an abnormal inflorescence and spikelet (Lu et al., 2015; Tanaka et al., 2015). *HEADLESS*, a

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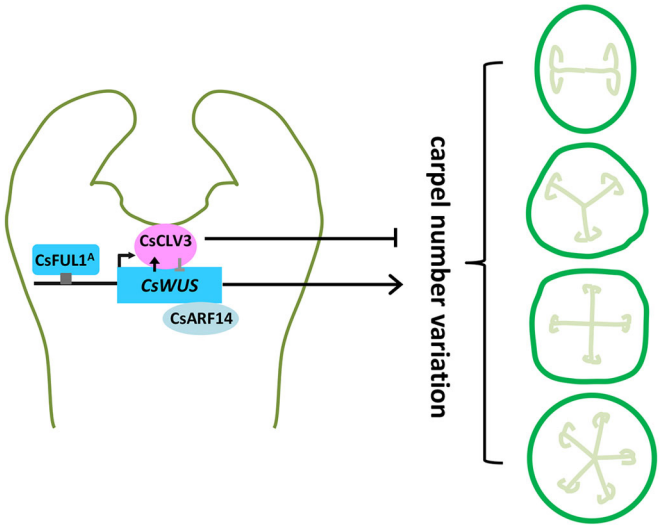

Fig. 6. A regulatory model involving CsCLV3, CsWUS, CsFUL1 and CsARF14 in specifying carpel number in cucumber. CsCLV3 acts as a repressor, while CsWUS functions as an activator for carpel number variation in cucumber. CsWUS directly binds to the promoter of *CsCLV3* and activates *CsCLV3* expression, while *CsCLV3* may suppress *CsWUS* activity indirectly. CsFUL1^{A} positively contributes to carpel number increase through direct binding to the promoter of *CsWUS* and promotes its expression. Auxin participates in carpel number regulation through physical interactions between CsARF14 and CsWUS.

WUSCHEL homolog in *Medicago truncatula*, is required for shoot meristem regulation and leaf blade development (Meng et al., 2019). Here, dramatic elevation of *CsWUS* expression is accompanied by increased number of flower organs (Fig. 3), suggesting that *CsWUS* acts as an activator in cucumber carpel number variation (Fig. 6). Taken together, the functions of WUS-CLV3 pathway in shoot apical meristem maintenance and floral organ development appear to be highly conserved, despite the variation in specific floral phenotypes in different plant species.

Expression patterns and regulatory network of *CsCLV3* and *CsWUS* in cucumber

In *Arabidopsis*, the WUS-CLV3 feedback loop has been established for controlling the stem cell niche in the SAM, in which WUS activates *CLV3*-expressing stem cells in the meristem apex, and in turn, the CLV3 signaling pathway involving CLV1, CLV2 and other receptors restrict WUS activity in the OC (Brand et al., 2000, 2002; Fletcher et al., 1999; Müller et al., 2006; Schoof et al., 2000). WUS was found to be synthesized in OC cells, and WUS protein migrates into *CLV3*-expressing stem cells and binds to the promoter region of *CLV3* to stimulate its expression (Rodriguez et al., 2016; Yadav et al., 2011). When CLV3 protein accumulates above a certain threshold, it is secreted to the outside of the cell in the form of a small peptide, which will transmit signals to the *WUS* expression region to inhibit the *WUS* expression, thus forming a negative-feedback regulatory loop to maintain the stem cell homeostasis in *Arabidopsis* (Brand et al., 2000; Schoof et al., 2000). However, in rice, the WUS ortholog *TILLERS ABSENT1* (*TAB1*) does not affect SAM maintenance and its expression is detected in premeristem zone and absent in the shoot apex (Tanaka et al., 2015). In maize, *ZmWUS1* is expressed in the center of SAM, whereas *ZmWUS2* is expressed in leaf primordia (Nardmann and Werr, 2006). Here, we have found that *CsWUS* expression is restricted in the central region underneath the central zone in the SAM and FM in cucumber (Fig. 1), which is similar to that in *Arabidopsis* and tomato (Chu et al., 2019).

In *Arabidopsis*, *CLV3* is specifically expressed in the stem cells and serves as a stem cell marker of the SAM (Müller et al., 2006). In tomato, *SlCLV3* is not expressed in the L1 layer and partially overlaps with the *SlWUS* expression domain (Chu et al., 2019). *ZmFCP1* transcripts are accumulated in the leaf primordia and flank of SAM in maize (Je et al., 2016). *LjCLV3* and *GmCLV3* are expressed in the inner layer of the SAM in lotus and soybean, respectively (Okamoto et al., 2011; Wong et al., 2013). Here, we found that *CsCLV3* was expressed in the basal domain of SAM and FM, a region that overlaps with *CsWUS* expression domain in cucumber (Fig. 1). Therefore, the specific expression domains of *WUS* and *CLV3* are quite divergent in different plant species.

Furthermore, our data show that downregulation of *CsCLV3* in the *CsCLV3*-RNAi lines or GFC mutant leads to increased *CsWUS* expression (Figs 2 and 5), whereas overexpression of *CsWUS* results in elevated *CsCLV3* accumulation in *CsWUS-OX* plants (Fig. 3), which is consistent with the classical feedback loop between WUS and CLV3 (Brand et al., 2002; Fletcher et al., 1999; Müller et al., 2006; Schoof et al., 2000; Somssich et al., 2016; Zhou et al., 2018). Biochemical analyses indicated that CsWUS can directly bind to *CsCLV3* promoter to stimulate its expression (Fig. 3). Considering the overlapping expression domains of *CsWUS* and *CsCLV3* (Fig. 1), CsWUS protein may skip migration to promote *CsCLV3* transcription in cucumber. In addition, overexpression of *CsFUL1^{A}* led to increased *CsWUS* expression while decreased *CsCLV3* transcription (Fig. 4G) and CsFUL1^{A} could directly promote *CsWUS* expression (Fig. 4N-Q), implying that there are additional players other than CsWUS that regulate *CsCLV3* transcription during cucumber floral organ development.

CsFUL1 and CsARF14 are new players in the CLV3-WUS pathway, regulating CN variation in cucumber

Several regulators have been reported to mediate floral organ numbers through the WUS-CLV pathway, such as HEC1, HAM and HAN in *Arabidopsis* (Schuster et al., 2014; Zhang et al., 2013; Zhao et al., 2004; Zhou et al., 2015, 2018), CT2 in maize (Bommert et al., 2013a), and RRA and FIN in tomato (Xu et al., 2015). The complex of INHIBITOR OF MERISTEM ACTIVITY (IMA) and KNUCLES (KNU) has been shown to bind to the WUS promoter region to repress its activity during flower development in both *Arabidopsis* and tomato (Bollier et al., 2018). In this study, we found that the MADS-box transcription factor *CsFUL1* positively contributes to petal and carpel development via direct binding to the CArG-box at the promoter of *CsWUS* (Fig. 4); thus, the activation of *CsWUS* by CsFUL1^{A} is strongly associated to carpel number variation in cucumber. *CsFUL1^{A}* was shown to be an important regulator for fruit length in cucumber (Zhao et al., 2019), which is consistent with the mapping data indicating the association of carpel number with fruit size, shape and weight (Li et al., 2016). Therefore, CsFUL1^{A} is a new player in the WUS-CLV pathway mediating carpel number variation and fruit elongation in cucumber (Fig. 6).

Auxin has been shown to be essential for multiple developmental processes, including organogenesis, apical dominance and flowering (Zhao, 2010). The auxin efflux carrier PIN-FORMED1 (PIN1) mediates the local auxin accumulation together with AUX/IAA and ARF family proteins during organogenesis from the SAM (Vernoux et al., 2011). *AUXIN RESPONSE FACTOR3* (*ARF3*) can restrict *WUS* expression by binding to its promoter in *Arabidopsis* (Liu et al., 2014). Here, we have found that auxin content is significantly reduced in the ovary of GFC (CN=5) (Fig. 5),

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suggesting a negative role for auxin accumulation in carpel number variation. We further showed that CsARF14 can physically interact with CsWUS protein (Fig. 5), and *CsARF14* displayed overlapping expression with *CsWUS* in the developing ovaries (Fig. 1). *CsARF14* transcription was significantly increased in *CsWUS-OX* plants (Fig. 5J), suggesting that CsWUS may interact with CsARF14 to stimulate *CsARF14* expression. Therefore, *CsARF14* appears to be another player in the WUS-CLV pathway mediating carpel number variation in cucumber (Fig. 6). Similar to the reduced auxin content, the ABA level was less accumulated in the GFC (Fig. S4), but the specific roles of ABA in cucumber carpel number variation need further investigation. Future studies using the CRISPR/CAS9 system to obtain knockout transgenic lines of *CsCLV3-CsWUS* pathway genes and regulators would be promising to shed light on the regulatory network of SAM maintenance, floral organ specification and fruit development in cucumber.

MATERIALS AND METHODS

Plant materials and growth conditions

Cucumber inbred line R1461 was used for expression analysis and genetic transformation. Inbred line GFC is a spontaneous mutant derived from line 32X. Cucumber cultivars R1461, HP, Q60 and JB were selected for different carpel numbers, and selfed for three generations prior to this study. The cucumber seedlings were germinated in an incubator at 28°C in the dark overnight and grown in a growth chamber at 16 h light at 25°C and 8 h dark at 18°C. Seedlings were then transferred to the greenhouse at China Agricultural University in Beijing under standard growth conditions at the two true-leaf stage.

Gene cloning and phylogenetic analysis

Total DNA was extracted from the female bud of R1461, 32X and GFC using a DNA extraction kit (Waryoung). The whole-length genomic sequence of CsCLV3 and CsWUS were obtained using gene-specific primers (Table S3). Total RNA of female buds was extracted using a Quick RNA Isolation Kit (Waryoung, Beijing, China) and cDNAs were synthesized using TianScript II RT Kit (Tiangen Biotech, Beijing, China). The coding sequence of *CsCLV3* and *CsWUS* was cloned using gene-specific primers (Table S3). The amino acid sequence of CsCLV3 and CsWUS homologs in other species was obtained from the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov). Protein alignment was performed using the ClustalW in the MEGA5 software package, and the conserved domains were compared using the BoxShade website (www.ch.embnet.org/software/BOX_form.html). The cucurbit CLE protein sequences were obtained from the Cucurbit Genomic Database (www.icugi.org/) and the GenBank database. The phylogenetic tree was analyzed by Neighbor Joining method with 1000 bootstraps in the MEGA5.2 software.

Quantitative real-time RT-PCR (qRT-PCR)

The cucumber fruits at different stages were used for RNA extraction and the synthesized cDNA was used as the template for real-time RT-PCR. The SYBR Premix Ex Taq Mix (Takara) was used for qRT-PCR analysis on an Applied Biosystems 7500 real-time PCR system. The cucumber *UBIQUITIN EXTENSION PROTEIN* (*UBI-EP*, Csa000874) was used as an internal reference gene. Three biological replicates and three technique replicates were performed for each qRT-PCR analysis. The gene-specific primers are listed in Table S3.

In situ hybridization

Cucumber shoot apex, female buds, male buds and young fruits were sampled from 14-day-, 16-day-, 18-day- and 20-day-old seedlings of inbred line R1461. All samples were fixed, sectioned and hybridized as previously described (Liu et al., 2018). Sense and antisense probes of *CsCLV3* and *CsWUS* were designed using the whole coding sequence (Table S3).

Cucumber transformation

To generate *CsCLV3*-RNAi construct, the full-length of *CsCLV3* coding sequence was cloned and inversely inserted into the pFGC1008 vector. The full-length CDS of *CsWUS* was inserted into the PBI121 vector to generate the overexpression construct. The *CsCLV3*-RNAi and *CsWUS*-OX construct was introduced into *Agrobacterium* by electroporation and then transformed into cucumber as previously described (Ding et al., 2015a). The gene-specific primers used for vector construction are listed in Table S3.

Yeast two hybrid assay

Full length coding sequence of CsWUS and CsARF14 genes were inserted into pGADT7 (bait vector) and pGBKT7 (prey vector) (Table S3). All constructs were confirmed by sequencing before transformation into yeast strain AH109. Yeast two hybrid assays were performed according to the description of Matchmaker TM GAL4 Two-Hybrid System 3 & Libraries (Clontech). AtHAN-AD and AtHAN-BD were used as positive controls (Zhang et al., 2013).

Bimolecular fluorescence complementation (BiFC) assay

The coding sequence of CsWUS and CsARF14 (without stop codon) were transfected into the YFP vectors (pSPYCE-35S and pSPYNE-35S). The resultant constructs were transformed into the *Agrobacterium tumefaciens* strain GV3101. Each combination of two genes was transfected to the mature leaves of 1-month-old *Nicotiana benthamiana* as previously described (Ding et al., 2015a). The infected tobacco leaves were cut down and observed using a Zeiss LSM 510 Meta confocal laser microscope under 488 nm excitation wavelength. A combination of AtIND-YFPC and AtSPT-YFPN was served as a positive control (Girin et al., 2011). The gene-specific primers used for BiFC are listed in Table S3.

IAA immunolocalization

Young fruits of cucumber inbred lines 32X and GFC were sampled in pre-cooled 3% N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) for 1 h dark at 4°C, and then put in paraformaldehyde for fixation overnight. The fixed samples were dehydrated, embedded, sectioned, dewaxed and incubated with the primary anti-auxin antibody (mouse monoclonal; Sigma-Aldrich, 1:1000, A0855) as described previously (Liu et al., 2018). The secondary antibody (DylightTM 488-labeled antibody to mouse IgG; Sigma-Aldrich,1:250, 072031806) was applied on slides and incubated for 4 h at room temperature in dark. After washing with 10 mM phosphate-buffered saline (PBS) twice for 5 min, specimens were mounted with 50% glycerin and imaged under a LSM 510 fluorescent microscope (Zeiss). The negative control images of ovary without anti-auxin antibody and a positive control of the ovule in wild type were taken under the same exposure time and setting (Liu et al., 2018).

Transcriptome analysis

Fruits at anthesis from inbred lines 32X and GFC were used for RNA-seq. Three biological replicates were prepared for each sample. The sequencing information was displayed in Table S1. The RNA-seq library was prepared following the manufacturer's instructions as previously described (Jiang et al., 2015). RNA-seq was performed on an Illumina HiSeq PE150 platform. Bioinformatic analysis was performed as previously described (Zhao et al., 2016). Gene Ontology (GO) term enrichment analysis and MapMan category enrichment were performed using the R package topGO and JAVA software MapMan, respectively (Alexa et al., 2006). The enriched GO terms or categories with a *P*-value less than 0.05 were identified as significant. Sequence data have been deposited in GEO under accession number GSE125899.

Yeast one hybrid assay

The *CsFUL1*^{A} and *CsWUS*-coding sequences were cloned into the pGADT7 vector (Clontech). The CArG-box fragments at the promoter of *CsWUS* and the TAAT-box fragments at the promoter of *CsCLV3* were cloned with the pAbAi vector (Clontech). The resultant constructs were transformed into the yeast Y1H Gold strain according to manufacturer's instructions and selected by optimal AbA (Aureobasidin A) concentration on the SD/-LEU (Synthetic Dropout Medium/-Leucine) medium

(Clontech). AtIND-AD and AtPID-Ebox were used as positive controls (Zhao et al., 2019). Primers for oligonucleotide synthesis were listed in Table S3.

LUC assay

The 2000 bp upstream sequence of *CsWUS* was cloned and linked with pCAMBIA 1381 and introduced into *Agrobacterium* GV3101 together with pSuper1300 promoter connecting $CsFUL1^A$ and $CsFUL1^C$. The ∼2000 bp upstream sequence of *CsCLV3* was linked with pGreen II 0800-LUC as a reporter and *CsWUS* CDS was linked with pGreen 62SK as effector. The bacterial fluid was injected into young leaves of *Nicotiana benthamiana*. The GUS/LUC ratio reflected the intensity of protein and DNA binding. GUS activity was measured using the methyl umbelliferyl glucuronide (Sigma-Aldrich). Luciferase (LUC) was measured using luciferin (Promega) and was used as an internal control. Primers for oligonucleotide synthesis are listed in Table S3.

ChIP-qPCR

ChIP-qPCR was performed as described previously (Ding et al., 2015b). The sonicated chromatin from vector control, *35S:CsWUS*, $35S{:}CsFUL1^A$ and $35S{:}CsFUL1^C$ transgenic cucumber lines was used as input and stored at −20°C. An anti-myc antibody was used in the immunoprecipitation reactions, and the complex of chromatin antibody was captured by protein A beads (Abcam). The final DNA was purified using a QIAquick PCR Purification Kit (QIAGEN, Germany) and used in qRT-PCR. Three technical repeats and three biological replicates were performed for each sequence segment. The primer pairs used in ChIP-qPCR are listed in Table S3.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Methodology: G.C., R.G.; Validation: G.C., R.G.; Formal analysis: J.Z.; Investigation: G.C., R.G., J.Z., X.L., H.Z., Z.C., J.S., Z.W.; Resources: X.S., R.L., L.Y., Y.W.; Writing - original draft: G.C.; Visualization: G.C.; Supervision: X.Z.

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Data availability

Sequence data have been deposited in GEO under accession number GSE125899. All sequences of the genes used in this study can be found in NCBI, the Cucurbit Database or GenBank under the accession numbers listed in Table S4.

Supplementary information

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.184788.supplemental

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