Genome-wide identification of CLE gene family and their potential roles in bolting and fruit bearing in cucumber (Cucumis sativus L.)

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Abstract

Background: Signal peptides are essential for plant growth and development. In plants, biological processes including cell-cell communication, cellular proliferation and differentiation, cellular determination of self-incompatibility, and defensive responses, all depend heavily on peptide-signaling networks such as CLE (CLAVATA3/Embryo surrounding region-related). The CLEs are indispensable in different periods of plant growth and development, especially in maintaining the balance between proliferation and differentiation of stem cells in various meristematic tissues.

The working system of CLE genes in cucumber, an important economical vegetable (Cucumis sativus L.), has not been fully studied yet. The distributional patterns of chromosome-level genome assembly in cucumber provide a fundamental basis for a genome-wide comparative analysis of CLE genes in such plants.

Results: A total of 26 individual CLE genes were identified in Chinese long ‘9930’ cucumber, the majority of which belong to unstable short alkaline and hydrophilic peptides. A comparative analysis showed a close relationship in the development of CLE genes among Arabidopsis thaliana, melon, and cucumber. Half of the exon-intron structures of all CsCLEs genes are single-exon genes, and motif 1, a typical CLE domain near the C-terminal functioning in signal pathways, is found in all cucumber CLE proteins but CsCLE9. The analysis of CREs (Cis-Regulatory Elements) in the upstream region of the 26 cucumber CLE genes indicates a possible relationship between CsCLE genes and certain functions of hormone response elements. Cucumber resulted closely related to Arabidopsis and melon, having seven and 15 orthologous CLE genes in Arabidopsis and melon, respectively. Additionally, the calculative analysis of a pair of orthologous genes in cucumber showed that as a part of the evolutionary process, CLE genes are undergoing a positive selection process which leads to functional differentiation. The specific expression of these genes was vigorous at the growth and development period and tissues. Cucumber gene CLV3 was overexpressed in Arabidopsis, more than half of the transformed plants in T1 generation showed the phenomena of obvious weakness of the development of growing point, no bolting, and a decreased ability of plant growth. Only two bolted strains showed that either the pod did not develop or the pod was short, and its development was significantly inferior to that in the wild type.

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Conclusions: In this study, 26 CLE genes were identified in Chinese long ‘9930’ cucumber genome. The CLE genes were mainly composed of alkaline hydrophilic unstable proteins. The genes of the CLE family were divided into seven classes, and shared close relationships with their homologs in Arabidopsis and melon. The specific expression of these genes was evaluated in different periods of growth and tissue development, and CLV3, which the representative gene of the family, was overexpressed in Arabidopsis, suggesting that it has a role in bolting and fruit bearing in cucumber.

Keywords: CLE peptides, Cucumber, Gene family, Phylogenetic analysis

Background
Signal peptides are short peptide chains that guide the transfer of newly synthesized proteins to the secretory pathway, and are generally 5–30 amino acids in length. Plant small signaling peptides play important roles in eukaryotic growth and development process, for instance, stress response [1–3], apical meristem maintenance [4–6], vascular bundle development [7], stomata development [8], reproductive growth [9], cell division [10], and mineral element absorption [11], as well as cell-cell communication, self-incompatibility determination and other molecular-level roles [12]. The CLAVATA3(CLV3)/EMBRYO SURROUNDING REGION (CLE) peptides, a better studied class of signal peptide molecules, consist of 12 or 13 amino acids, including hydroxylated proline residues that may or may not contain sugar modifications, and function in a non-cell-autonomous fashion. They function in the extracellular space as intercellular signaling molecules and bind to cellular surface receptor-like proteins to transmit a signal. The CLE polypeptide hormones play a key role in many important processes of higher plant growth and development, especially in maintaining the balance between proliferation and differentiation of stem cells in different meristematic tissues [13, 14]. The CLE peptide hormones, in regulating stem meristem of transcription factors in the cell nucleus WUSCHEL (WUSCHEL), promote the expression of stem cell marker genes CLV3, maintain stem cell properties, while the expression of CLV3 inhibiting WUS transcription level promote stem cell differentiation, and form a very precise feedback control loop that controls the number of stem cells in the stem meristem [13]. In the distal root meristem, a self-regulating interaction feedback loop is also formed between the ACR4-CLE40-mediated signal and the WUS family transcription factor WOX5 to regulate the proliferation and differentiation of the distal root meristem [13, 14]. Vascular cambium, as the initial center of plant vascular phylogeny, has the characteristics of pluripotent stem cells. Hirakawa, Fisher, Turner et al. [15–17] found that there is a regulatory mechanism whereby vascular cambium cell division is regulated by the PXY/TDR gene expressed in the Arabidopsis cambium cells and the CLE41/ TDIF gene expressed in the phloem element differentiation inhibitory factor. Hirakawa et al. [16] found that vascular bundles of Arabidopsis that overexpressed CLE41 and CLE44 were fractured, resulting in excessive division of procambium cells and significant enlargement of vascular regions. Gene CLE41/TDIF expressed specifically in the phloem of Arabidopsis can promote the proliferation of procambium cells and inhibit the differentiation of procambium cells into conduit molecules. Except for CLE41/44 and CLE42, all other CLE polypeptides, including CLV3, do not perform this function. The PXY/TDR mutation of the receptor of CLE41/44 would make the procambium cells disoriented during division, reducing the differentiated xylem and phloem cells, while retaining more undifferentiated procambium cells [15–17]. The expression level of WUS homologous gene WOX4 in the procambium was shown to be regulated by CLE41/44 [16].

Cucumber (Cucumis sativus L.; 2n = 2x = 14), is an economically important vegetable crop worldwide. It is consumed in the immature or mature, fresh or processed form. Cucumber genome was sequenced early in 2009 due to its small genome size (around 367 Mb) and agricultural importance, providing insight into traits such as its sex expression, disease resistance, biosynthesis of cucurbitacin, and ‘fresh green’ odor [18]. This draft genome was assembled using low-coverage Sanger sequences and high-coverage short Illumina sequences over a 70-fold genome coverage for the Chinese long inbred line ‘9930’, 722-fold genome coverage. For years, researchers have shown interest in whole-genome and whole-transcriptome sequencing that resulted in several versions of the cucumber genome and provided genetic information. Scaffold assemblies of three cucumber lines (Chinese long ‘9930’, Gy14 [19], and B10) are available so far. Mainstream genome assembly and gene annotation of cucumber cultivar Chinese long ‘9930’ have been updated to version 3. Version 3 of the cucumber genome was assembled using PacBio long reads, 10X Genomics, and high-throughput chromosome conformation capture (Hi-C) data. This accurate genome assembly, containing 174 contigs with a total length of 226.2 Mb and an N50 of 8.9 Mb, identified 1078 novel genes, including 239
tandemly-duplicated genes and 1374 full-length long terminal retrotransposons [20]. Additionally, many cucumber genome and transcriptome databases were constructed to provide user-friendly query interfaces for genomic research and breeding. In 2012, the first draft genome of melon became available. The double-haploid line DHL92 was sequenced and 375 Mb (83.3%) of melon genome has been assembled, including 27,427 protein-coding genes. Since the ancient eudicot triplication, the recent whole-genome duplications were absent in the melon lineage, which might have resulted in the increased size of melon genome compared with cucumber [21]. The genome-wide identification of CLE genes in melon and further comparison with cucumber homologs will help elucidate the evolutionary pattern within cucurbit species. The available cucumber genome assembly and abundant tools have accelerated genomic research, including gene family analysis through a bioinformatic approach. Genome-wide gene family analysis has been implied in numerous cucumber gene families, including MADS-box [22], SWEET [23], auxin response factor [24], lipoxygenase [25], calcium-dependent protein kinase [26], metacaspase [27], glutathione peroxidase [28], and aquaporin [29], but the specific biological and functional roles of the CLE genes in cucumber remain unknown.

The present study focuses on a genome-wide analysis of CLE genes in cucumber. We analyzed the exon-intron structure, gene phylogeny and synteny, and the tissue-specific expression of the identified CLE genes, as well as heterologous overexpression of cucumber CLV3 gene in Arabidopsis. The objective of this study was to provide insights into the genetic network of signaling peptides in cucumber and other cucurbit crops which should in the future facilitate improvement in yield, nutritional value, and commercial quality.

Results

Genome-wide identification of CLE genes in cucumber

The genome-wide search for CLE genes in cucumber was done using alignment. We first downloaded the known CLE proteins of Arabidopsis to self-construct a CLE Markov model. By scanning all 24,317 cucumber coding proteins in the genome assembly version 3 using the designed CLE Markov model, we identified 26 CLE transcription factors (S1 Table). Manual check revealed well conserved CLE transcription factors (referred by the term ‘CsCLE’ with a serial number and sorted by the E-value of the CLE domain; Table 1). Many cucumber CLE peptides are short peptides (90 amino-acid long) except CsCLE24 (1021 amino acids), CsCLE5 (416 amino acids), and CsCLE6 (404 amino acids). The molecular weight of the cucumber CLE proteins ranged from 5293.84 (CsCLE9) to 113,323.11 (CsCLE24), which is very similar to those in Arabidopsis. Most cucumber CLE proteins are alkaline with a theoretical pI ranging from 7.09 (CsCLE17) to 12.11 (CsCLE10), except for one weakly acidic protein, CsCLE24 (pI = 6.52). The CsCLE4 protein is stable whereas others are unstable. The aliphatic index of cucumber CLE proteins ranged from 49.03 (CsCLE6) to 106.78 (CsCLE17), and the GRAVY index ranged from −0.935 (CsCLE9) to 0.17 (CsCLE21).

The 26 CLE genes remained distributed across chromosomes, including 7 on chr5, 6 on chr6, 5 each on chr1 and chr2, and one each on chr3, chr4, and chr7 (Fig. 1). The CLE genes CsCLE24 and CsCLE25 were located extremely close with a short intergenic location of 1 kb. Besides, CsCLE13 and CsCLE14 were also located in close proximity; this finding indicated that these genes might be tandemly duplicated. In addition, one gene pair, CsCLE5 and CsCLE6, with segmental duplication was found within the CsCLE family, and the identified segmental duplication gene pairs were distributed on different chromosomes in cucumber (Fig. 2).

Phylogenetic relationships and divergence of cucumber CLE proteins

In the present study, we primarily use the genome assembly of Chinese long ‘9930’.

Only 31 CLE genes were identified (S2 Table) in this report, while a previous report showed that Arabidopsis contained 32 CLE genes [30]. A total of 25 CLE genes were identified in melon (S3 Table) using the computational approach. To understand the evolution of CLE genes in Cucurbitaceae and the model plant Arabidopsis, we compared the genes from Arabidopsis, melon, and the cucumber cultivar Chinese long ‘9930’ (Fig. 3). These CLEs were divided based on their phylogenetic relationship into seven paraphyletic groups (Groups 1 to 7). All groups except for Group 2 and Group 6 were composed of CLEs from both cucumber and other plant species, indicating the close relationship between cucumber CLEs and those of other plants. The distribution of cucumber CLEs was uneven in these groups. Group 7 was the largest group with 12 members, accounting for nearly half of the cucumber CLEs, followed by Groups 1 and 5 containing 6 and 5 cucumber CLEs, respectively. The least represented subfamilies were Groups 2 and 6 with no cucumber CLEs.

Gene structure of cucumber CLE genes

In order to compare the cucumber genes, their exon-intron structures were predicted (Fig. 4). The phylogenetic tree was constructed by the Maximum-Likelihood method using the conserved protein sequence of the cucumber CLE genes. Fourteen CLE genes were single-exon genes, ten were double-exon genes, and one was a
| Gene  | Number of amino acids | Molecular weight | Theoretical pI | Formula               | Instability index | Stability index | Aliphatic index | Grand average of hydrophathicity (GRAVY) | Gene ID | Protein ID  | Chromosome | Start | End |
|-------|-----------------------|------------------|----------------|-----------------------|------------------|-----------------|----------------|------------------------------------------|---------|-------------|------------|-------|-----|
| CGLE1 | 98                    | 10,680.15        | 9.62           | C468H748N136O14254    | 45.68            | unstable        | 74.69          | −0.347                                   | CsaV3_  | CsaV3_      | chr1       | 1     | 1   |
|       |                       |                  |                |                       |                  |                 |                |                                          | 1G002320 | 1G002320    |             | 490   | 477 |
| CGLE2 | 95                    | 10,833.66        | 9.23           | C491H771N137O13055    | 56.01            | unstable        | 95.47          | −0.064                                   | CsaV3_  | CsaV3_      | chr1       | 3     | 3   |
|       |                       |                  |                |                       |                  |                 |                |                                          | 1G005510 | 1G005510    |             | 590   | 463 |
| CGLE3 | 109                   | 12,477.54        | 9.89           | C558H873N161O14958    | 69.27            | unstable        | 66.15          | −0.504                                   | CsaV3_  | CsaV3_      | chr1       | 10    | 10  |
|       |                       |                  |                |                       |                  |                 |                |                                          | 1G015250 | 1G015250    |             | 838   | 630 |
| CGLE4 | 110                   | 12,200.05        | 8.68           | C527H850N150O162510   | 30.58            | stable          | 80.55          | −0.176                                   | CsaV3_  | CsaV3_      | chr1       | 19    | 19  |
|       |                       |                  |                |                       |                  |                 |                |                                          | 1G032100 | 1G032100    |             | 100   | 195 |
| CGLE5 | 416                   | 45,422.36        | 8.55           | C1974H3088N566O643S12 | 62.6             | unstable        | 55.79          | −0.701                                   | CsaV3_  | CsaV3_      | chr2       | 24    | 24  |
|       |                       |                  |                |                       |                  |                 |                |                                          | 2G006930 | 2G006930    |             | 073   | 345 |
| CGLE6 | 404                   | 43,809.33        | 9.25           | C1888H2967N555O626S11 | 61.62            | unstable        | 49.03          | −0.825                                   | CsaV3_  | CsaV3_      | chr2       | 3     | 3   |
|       |                       |                  |                |                       |                  |                 |                |                                          | 2G010680 | 2G010680    |             | 452   | 939 |
| CGLE7 | 87                    | 9948.6           | 11.09          | C426H723N141O12355    | 94.63            | unstable        | 99.66          | −0.363                                   | CsaV3_  | CsaV3_      | chr2       | 7     | 7   |
|       |                       |                  |                |                       |                  |                 |                |                                          | 2G011430 | 2G011430    |             | 846   | 926 |
| CGLE8 | 66                    | 7406.7           | 7.86           | C337H518N86O90S6      | 57.75            | unstable        | 76.82          | 0.1                                      | CsaV3_  | CsaV3_      | chr2       | 8     | 8   |
|       |                       |                  |                |                       |                  |                 |                |                                          | 2G0117950 | 2G0117950   |             | 729   | 911 |
| CGLE9 | 49                    | 5298.84          | 8.04           | C217H60N78O73S52      | 61.51            | unstable        | 59.8           | −0.935                                   | CsaV3_  | CsaV3_      | chr2       | 14    | 14  |
|       |                       |                  |                |                       |                  |                 |                |                                          | 2G026370 | 2G026370    |             | 938   | 987 |
| CGLE10| 94                    | 10,354.26        | 12.11          | C461H778N144O12252    | 58.84            | unstable        | 97.45          | −0.296                                   | CsaV3_  | CsaV3_      | chr2       | 18    | 18  |
|       |                       |                  |                |                       |                  |                 |                |                                          | 2G026370 | 2G026370    |             | 057   | 265 |
| CGLE11| 197                   | 22,244.05        | 10.54          | C963H1557N303O296S54  | 82.23            | unstable        | 70.86          | −0.795                                   | CsaV3_  | CsaV3_      | chr3       | 25    | 25  |
|       |                       |                  |                |                       |                  |                 |                |                                          | 3G029850 | 3G029850    |             | 594   | 596 |
| CGLE12| 87                    | 9737.34          | 9.39           | C433H706N120O12654    | 84.37            | unstable        | 96.32          | −0.216                                   | CsaV3_  | CsaV3_      | chr4       | 23    | 23  |
|       |                       |                  |                |                       |                  |                 |                |                                          | 4G033240 | 4G033240    |             | 527   | 528 |
| CGLE13| 89                    | 9650.95          | 8.4            | C432H676N118O129S52   | 60.32            | unstable        | 74.61          | −0.287                                   | CsaV3_  | CsaV3_      | chr5       | 1     | 1   |
|       |                       |                  |                |                       |                  |                 |                |                                          | 5G003050 | 5G003050    |             | 933   | 988 |
| CGLE14| 80                    | 8694.96          | 8.31           | C391H603N113O107S53   | 21.9             | unstable        | 95            | 0.099                                    | CsaV3_  | CsaV3_      | chr5       | 1     | 1   |
|       |                       |                  |                |                       |                  |                 |                |                                          | 5G003100 | 5G003100    |             | 951   | 951 |
| Gene      | Number of amino acids | Molecular weight | Theoretical pI | Formula                        | Instability index | Stability | Aliphatic index | Grand average of hydropathicity (GRAVY) | Gene ID         | Protein ID      | Chromosome | Start | End  |
|-----------|-----------------------|------------------|----------------|-------------------------------|-------------------|-----------|-----------------|----------------------------------------|----------------|----------------|------------|-------|------|
| CsCLE15   | 99                    | 10,877.5         | 11.29          | C496H763N141O132S52           | 58.3              | unstable  | 71.11           | -0.205                                 | CsV3_5G028390  | CsV3_5G028390 | chr5       | 231   | 452  |
| CsCLE16   | 108                   | 11,738.69        | 9.69           | C527H839N151O143S55           | 35.76             | unstable  | 85.65           | -0.193                                 | CsV3_5G032070  | CsV3_5G032070 | chr5       | 26    | 449  |
| CsCLE17   | 115                   | 12,571.58        | 7.09           | C562H903N153O163S55           | 63.43             | unstable  | 106.78         | 0.15                                    | CsV3_5G033190  | CsV3_5G033190 | chr5       | 26    | 449  |
| CsCLE18   | 107                   | 12,110.72        | 8.82           | C536H827N157O155S55           | 74.14             | unstable  | 62.99           | -0.67                                   | CsV3_5G034340  | CsV3_5G034340 | chr5       | 27    | 332  |
| CsCLE19   | 90                    | 10,637.26        | 10.27          | C487H739N139O125S55           | 48.41             | unstable  | 75.78           | -0.359                                  | CsV3_5G037710  | CsV3_5G037710 | chr5       | 29    | 866  |
| CsCLE20   | 148                   | 16,584.49        | 10.74          | C743H1235N209O210S54          | 50.33             | unstable  | 98.85           | -0.307                                  | CsV3_6G004590  | CsV3_6G004590 | chr6       | 3     | 923  |
| CsCLE21   | 88                    | 9875.56          | 10.04          | C453H704N130O113S55           | 37.63             | unstable  | 105.34         | 0.17                                    | CsV3_6G018750  | CsV3_6G018750 | chr6       | 13    | 318  |
| CsCLE22   | 104                   | 11,124.83        | 9.64           | C484H780N146O141S57           | 47.5              | unstable  | 72.12           | -0.3                                    | CsV3_6G036050  | CsV3_6G036050 | chr6       | 20    | 20   |
| CsCLE23   | 92                    | 10,326.16        | 9.81           | NOT SURE                     | 65.39             | unstable  | 73.26           | -0.266                                  | CsV3_6G037620  | CsV3_6G037620 | chr6       | 21    | 21   |
| CsCLE24   | 1021                  | 113,323.11       | 6.52           | C4870H7888N141O161D2540       | 47.03             | unstable  | 72.78           | -0.708                                  | CsV3_6G030690  | CsV3_6G030690 | chr6       | 29    | 541  |
| CsCLE25   | 80                    | 8987.28          | 8.89           | C411H623N113O115S52           | 48.13             | unstable  | 86.75           | -0.125                                  | CsV3_6G050700  | CsV3_6G050700 | chr6       | 29    | 552  |
| CsCLE26   | 106                   | 11,610.31        | 9.19           | C532H803N130O146S54           | 56.02             | unstable  | 76.42           | 0.122                                    | CsV3_7G025870  | CsV3_7G025870 | chr7       | 15    | 350  |

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**Fig. 1** Chromosome distribution of CLE genes in cucumber. Different line colours represent different chromosomes and marks on them are the corresponding genes and their locations.

**Fig. 2** Distribution of segmental duplication CLE genes CLE5 and CLE6 in the cucumber genome. Two genes of the same segmental duplicated gene pair are labelled in red.
triple-exon gene. The exon-intron patterns within the same phylogenetic classification group showed similarity. Cucumber gene CLE24 was unique with its discrete coding regions split by 19 introns.

Conserved cucumber CLE protein motifs

The MEME software was used to explore the conserved domains and motifs of the CLE proteins in cucumber. The motifs were listed from motif 1 to 10 according to the ascending E-value of the alignment (Fig. 4). Almost all cucumber CLE proteins contained motif 1, which represents a typical CLE domain near the C-terminal, except for CsCLE9. Proteins CsCLE5 and CsCLE6 were different at the C-terminal; both contained motif 4 near the C-terminal CLE domain. Protein CsCLE11 contained four tandem located motif 6 and one CLE domain, while CsCLE24 contained two motif 2 in tandem. These results indicated that the sequence characterization and biological function widely differed among cucumber CLE proteins in each branch of the phylogenetic tree. These proteins also possessed other functional domains at the N-terminal or in the middle, while their CLE domain was functional in signal pathways.

Upstream cis-regulatory elements (CREs)

In the PlantCARE database, the sequence of 1500 bp in the upstream promoter was used to locate the cis-regulatory element in the upstream regulatory region. By analyzing the biological functions of CREs in the upstream region of 26 cucumber CLE genes, it can be concluded that most of the components other than those necessary for the normal expression of promoters, such as AT~TATA-Box and CAAT-Box, can be divided into the following four categories. 1) The first type of element is hormone response elements, such as ABRE (ABA response element), the TCA-element (salicylic acid response element), the TATC-Box (gibberellin response element), and the TGA-element (auxin response element), among others. These elements were plotted in this experiment (Fig. 5). 2) The second type of element is light-responsive elements, such as ACE, G-box, ATA-motif, LAMP-element, etc. 3) The third type of element is the meristematic expression elements. For example, HD-zip1 (elements related to mesophyll cell differentiation) and TGACG-motif (elements related to meristem development) play an important role in CLE gene regulation of plant meristem maintenance and organogenesis. 4) The fourth type is stress response elements, such as ARE, MBS, TC-rich repeats, LTR, etc., as well as other regulatory factors that play a role in metabolic regulation.

Homologous pairs of cucumber CLE proteins

To understand the vertical descent from a single ancestral gene and duplication, a comparative analysis was performed to identify the orthologous, co-orthologous, and paralogous gene pairs in Arabidopsis, cucumber, and melon.

The OrthoMCL software identified 24 orthologous, 23 co-orthologous, and 8 paralogous CLE gene pairs among Arabidopsis, cucumber, and melon. This homologous gene pair network was highly consistent with the phylogenetic tree, which is clearly divided into three parts. The orthologous genes between these three species are shown in Fig. 6. Out of 26 cucumber CLE genes, 15 orthologous genes in melon and 7 in Arabidopsis were found.

Selection and divergence time

In order to investigate the selection mechanisms of CLE genes during evolution, we calculated Ka, Ks, and divergence time of the homologous pair CsCLE24-CsCLE25 (S4 Table). This pair had a Ka of 1.08 and Ks of 0.82, and Ka/Ks ratio of 1.31 with a P-value of 0.0098, indicating this gene pair is undergoing a positive selection which led to functional differentiation.

Expression pattern of cucumber CLE genes during development

We investigated the expression of each CLE gene using published RNA-seq data (S5 Table) for 23 different cucumber tissues during vegetative and reproductive development. The expression level of each gene was normalized using the RPKM method. Ten cucumber CLE genes including CsCLE1, 2, 4, 5, 14, 25, 24, 23, 15,
and 17 showed relatively low levels of expression in all 23 samples, showing that they played a negligible role in developmental processes in cucumber. Genes CsCLE9, 10, 18, and 22 showed relatively high levels of expression across multiple samples (Fig. 7a).

Using qPCR, we analyzed the expression of cucumber CLE genes (S6 Table) in different tissues of Chinese long '9930' seedlings. Out of 26 cucumber CLE genes, 24 genes were detected in 21 samples. Genes CLE1, 2, 7, 8, 9, 17, 18, 19, and 26 were expressed at relatively low levels across all samples, and others were expressed at relatively high levels in at least one sample. Cucumber CLE genes showed relatively high levels of expression in the growing tissues, including point, stem, and flower, and relatively low levels of expression in the root. The expression level of CsCLE4, 6, 11, 14, 21, 23, 24, and 25 was the highest (Fig. 7b). The qPCR results are highly consistent with the former RNA-seq data available in the public database.
Heterologous overexpression of CsCLV3 gene in Arabidopsis

The expression vector was successfully constructed by carrying the target gene on PHB plasmid, and then CsCLV3 gene, the representative gene of the CLE family, was overexpressed in Arabidopsis; we refer to these transformed plants as positive strain. The results showed that in the phenotype of positive strain weakness developed at the growing point. Compared with wild type, the growing point diameter of wild type Arabidopsis was about 0.66 mm (Fig. 8a), and that of CsCLV3 gene overexpression positive strain was about 0.54 mm (Fig. 8b).

Under the same conditions, the growing point of the positive strain was smaller than that of the wild type.

After transplanting, almost all of the transformed plants in the T1 generation showed the phenomena of obvious weakness of the development of growing point, no bolting, and a decreasing capability of plant growth (Fig. 9). Heritable seeds obtained from T1 plants were extremely few, making functional analysis in subsequent generations unfeasible. Besides, few of the positive strains completed the processes of growth and development. In this study, only two bolted strains showed that either the pod did not develop or the pod was short, and

![Fig. 5](image_url) Hormone response elements upstream of cucumber CLEs. The elements which respond to hormones are displayed in differently coloured boxes

![Fig. 6](image_url) Homologous gene pairs in Arabidopsis, cucumber, and melon. The gray lines represent all the homologous pairs between different genomes, and the red lines represent the homologous pairs of the CLE gene family
its development was significantly inferior to that in the wild type (Fig. 10a). Ten transformants were randomly selected for fluorescence quantitative PCR detection (Fig. 10b). The results (S7 Table) showed that the target genes were expressed in all transgenic materials, and the phenotype of No. 15 and No. 8 with lower expression was the weakest, which could bear fruit but not great fruiting ability. At the same time, No.1, 12, and 13, which had high expression levels, had capable phenotypes and high degree of abnormality, and were unable to bolt or fructify. Generally speaking, the expression level of CsCLV3 gene is consistent with its phenotype. The experimental results proved that cucumber CLV3 genes function in a way essential in controlling the development of growing points and fruits, and plant bolting.

Discussion

Our study investigated the CLE family genes in cucumber in detail. The spatiotemporal expression characteristics of all family genes in cucumber were also verified quantitatively. Gene CsCLV3, the representative gene of this family, was overexpressed in Arabidopsis. This work provides a new direction and idea for understanding the
possible role of cucumber CLE proteins in plant growth and development, especially in fruit setting.

**Discovery of the CLE gene family in cucumber**

A newly designed CLE Markov model was used in this study on the basis of which 26 new CLE genes were identified, proving that a CLE domain composed of 14 amino acids at the C-terminal is the conserved motif of the cucumber CLE gene family.

The replacement experiment of CLE domain in CLV3 revealed that the expression of fusion protein retained full physiological activity even after the upstream sequence of the CLE domain was replaced with an unrelated sequence [31]. The elimination experiment of variable domains in CLV3 demonstrated that the function of CLV3 is not significantly influenced by the absence of variable domains [32]. No MCLV3 activity was identified through the artificially synthesized CLE peptides lacking either Arg at the N-terminal or His at the C-terminal in the research of Kondo et al. [33]. In treating synthetic CLV3 peptides, Fiers et al. managed to totally inhibit the mutant phenotype of CLV3 [34]. Previous studies such as the abovementioned prove that the CLE domain is a key domain for the functioning of CLE peptides [35].

In this study, nearly every cucumber CLE protein containing motif 1 represented a typical CLE domain with CsCLE9 as an exception in which no typical CLE domain was detected. In maize, a CLE peptide expressed itself in primordial cells of organs but was manipulated and regulated by functional genes of FASCIATED EAR3 in stem cells, the regulatory function of which was achieved through leucine-rich-repeat receptors [36]. In the initial phase of flower development, stem cell niches are maintained through a negative feedback loop between the homeodomain transcription factor WUSCHEL (WUS) and the ligand-receptor system of CLAVATA3 (CLV3) [37]. The properties of stem cells and the size of FMs (floral meristem) are consequently affected when either of the genes above loses its activity [38]. The expression level published in both RNA-seq data and in the data of Chinese long ‘9930’ indicates a possible high expression of CsCLE9 in tissues with high meristematic activity, which leads us to predict that CsCLE9 may function in the CLAVATA–WUSCHEL feedback signaling between stem cells at the tip of the meristem and the underlying organizing center. This calls for further verification in later studies.

The 26 cucumber CLE family genes identified in this experiment exhibit a high degree of gene expression in

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**Fig. 9** Heterologous overexpression of CsCLV3 gene in Arabidopsis. Wild type (WT) strain has normal growth and development cycle, and phenotypic structure of rosette leaves, stems, leaves, pods and so on. The positive strain (4910–13) was the phenomenon of heterologous overexpression of CsCLV3 gene, and it has no stem, leaf, fruit pod or other morphological structures, showing only wrinkled rosette leaves.
meristem tissues, vascular bundles, and sites of vigorous growth as is shown in the analysis of public transcriptome data and real-time fluorescence quantitative analysis. However, only one of the 26 cucumber CLE family genes, i.e. CsCLE6, manifests a high degree of expression in plant roots, a finding inconsistent with those of previous studies. The tissue distribution of CLE genes influences their functional specificity to a large extent, as revealed in many tests of CLE protein ectopic expression at stem and root ends, but the location of gene expression is not the sole determinant of the functions of CLE genes [39], as shown in the cases of CsCLE6 and CsCLE9 in the current study. The CLE polypeptide family is a group of secreted peptide hormones that function among cells and play an essential role in the formation of stems, roots, vascular tissues, and pod nodules of plants [40, 41]. Numerous studies have demonstrated that CLE polypeptides are a significant kind of regulatory factors for keeping the balance between proliferation and differentiation of stem cells [34, 42].

**Phylogenetic analysis and evolution of cucumber CLE genes**

According to the types of phylogenetic relationships, CLEs from three different plant species were divided in this research into seven groups within each group containing at least one Arabidopsis sequence. This provides necessary evidence for the identification of the conservation of CLEs in different plants. However, CLE genes do not distribute uniformly in the three different species. In cucumber, there are 12 CLE genes and the highest number of Arabidopsis CLE genes is found in Group 7, in which the largest number of CsCLE genes was identified. However, in neither Group 2 nor Group 6 were cucumber- or melon CLE genes found. Such difference may imply certain physiological significance. Rapid adaptive evolution is more likely to happen in groups with more genetic members than in groups with fewer genetic members, as hypothesized in a previous study [43]. In terms of the physiological functions of CLE peptides [44], the Arabidopsis CLE genes in Group 7 are classified as class A genes which function through the inhibition of the proliferation and division of apical meristem stem cells and root meristem stem cells. Cucumber and melon CLE genes that are classified into the same group can both be analyzed with reference to similar functions, the finding of which lays a foundation for future researches on the gene functions of CLE in Cucurbitaceae.

As shown in Fig. 11, we compared the evolutionary relationship and the species relationship and obtained the relationship among orthologous genes. It can be seen that the positions of orthologous genes in the phylogenetic tree are quite close, which indicates that the evolutionary relationship of CLE family genes among the
three species is relatively close. Based on this, we can infer the functions of the related genes of cucumber and melon that are orthologous to those in *Arabidopsis*, as the latter has been studied in a relatively thorough manner. If *CsCLE19* is orthologous to *AtCLE26*, it can be inferred that its function is similar to *AtCLE26*; *AtCLE26* can inhibit the division of stem cells in the apical meristem and root meristem but cannot inhibit the division and differentiation of the procambium nor can it inhibit the differentiation of primary xylem vessels. This predicted function is different from the predictions of several other orthologous gene pairs, namely, *CsCLE3/CmCLE23/AtCLE17, CsCLE15/CmCLE15/AtCLE13/AtCLE16* and *CsCLE17/CmCLE20/AtCLE10*, which can inhibit the stem cells of apical meristem and root meristem. This difference can also be verified by measuring the gene expression. Gene *CsCLE19* exhibited a significantly different temporal and spatial expression pattern from *CsCLE15* and *CsCLE17*. In terms of the expression in the non-stem apex and root tip meristems, *CsCLE19* also had a higher expression.

Considering that a homologous gene of *CsCLE26* was found in *Arabidopsis* but not in melon, it is assumed that *CsCLE26* was retained in ancient cucurbits but was eliminated in melon in the process of evolution.

**Putative functions of CsCLE genes in hormone response and plant growth**

The identification of cis-regulatory elements such as ABRE, AuxRR-core, and TCA-elements in response to hormones in 26 *CsCLE* genes indicated a dynamic mutual interaction between *CsCLE* polypeptides and hormones of other kinds. The frequent occurrence of cis-regulatory elements in either ABRE or ABA-dependent response uncovers the function of *CLE* genes in ABA dependent reactions under stringent conditions [45]. The gene name of *CLE* is a compound derived from the name of *CLV3* genes of *Arabidopsis* and that of Embryo Surrounding Region (ESR) genes of maize [46]. All the
CLEs identified up to date share quite similar protein structures [47], usually with a short secretory signal peptide at the N-terminal, a long variable domain in between, and a conserved CLE domain at the C-terminal of the protein. Such conserved CLE domain is generally composed of 12–13 amino acid residues and is crucial for the functional execution of CLE proteins [48, 49]. One of the main features of CLE genes is their highly conserved structural domain at the C-terminals. Moreover, it is impossible to identify those CLE members that are expressed at relatively low levels using high-throughput sequencing. At the early stage of gene studies, the genome-wide identification of CLE genes in plants was conducted using BLASTP. Hidden Markov models and position-specific iterative BLAST method were employed by Oelkers et al. and more than 100 new CLE members were identified in 19 different species [46].

Essential elements such as the CAT-box and others that are crucial for CLE gene regulation in the process of maintenance of plant meristem and organogenesis, were definitely detected. Besides the analysis of the effect of overexpression of *Raphanus sativus* CLEs encoded with CLE peptides on secondary root structure in *Raphanus sativus*, Gancheva et al. [50] investigated the effect of synthetic CLE peptides treatment on *Raphanus sativus* and *R. raphanistrum* plants. For plants with a changing quantity of CLE peptides in roots resulting from either CLE overexpression or exogenous CLE treatment, the development of tissues in store roots alters accordingly. Additionally, a group of essential elements exist in numerous light regulation genes and groups of essential elements relate to environmental stimuli include responses to biotic and abiotic stresses. The existence of all four kinds of essential elements discussed above unveils the possible functions of CLE genes in corresponding responsive mechanisms.

**Cucumber CLV3 genes act as critical regulatory in cucumber bolting and fruit bearing**

Overexpression of CLE genes often shows a common dwarf and short-root phenotype [51], but in this study the overexpression of CsCLV3 gene showed internode shortening but no obvious dwarfism in total stem length. Brand et al. [34] used transgenic plants overexpressing CLV3 to show that the shoot meristem arrests after initiation of the first leaves (arrow), and the phenotypic results were similar to those in this study. Stamens and carpels are missing in the flower of the transgenic, at the same time, they also inspected the phenotype of clv3 mutants, and the mutant flower showed organ number increased particularly carpel. CLV3 is a specific regulator of shoot and floral meristem development [52]. In *Brassica rapa*, it was proven that the multilocular mutant contained more stamens and carpels in the functional characterization of the multilocular silique gene *BrCLV3*, and most of its siliques had 4 locules with a shorter, rounder and thicker shape and extra gynoecium inside [53]. Knockout of rapeseed homologues of *CLV3* using the CRISPR/Cas9 system, and the double mutation of *BnCLV3* produced more leaves and multilocular siliques with a significantly higher number of seeds per silique and a higher seed weight than the wild-type and single mutant plants [54]. The gene of *CsCLV3*, a homolog of the *Arabidopsis* gene *CLV3*, fine genetic mapping in F2 and RIL populations and association analysis in natural populations confirmed *CsCLV3* as the candidate gene for Cn (carpel gene) [55]. However, as for the *paeonia rockii* carpel, recent genetic studies [56] have shown that the phenotypic carpel is the complex of gene regulation networks. There are multiple candidate genes, and we are also actively verifying CLV3 as a candidate gene for cucumber carpel numbers. It is believed that the specific relationship between the *CsCLV3* gene and cucumber carpel number phenotype will be clearly depicted in the near future.

The phenotype of weak growth point and loss of bolting and fruit bearing ability was obvious in the T1 generation. Our experiment of heterologous overexpression of representative CLV3 gene in *Arabidopsis* revealed that more than half the number of transformed light-demanding plants in the T1 generation manifested responses such as stunted growth points, lack of bolting, plant shrinkage, etc., and some bolted lines grew to be short and stunted strains that were even significantly less developed compared to the wild-type strains. In contrast, the double mutation of *BnCLV3* produced more leaves and multilocular siliques with a significantly higher number of seeds per contributing to increased seed production. Results above demonstrate that *CsCLV3* gene function as a regulatory factor, most likely controlling plant development at different growing points and the processes of bolting and fruit bearing.

**Conclusions**

Twenty-six CLE family genes were identified in the genome of Chinese long ‘9930’ cucumber. Almost all of the family members contained motif 1, a typical CLE domain, at the C-terminal. Analysis of CREs in the upstream promoter region of the genes predicted that this gene family might be related to hormone response in cucumber growth and development. The results of interspecies evolutionary analysis showed that the genes of the CLE family in cucumber were closely related to the model plants *Arabidopsis* and congener melon, with cucumber sharing seven orthologous CLE genes with *Arabidopsis* and 15 with melon. The analysis of a pair of orthologous genes in cucumber showed that as a part of the evolutionary process, CLE genes are undergoing a
positive selection process which leads to functional differentiation. The family representative gene CsCLV3 acts as a significant factor in the process of the development of growth points and functions in the growth of plant bolting and pod of those plants of positive transformation in which the expression was relatively low.

**Methods**

**Identification of cucumber CLE genes**

The genome, gene sequences, and corresponding protein sequences of cucumber were downloaded from cucumber genome database (CuGenDB, http://cucurbitgenomics.org/). The Markov models of two identical domains for the CLE genes were constructed using known Arabidopsis CLE proteins. All cucumber proteins were aligned with these Markov models using the HMMER program (http://www.hmmer.org/) with a domain cut-off E-value of $e^{-3}$. The CLE genes located at a distance of 10 kb apart on the same chromosome or scaffold were considered as tandemly duplicated genes.

The genome and annotated proteins of the model plant Arabidopsis were downloaded from TAIR database (https://www.arabidopsis.org/) [57]. The genome and annotated proteins of Cucumis melo were downloaded from CuGenDB database. Candidate proteins with only the CLE domains were eliminated manually. The ProtParam (http://web.expasy.org/protparam/) [58] was employed to analyze the physical and chemical characteristics, including the molecular weight, theoretical isoelectric point (pI), atomic composition formula, instability index, aliphatic index, and grand average of hydropathicity (GRAVY), of the CLE proteins in these analyzed species.

**Phylogenetic analysis**

The putative CLEs were determined by searching the melon genome database with HMM (Hidden Markov Model) profiles constructed using CLE sequences from Arabidopsis as queries. All the putative CLEs were further subjected to Pfam and SMART analyses to identify their conserved domains and signature sequences. Protein sequences from multiple species were used for a phylogenetic analysis in planta. Pairwise alignment and multiple alignment were performed using the ClustalX2 program with Gonnet protein weight matrix [59], and a maximum likelihood phylogenetic tree was built with the MEGA program (v6.06) using the conserved protein sequence of the cucumber CLE genes. Uniform rates and homogeneous lineages were adopted, and the partial deletion with a site coverage cutoff of 70% was used for gaps/missing data treatment. The frequency of each divergent branch higher than 50% was displayed. The figure was processed using the Adobe Illustrator software.

**Gene structure and motif analysis**

The gene structure was analyzed using Gene Structure Display Server tool (http://gsds.cbi.pku.edu.cn/, v2.0) [60]. The MEME software (http://meme.nbcr.net/meme/, v4.12.0) was used to search for motifs (10 to 100 bp long) in the proteins [61]. Only widely distributed motifs that occurred in at least three protein sequences were retained. These motifs are represented in two separate figures, in accordance with the phylogenetic trees. The top ten motifs (with the lowest E-values) were reported, and statistically significant (lower E-value) motifs came first in the display.

**Upstream Cis-regulatory elements (CREs)**

A 1500 bp fragment located before the initiation codon ATG of CLE gene family was captured use perl script from the cucumber genome sequence, and the online website PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to analyze CREs of them. The figure was drawn using the online software GSDS (http://gsds.cbi.pku.edu.cn/).

**Identification of orthologous and paralogous genes**

OrthoMCL software (v2.0.3) [62] was employed to search for orthologous, co-orthologous, and paralogous genes in Arabidopsis, cucumber, and melon using entire CLE protein sequences. An E-value cut-off of an all-against-all BLASTP alignment process was set at $1e^{-5}$, and the alignment with a match cut-off value lower than 50 was eliminated. Nonsynonymous (amino acid-replacing, Ka) and synonymous (Ks) substitution rates among protein-coding sequences were used to reveal the DNA sequence evolution. The Ka/Ks ratio (Ka/Ks) was used to estimate the selective strength for DNA sequence evolution; Ka/Ks > 1 indicates positive selection, Ka/Ks < 1 indicates purifying (negative) selection, and Ka/Ks close to 1 indicates neutral mutation [63]. The ParaAT software (http://cbb.big.ac.cn/software) is capable of constructing multiple protein-coding DNA alignments in parallel for a large number of homologous genes [64]. To evaluate the divergence of duplicated cucumber CLE genes, synonymous rate (Ks), nonsynonymous rate (Ka), and evolutionary constraint (Ka/Ks) between paralogous pairs of genes were calculated with the KaKs_calculator tool and paraAT software using the method developed by Nei and Gojobori (http://cbb.big.ac.cn/software). The divergence time was calculated for the homologous pair using the formula $T = Ks/2R$, where $R$ is the rate of divergence for nuclear genes from plants, and is $R = 1.5 \times 10^{-8}$ synonymous substitutions per site per year for dicotyledonous plants [65]. The selected homologous pairs of Arabidopsis, cucumber, and melon were gathered and displayed using Cytoscape software (http://www.cytoscape.org, v2.8.3) [66].
Tissue-specific gene expression analysis of CsCLEs

# Tissue-specific gene expression analysis of CsCLEs

The gene expression data of cucumber CLE genes were gathered from CuGenDB (http://cucurbitgenomics.org/rnaseq/cu/17) (NCBI accession: PRJNA312872) [67]. The RNA-seq data from Cotyledon_4Week_Seedlings, FemaleFlower, Flesh_2Week_Fruit, Flesh_3Week_Fruit, Flesh_UnfertilizedOvary, Flesh_Week_Fruit, Hypocotyl_4Week_Seedlings, MaleFlower_Bud, MaleFlower, OldLeaf, Peel_2Week_Fruit, Peel_3Week_Fruit, Peel_UnfertilizedOvary, PeelWeek_Fruit, Petiole_OldLeaf, Petiole_YoungLeaf, Root_4Week_Seedlings, Root Stem, Tendril, TrueLeaf_4Week_Seedlings, UnfertilizedOvary and YoungLeaf of cucumber cultivar Chinese long 9930 were used. The TopHat program (https://ccb.jhu.edu/software/tophat/index.shtml, v2.1.0) was used to map the reads to cucumber genome; expression profile of all the cucumber genes was then obtained with the FPKM (Fragments Per Kilobase of exon per million fragments Mapped) value using Cufflinks software (http://cole-trapnell-lab.github.io/cufflinks, v2.2.1) under the guidance of annotated gene models with a GFF file. The cucumber CLE gene expression profile from each sample was analyzed using the Heml program. The gene expression profile in each sample was standardized using RPKM (reads per kilobase per million reads) method. The expression profile of cucumber CLE genes from each sample was clustered and the heatmap was drawn using the Heml program (http://hemi.biocuckoo.org/).

After normalization using the default linear method, expression data were clustered using hierarchical average linkage algorithm and Euclidean distance similarity metric algorithm in both the horizontal axis and the vertical axis.

Tissue-specific gene expression in the cucumber line Chinese long ‘9930’

The cucumber line Chinese long ‘9930’ was used as the plant material to explore CsCLE expression differentia-

tion in different tissues. Seeds were soaked for 15 min in water at 55°C and placed overnight at 28°C for germination. The seedlings were allowed to grow in a controlled-environment growth chamber programmed for a photoperiod of 16/8 h (light/dark) and an air temperature of 28/18°C (day/night). At the four-leaf stage, roots, stems, mature leaves, young leaves, growth points, and cotyledons were harvested and snap-frozen in liquid nitrogen. At the flowering stage, flowers were harvested and snap-frozen before storage at −80°C for further analysis.

An RNA kit (Takara, Kyoto, Japan) was used to extract total RNA from the isolated plant tissues according to the manufacturer’s instructions. The Prime Script RT reagent kit (Takara, Kyoto, Japan) was used to reverse transcribe RNA into cDNA. The Primer 5.0 software was used to design specific primers according to the CLE gene sequences. Cucumber actin gene (CuGenDB name: Csa6G484600) was used as an internal control to normalize the expression level of the target genes among different samples. Three biological and three technical replicates were used. Each reaction was performed in a 20 μL reaction mixture containing diluted cDNA sample as the template, SYBR Pre-mix Ex Taq (2x) (Takara), and gene-specific primers. Quantitative real-time PCR (qPCR) was performed using a CFX96 Single color Real-Time PCR Detection System (BioRad, Hercules, CA, USA) with the following cycling profile: 95°C for 20 s followed by 40 cycles at 95°C for 3 s and 60°C for 30 s; and a melting curve (61 cycles at 65°C for 10 s) was generated to check the specificity of the reaction. The comparative Ct value method was employed to analyze the relative gene expression level. The RNA level was expressed relative to the actin gene expression level following 2^−ΔΔCt method. The CLE gene expression cluster was analyzed via the TBTOOLS software [68].

Heterologous overexpression of CsCLV3 gene in Arabidopsis

The gene name of CLE is composed of the CLV3 gene of Arabidopsis and the Emeryo Surrounding Region (ESR) gene of maize. In this experiment, the representative gene CsCLV3 of the CLE family was heterologously overexpressed in Arabidopsis to explore the possible gene function of CsCLV3. Variety ‘GY14’ was employed as test material to clone CsCLV3 gene, and its cDNA was obtained by the same method as for that of Chinese long ‘9930’ as mentioned above. The reference sequence was obtained from the cucurbit genomics database (CuGenDB, http://cucurbitgenomics.org/feature/gene/CsGy1G014910). The primers were designed by SnapGene, and the target gene was amplified. The gene cloning product and expression vector PHB were transformed into E. coli DH5α competent cells respectively, digested by double digestion method (the digestion sites were Hind III and Xba I), ligated by T4DNA ligase, cultured overnight at 37°C, and the positive clones were screened by bacterial PCR. The expression vector with the target gene correctly loaded as a result of sequencing was obtained.

Transgenic plants of Arabidopsis were obtained by floral dip. The connected PHB expression vector was transferred into the competent cells of Agrobacterium tumefaciens GV3101 by freeze-thaw method. The bacterial suspension was prepared by the identified A. tumefaciens monoclonal using transformation buffer, and was used to infect plants of Arabidopsis when the OD value was about 1.0. Water was poured on the flowering plants 1 day in advance, and all inflorescences were inverted into the bacterial liquid suspended in advance by the
transformation buffer for about 30 s; the transformation as repeated once after 7 days. After growing for 2–3 weeks, less nutrient solution was poured to aged, and mature seeds were harvested and dried for later determinations. Half-strength MS (Murashige and Skoog medium) (pH 5.8) solid medium with 30 μg mL⁻¹ hygromycin was used to screen positive seedlings of transformed plants. After disinfection, the seeds were sown on the screening medium, placed at 4°C for 48 h, and then transferred to an incubator. The conditions for germination and growth were 25°C, 16 h/8 h (light/dark), 30,000 lx light intensity, and 60% relative humidity. Positive strains could be distinguished after about 2 weeks, when they were transplanted and followed up.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12870-021-02900-2.

Additional file 1: S1 Table. Location and sequences of cucumber CLE genes. S2 Table. Location and sequences of Arabidopsis CLE genes. S3 Table. Location and sequences of melon CLE genes. S4 Table. Ka/Ks and divergence time of the homologous pair. CgCLE24-CiCLE25. S5 Table. RKPM of cucumber CLE genes during different developmental stages. S6 Table. Expression of cucumber CLE genes during different tissue in Chinese long ‘9930’. S7 Table. Expression of heterologous overexpression of CiCLV3 gene in Arabidopsis.

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Authors’ contributions
Nannan Qin conceived and designed the experiments, analyzed the data, wrote the manuscript, prepared the figures and tables, and reviewed the manuscript drafts. Guoming Xing and Sen Li contributed reagents/materials/analysis tools and revised the manuscript drafts. Yang Gao analyzed the data. Xiaojing Cheng, Jiang Wu, Yang Yang, and Jinyao Wang cultivated the experimental material. The author(s) read and approved the final manuscript.

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Availability of data and materials
The datasets supporting the conclusions of this article are available in the Treebase repository, [unique persistent identifier and hyperlink to datasets in http://purl.org/phylo/treebase/phylo/lookup/study/TB2:527518?x-access-code=a142631f6de4f4d2f968ba893afcc838f8format=html].

Declarations
Competing interests
The authors declare no competing interests.
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