Genome-wide identification of CUC gene family and functional analysis of HcCUC1 in kenaf

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Research Article

Keywords: Kenaf (Hibiscus cannabinus L.), CUP-SHAPED COTYLEDON (CUC), Growth and development, Lateral branches, Flowering

Posted Date: April 3rd, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2607938/v1

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**Version of Record:** A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on July 7th, 2023. See the published version at [https://doi.org/10.1007/s11240-023-02555-x](https://doi.org/10.1007/s11240-023-02555-x).
Abstract

*CUP-SHAPED COTYLEDON (CUC)* is a plant-specific transcription factor that plays an important regulatory role in many aspects of plant growth and development. However, its role in kenaf (*Hibiscus cannabinus L.*) is largely unknown. In this study, we identified six *CUC* genes from the kenaf genome and performed bioinformatics analysis. Conserved motif and gene structure analysis showed that the kenaf *HcCUC* genes had similar conserved motifs and highly conserved gene structure. Phylogenetic analysis showed that the six *HcCUC* genes could be divided into two categories: homologous to *CUC2* or *CUC3* of other species. Collinearity analysis showed that 6 pairs of syntenic gene pairs were formed between *HcCUC* genes, and 8 pairs of homologous gene pairs were formed with three *AtCUC* genes of *Arabidopsis*. Tissue specific expressions of the *HcCUC* genes was analyzed by using transcriptome data. The results showed that the *HcCUC* genes were differentially expressed in various tissues, mainly in the leaves of the seedling stage, buds of the mature period, and anthers at the dual-core period. Overexpression of *HcCUC1* in *Arabidopsis* resulted in increased cotyledon length, petiole absence, and a significant increase in the number of rosette leaves and lateral branches. The qRT-PCR analysis showed that the *HcCUC1* might affect leaf or lateral branch development by up-regulating the expression of auxin-related genes (*YUC2, YUC4, PIN1, PIN3, PIN4*) and leaf shape-related genes (*KNAT2, KNAT6*); In addition, overexpression *HcCUC1* down-regulated the expression of flowering-related genes (*FT, AP1, LFY, FUL*) caused the flowering delay. Taken together, these results suggest that *HcCUC1* is involved in regulating leaf and lateral branch growth and development and flowering time, which can help us to understand the function and genetic regulation of *HcCUC* genes.

Key Message

Six *HcCUC* genes were identified from the kenaf genome, of which *HcCUC1* is involved in regulating the growth and development of leaves and lateral branches and regulating flowering time.

Introduction

*CUP-SHAPED COTYLEDON (CUC)* is a plant-specific transcription factor belonging to the NAC (*NAM/ATAF/CUC*) family. It has a highly conserved N-terminal domain and plays an essential regulatory role in plant growth and development (Aslam et al. 2022; Taoka et al. 2004). The *CUC* gene was first identified from *Arabidopsis*, and it was found that mutations in *Arabidopsis CUC1* and *CUC2* resulted in defects in the separation of cotyledons, sepals, and stamens and the formation of shoot apical meristem (Aida et al. 1997). Subsequent studies have found that *miR164* may also regulate its transcription level (Laufs et al. 2004; Mallory et al. 2004). With the deepening of research, more biological functions of *CUC* involved in plant growth and development have been further revealed. During the growth and development of buds, *CUC* can promote the regeneration of adventitious buds from the callus (Daimon et al. 2003; Kareem et al. 2015). *CUC2* and *CUC3* can directly bind to the promoter of the ubiquitin-dependent peptidase DA1 (the substrate is UBIQUITIN-SPECIFIC PROTEASE15 (UBP15)) and activate its expression. The regulatory module composed of *CUC2/CUC3-DA1-UBP15* controls the number of lateral branches in *Arabidopsis* and affects plant structure by regulating the initiation of the lateral meristem (Li et al. 2020). Research has demonstrated that *CUC* also plays an important role in forming leaf morphology. The regulatory module formed by *miRNA164-CUC2* controls the serration depth at the leaf edge of *Arabidopsis*. The leaves of *Arabidopsis* overexpressing *miR164* have
smooth edges, but enhanced leaf serrations were observed after the expression of miR164-resistant CUC2. In addition, the inactivation of CUC2 eliminates miR164a mutant and wild-type serrations (Nikovics et al. 2006). Hasson et al. (Hasson et al. 2011) found that CUC3 also plays a role in the formation of serrated leaves, but it is different from CUC2. CUC2 is necessary for the beginning and early stages of serrated development, while CUC3 plays a later role in maintaining its growth, and the effect of CUC3 is lower than that of CUC2. During floral organ development, miR164c controls the number of petals in a non-redundant manner by regulating the transcription levels of CUC1 and CUC2 (Baker et al. 2005). The number of floral organs can be regulated owing to the collaborative efforts of HWS (HAWAIIAN SKIRT), CUC1, and CUC2 (Gonzalez-Carranza et al. 2017). In addition to Arabidopsis, CUC has also been involved in regulating plant growth and development in other species. MiR164a affects internode growth and leaf shape by targeting BpCUC2 during internode development in Betula pendula (Liu et al. 2019). The regulatory module formed by FvemiR164 and FveCUC2 in strawberries regulates leaf morphology and floral organ development (Zheng et al. 2019). OsCUC1 was found to interact leaf-rolling protein CURLED LEAF AND DWARF1 (CLD1) to control leaf morphology in rice (Wang et al. 2021). The above results indicate that CUC plays an important role in regulating plant growth and development.

Kenaf (Hibiscus cannabinus L.) is an annual dicotyledonous plant in the Malvaceae family and is widely cultivated, especially in China, India, Bangladesh, and Thailand (Ramesh 2016). This plant has the advantages of being fast-growing, having large biomass, and wide adaptability (Chen et al. 2021). It is widely used in textiles, clothing, papermaking, rope and wire materials, building materials, automobiles, and other industries. Due to its strong tolerance, it can also be used for the remediation of saline-alkali and heavy metal-contaminated soil (Danalatos and Archontoulis 2010; Ramesh 2016; Wei et al. 2019; Yue et al. 2022). The CUC genes of kenaf have yet to be identified. Identifying and analyzing the CUC genes of kenaf and elucidating its gene function is of great significance for understanding the regulation of kenaf growth and development.

In the present study, six HcCUC genes were identified in kenaf using bioinformatics analysis methods. HcCUC overexpression plants were generated in Arabidopsis, and the phenotype of transgenic lines and the expression level of related metabolic regulatory genes were analyzed to study their gene function. The results showed that the HcCUC genes were identified differentially expressed. Overexpression of HcCUC1 resulted in abnormal phenotypes in transgenic Arabidopsis, such as longer cotyledons, and missing petioles, significantly increased rosette leaves and lateral branches, and delayed flowering time. The underlying mechanism has also been discussed by investigating the related genes expression based on RT-qPCR analysis.

**Materials And Methods**

**Plant materials and growth conditions**

Kenaf cultivar of FuHong952 was used in this study. The sterilized seeds were put into a plastic seedling basin covered with filter paper. After growing in the culture room for about 4 days, seedlings with grew evenly were selected and transferred to a seedling basin containing 0.5×Hoagland solution for hydroponics cultivation. The temperature of the culture room was 25°C, 16h light/8h dark. The leaves of kenaf seedlings about 3 weeks old were used for RNA extraction. Arabidopsis thaliana WT (Col-0) was used in this work. After disinfection, its seeds were sown in 1/2MS medium containing 0.7% (w/v) agar and 3% (w/v) sucrose and
vernalized in dark conditions at 4°C for two days, then transferred to the growth chamber and transferred to
the soil for growth after about 10 days. *Arabidopsis* plants were grown in soil under controlled environmental
conditions at 22°C, and a 16h light /8h dark photoperiod to observe natural senescence.

**Identification of HcCUC genes in kenaf**

The kenaf genome and annotation files were downloaded from the National Genomics Data Center, and the
accession number was GWHACDB00000000.1(Zhang et al. 2020). The protein sequences of *CUC* genes from
*Arabidopsis* and other species were downloaded from The National Center for Biotechnology Information
(NCBI) public database. The protein accession numbers of other species are shown in Supplementary table 1.

Using TBtools(Chen et al. 2020), the *CUC* sequence of *Arabidopsis* was used as a probe to search homologous
protein sequences in the kenaf genome. After removing the redundant sequence, the sequence with E-value<e^-5
was selected as the candidate sequence. Pfam database (http://pfam.xfam.org/) and NCBI CDD database
(https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) were used to check whether the candidate sequence
had a complete conserved domain. The ExPASy proteomics server database (https://www.expasy.org/) was
used to analyze the related physical and chemical properties such as molecular weight (MW), isoelectric point
(PI), grand average of hydropathicity (GRAVY), instability index.

**Conserved motif and gene structure analysis**

MEME(Bailey et al. 2015) online tool was used to analyze the conserved motifs of kenaf *CUC* family proteins.
The parameter setting was to identify the number of Motifs as 10, and other parameters were default. TBtools
was used to visualize conserved motif prediction results and gene structure.

**Construction of phylogenetic tree and collinearity analysis**

The phylogenetic tree of the kenaf *HcCUC* genes family was constructed by the maximum likelihood method
using MEGA-X(Kumar et al. 2018) software. The phylogenetic tree of the kenaf *CUC* proteins with other species
was constructed by the Neighbor-joining method. The bootstrap repeat number was set to 1000. The whole
genome protein database of kenaf and *Arabidopsis* was constructed by Tbtools software. After BLASTp
comparison, the collinearity analysis was carried out by MCScanX(Wang et al. 2012) software, and the
collinearity map of homologous genes was drawn on the chromosome.

**Tissue-specific expression analysis**

The tissue expression pattern of *HcCUC* genes was analyzed based on the raw RNA sequencing data, and the
data (Supplementary table 2) were obtained from the NCBI SRA database
(https://www.ncbi.nlm.nih.gov/sra/). Firstly, the RNA-seq data were standardized to TPM (transcripts per
million reads), and then log₂(TPM+1) conversion was performed to visualize in the form of heat maps.

**Gene cloning and transgenic plant generation**

The coding sequence of *HcCUC1* (*Hc.01G004330.t1*) was cloned from the cDNA of kenaf using primers
*HcCUC-F1/HcCUC-R1* (Supplementary table 3), and the amplified fragment was ligated to the *BamH I* and *Sac I*
sites of the pBI121-GUS vector by homologous recombination. The specific steps are described in the instructions of the ClonExpress II ® One Step Cloning Kit (Vazyme, China). Finally, the constructed expression vector was confirmed by sequencing. The constructed pBI121-HcCUC1 expression vector was transformed into Arabidopsis by GV3101 Agrobacterium tumefaciens using the inflorescence dipping method (Clough and Bent 1998). Pure and third-generation (T3) Arabidopsis plants were screened for subsequent research.

Expression analysis by RT-qPCR

According to the manufacturer's instructions, total RNA was extracted using RNA isolater Total RNA Extraction Reagent (Vazyme, China), and cDNA was synthesized by reverse transcription using HiScript ® II 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme, China). Quantitative PCR was performed using F ChamQ Universal SYBR qPCR Master Mix premix (Vazyme, China). Using Arabidopsis TIP41-like (AT4G34270.1) as an internal reference gene, the expression of the gene was detected using the primers in Supplementary table 3. The formula $2^{-\Delta\Delta CT}$ calculated the relative expression. All treatments used three independent biological replicates. IBM SPSS 25.0 was used for statistical analysis.

Results

Identification and basic physicochemical properties analysis of kenaf HcCUC genes

Through Blastp alignment, CDD and Pfam database for domain analysis, six HcCUCs were finally identified. In order to facilitate the distinction, they were renamed according to the chromosome position (Table 1). The predicted six CUC proteins ranged in length from 327 to 386 amino acid residues, and their relative molecular masses ranged from 36300.8 Da to 42780.41 Da. The isoelectric point (pI) values range from 5.81 to 8.48, with pI values of 7 for three members. The aliphatic index ranged from 58.5 to 69.51. The total average hydrophilicity (GRAVY) of the protein was between -0.388 and -0.554, which was a negative and hydrophilic protein. The instability coefficient ranged from 24.96 to 52.75. HcCUC2 and HcCUC5 were less than 40 stable proteins, and the rest were more than 40 unstable proteins.

Table 1 Basic information of CUC genes in kenaf

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene ID</th>
<th>Length of CDS (bp)</th>
<th>Length of protein (aa)</th>
<th>PI</th>
<th>MW (Da)</th>
<th>Instability index</th>
<th>Aliphatic index</th>
<th>GRAVY</th>
</tr>
</thead>
<tbody>
<tr>
<td>HcCUC1</td>
<td>Hc.01G004330.t1</td>
<td>984</td>
<td>327</td>
<td>8.48</td>
<td>36300.8</td>
<td>52.75</td>
<td>58.50</td>
<td>-0.554</td>
</tr>
<tr>
<td>HcCUC2</td>
<td>Hc.01G008340.t1</td>
<td>1056</td>
<td>351</td>
<td>6.21</td>
<td>39579.93</td>
<td>24.96</td>
<td>66.13</td>
<td>-0.404</td>
</tr>
<tr>
<td>HcCUC3</td>
<td>Hc.02G037940.t1</td>
<td>1050</td>
<td>349</td>
<td>6.61</td>
<td>38405.1</td>
<td>43.63</td>
<td>62.92</td>
<td>-0.423</td>
</tr>
<tr>
<td>HcCUC4</td>
<td>Hc.06G022950.t1</td>
<td>1065</td>
<td>354</td>
<td>8.25</td>
<td>39050.86</td>
<td>50.07</td>
<td>60.42</td>
<td>-0.491</td>
</tr>
<tr>
<td>HcCUC5</td>
<td>Hc.07G027740.t1</td>
<td>1161</td>
<td>386</td>
<td>5.81</td>
<td>42780.41</td>
<td>35.24</td>
<td>69.51</td>
<td>-0.388</td>
</tr>
<tr>
<td>HcCUC6</td>
<td>Hc.13G003690.t1</td>
<td>1062</td>
<td>353</td>
<td>7.64</td>
<td>38576.2</td>
<td>51.05</td>
<td>60.34</td>
<td>-0.418</td>
</tr>
</tbody>
</table>

Conserved motif and gene structure analysis
In order to better understand the structural evolution of HcCUCs, a phylogenetic tree was constructed based on the predicted full-length HcCUCs protein sequence. HcCUCs were divided into two groups (I-II) (Fig 1a), and the HcCUCs clustered in the same group contained highly similar conserved motifs and gene structures (Fig 1b, c). Motif 1-4 exists in all HcCUCs, where the region of Motif 1-3 is the region of the NAM conserved domain of HcCUCs, Motif 5~9 exists only in group I HcCUCs, while Motif 10 exists only in group II HcCUCs (Fig 1b). All HcCUCs did not have a 3’ non-coding region, except for HcCUC2, the remaining HcCUCs did not have a 5’ non-coding region (Fig 1c). The number and arrangement of coding regions of all HcCUCs are highly similar.

**Phylogenetic and collinearity analysis**

To further understand the phylogeny of HcCUCs, 22 CUC protein sequences from other species and six HcCUC protein sequences were used to construct a phylogenetic tree (Fig 2a). Six HcCUC genes were divided into two groups (I and V). HcCUC1, HcCUC3, HcCUC4, and HcCUC6 in group I was closely related to CpCUC2, while HcCUC2 and HcCUC5 in group V were most closely related to CpCUC3 and DcCUC3. Among the three AtCUC genes in the model plant Arabidopsis, AtCUC2 and AtCUC3 were divided into groups I and V containing HcCUC genes, respectively, indicating that the two AtCUC genes are more closely related to HcCUC genes, and the same cluster of CUC genes may have similar biological functions. Gene duplication events are essential for the evolution of gene families and often play an important role in gene amplification and the generation of new functional genes. Colinearity analysis showed that six pairs of fragment repeat gene pairs (HcCUC1/3, HcCUC1/4, HcCUC1/6, HcCUC2/5, HcCUC3/4, HcCUC4/6) were formed between the six HcCUCs (Fig 2b), indicating that fragment repeat plays an important role in the amplification of HcCUC gene family. Colinearity analysis showed that eight pairs of homologous gene pairs (HcCUC1/AtCUC1, HcCUC1/AtCUC2, HcCUC2/AtCUC3, HcCUC3/AtCUC1, HcCUC4/AtCUC1, HcCUC5/AtCUC3, HcCUC6/AtCUC1, HcCUC6/AtCUC2) were formed between six HcCUCs and three AtCUCs (Fig 2b). These homologous gene pairs may have evolved from a common ancestor and has a closer evolutionary relationship.

**Tissue-specific expression analysis of the HcCUC gene**

The expression patterns of HcCUC genes were analyzed using RNA-seq data from different kenaf organs. As shown in Fig3, different expression patterns exist among different members. For example, HcCUC6 is difficult to detect its expression in different tissues at different stages. HcCUC4 and HcCUC5 can be detected in different degrees in most tissues. HcCUCs were mainly expressed in leaves at the seedling stage, buds at the mature period, and anthers at the dual-core period, and all members could be detected in anthers at the dual-core period. At the same time, except for HcCUC6, all members could be detected in leaves at the seedling stage and buds at 2cm at the mature period, which indicated that HcCUCs might play a more important role in the development of these tissues.

**Overexpression of HcCUC1 regulated the growth and development of Arabidopsis leaves and lateral branches**

To further study the biological function of HcCUCs, we cloned HcCUC1, which is in the same group with AtCUC2 in phylogenetic analysis and has a collinearity relationship with AtCUC2, and is highly expressed in leaves at seedling stage, buds at mature period and anthers at dual-core period. HcCUC1 overexpression lines...
driven by cauliflower mosaic virus (CaMV) 35S promoter (35S: HcCUC1) were generated in Arabidopsis (Col-0). Two independent overexpression lines (OE1 and OE2) increased the transcriptional level of HcCUC1 for further study (Fig 4c).

**Overexpression of HcCUC1 delayed the flowering time of Arabidopsis**

In addition to the differences in leaves and lateral branches, the study also found significant differences in flowering time between wild-type and transgenic Arabidopsis. Compared with the wild type, the bolting and flowering time of HcCUC1 overexpression lines were about four days later (Fig 5a, b, d, e). Due to the late bolting time of the transgenic lines, the plant height of the transgenic lines was always lower than that of the wild-type in the early growth stage, but there was no significant difference between the transgenic lines and the wild-type plants in the whole growth period (Fig 5c, f). These results suggest that heterologous expression of HcCUC1 can delay the flowering time of Arabidopsis but does not affect the final height of the plant.

**HcCUC1 affects the expression of genes related to auxin, leaf development and flowering regulation**

Some studies have shown that auxin plays an important role in leaf and branch development (Xiong and Jiao 2019), and many genes also regulate plant flowering (Fornara et al. 2010). By detecting the transcription levels of some related genes in wild-type and transgenic Arabidopsis, it was found that the transcription levels of auxin synthesis-related genes YUC2 and YUC4 and auxin transport-related genes PIN1, PIN3, and PIN4 in transgenic Arabidopsis were up-regulated as a whole compared with wild-type plants (Fig 6a). In addition, the transcription levels of two leaf-related genes, KNAT2 and KNAT6, were also significantly higher than those in wild-type plants (Fig 6b). It is suggested that the overexpression of HcCUC1 may influence the growth and development of leaves or lateral branches by up-regulating the transcription levels of auxin and leaf-shape-related genes. However, Transgenic Arabidopsis showed dramatically reduced transcription levels of genes involved in flowering regulation (such as FT, AP1, LFY and FUL), as compared to wild-type plants (Fig 6c), which demonstrated that the heterologous expression of HcCUC1 may delay the flowering time of Arabidopsis by down-regulating the transcription level of genes involved in flowering regulation.

**Discussion**

Plant-specific transcription factor CUC is necessary for plant growth and development, such as establishing organ boundaries, forming shoot apical meristem, and developing leaves, lateral branches and floral organs (Aida et al. 1997; Aslam et al. 2022; Gonzalez-Carranza et al. 2017; Hasson et al. 2011). The transcription factor has been identified in many species, but no related research has been reported in kenaf. This study identified and characterized six kenaf HcCUC (HcCUC1~HcCUC6) genes from the kenaf genome. Changes in gene structure play a crucial role in the evolution of gene families (Guo et al. 2013). The six HcCUC genes of kenaf are very similar in structure, indicating that they are highly conserved in structural evolution (Fig 1c). The composition of motifs can reflect the similarities and differences between gene family proteins (Su et al. 2020). Combined with the conserved motifs and phylogenetic analysis of HcCUC genes, it can be found that HcCUC2/5 and HcCUC1/3/4/6 formed different branches in evolution. HcCUC2/5 and Arabidopsis AtCUC3 clustered together, while HcCUC1/3/4/6 and AtCUC2 clustered together. Compared with HcCUC2/5, HcCUC1/3/4/6 had more motifs (Fig 1b, Fig 2c), which corresponds to the results of Aslam et al.
(Aslam et al. 2022) and Hasson et al. (Hasson et al. 2011). It indicates that HcCUC1/3/4/6 may have similar functions to AtCUC2 of Arabidopsis and differentiate from HcCUC2/5. Further collinearity analysis can also reflect this speculation (Fig 2b). HcCUC2/5 and AtCUC3 form homologous gene pairs, while HcCUC1/6 and AtCUC1/2, HcCUC3/4 and AtCUC1 form homologous gene pairs. These highly homologous genes are more closely related in evolution and may be more conservative in function.

Further collinearity analysis can also reflect this speculation (Fig 2b). HcCUC2/5 and AtCUC3 form homologous gene pairs, while HcCUC1/6 and AtCUC1/2, HcCUC3/4 and AtCUC1 form homologous gene pairs. These highly homologous genes are more closely related in evolution and may be more conservative in function.

Tissue expression analysis showed that the HcCUC genes were mainly expressed in leaves at the seedling stage, buds at the mature period, and anthers at the dual-core period, especially in buds at the mature period, which was similar to the highest expression level of BpCUC2 in buds(Liu et al. 2018) and the significant expression of LcCUC2L in leaf buds(Wen et al. 2022). Functional redundancy often exists among family gene members. In Arabidopsis, both cuc1 and cuc2 single mutations lead to defects in the separation of cotyledons, sepals, and stamens and the formation of shoot apical meristem. However, these defects are more pronounced in double mutants(Aida et al. 1997). The three AtCUC genes significantly contribute to shooting meristem and cotyledon boundary formation, and the functional redundancy between AtCUC1 and AtCUC2 seems to be greater than that between AtCUC3 and AtCUC1 or between AtCUC3 and AtCUC2. In addition, AtCUC1 and AtCUC2 are involved in primordium development, but only AtCUC2 is involved in leaf margin development(Hibara et al. 2006). HcCUC2 and HcCUC5 were homologous to AtCUC3, and the expression of HcCUC5 was significantly higher than that of HcCUC2. HcCUC1, HcCUC3, HcCUC4, and HcCUC6 were more closely related to AtCUC2. Although HcCUC1 and HcCUC6 were homologous to AtCUC2, HcCUC6 was difficult to detect its expression in all tissues. At the same time, HcCUC1 had relatively high expression levels in leaves at the seedling stage, buds at the mature period, and anthers at the dual-core period. Therefore, it is speculated that HcCUC1 in the same group with AtCUC2 and HcCUC5 in the same group with AtCUC3 may play a more critical role in the growth and development of kenaf, but this needs further research to verify.

Based on the above analysis, we chose HcCUC1 for further functional study. The analysis of HcCUC1 overexpressing Arabidopsis lines showed that in terms of leaf development, overexpression of HcCUC1 led to abnormal cotyledon development, petiole deletion, and slender cotyledon leaves in transgenic Arabidopsis. At the same time, it also significantly increased the number of rosette leaves in transgenic Arabidopsis (Fig 4a, b, d). Transgenic Arabidopsis seedlings overexpressing AtCUC1 have a pair of symmetrical sinuses on both sides of their cotyledons rather than slender cotyledons(Hibara et al. 2003). Overexpression of AtCUC2 leads to leaf wrinkles(Laufs et al. 2004). The study of Hasson et al. (Hasson et al. 2011) found that overexpression of AtCUC2 or AtCUC3 after mutation of the miR164 target deepened the serrated depth of rosette leaves. It shows that the regulatory mechanism of CUC on leaf development in kenaf and Arabidopsis differs. In lateral branch development, DA1 mutation in Arabidopsis can lead to the decrease of lateral branches, and its direct substrate UBP15 can inhibit the initiation of the axillary meristem. At the same time, AtCUC2 and AtCUC3 directly bind to the DA1 promoter and activate its expression, thus affecting the number of lateral branches(Li et al. 2020). In our study, overexpression of HcCUC1 significantly increased the number of rosette leaf lateral branches in transgenic Arabidopsis but did not affect the final plant height (Fig 4e, Fig 5b, c, f). It shows a similarity in the regulation of CUC on lateral branch development in kenaf and Arabidopsis. In addition, overexpression of LcCUC2L in Arabidopsis observed cotyledon phenotypes similar to our study and significantly increased the number of rosettes leaves(Wen et al. 2022). Overexpression of GhCUC2m after mutation of miR164 target resulted in significantly fewer branches in transgenic Arabidopsis than in wild-type
and shortened branch length, which was opposite to the effect of OE-\textit{gh-pre164} line in \textit{Arabidopsis}. It can be seen that the regulation mechanism of \textit{CUC} on leaf and lateral branch development is both conservative and different among different species. At the early stage of growth, the plant height of \textit{HcCUC1} transgenic \textit{Arabidopsis} was consistently lower than that of wild-type plants, but there was no significant difference in their final plant height (Fig 5b, c, f). In the study of Hibara et al.(Hibara et al. 2003), it was observed that the growth of transgenic \textit{Arabidopsis} seedlings overexpressing \textit{CUC1} slowed down. Therefore, we speculate that the overexpression of \textit{HcCUC1} will lead to the slow growth of transgenic \textit{Arabidopsis}, so the early stage of transgenic \textit{Arabidopsis} is higher than that of wild-type plants, which may also be one of the reasons for the delay of bolting and flowering time of transgenic \textit{Arabidopsis} (Fig 5a, b, d, e).

Studies have shown that \textit{PIN-FORMED1} (\textit{PIN1}), which encodes an auxin efflux carrier, can regulate the expression of \textit{CUC2}(Aida et al. 2002). The \textit{CUC2} gene was actively expressed on an auxin-rich callus induction medium(Gordon et al. 2007). The regulatory network formed by auxin, \textit{PIN1}, and \textit{CUC2} has been shown to be involved in regulating leaf development(Bilsborough et al. 2011). At the same time, auxin has also been shown to be involved in regulating branch formation(Brewer et al. 2015; Muller and Leyser 2011). By detecting the transcription levels of auxin-related genes and leaf development-related genes in wild-type and transgenic \textit{Arabidopsis}, it was found that the transcription levels of auxin synthesis-related genes (\textit{YUC2}, \textit{YUC4}), auxin transport-related genes (\textit{PIN1}, \textit{PIN3}, \textit{PIN4}) and leaf development-related genes (\textit{KNAT2}, \textit{KNAT6}) were up-regulated in transgenic \textit{Arabidopsis} (Fig 6ab), which was consistent with the results of Wen et al.(Wen et al. 2022). \textit{HcCUC1} may regulate leaf and lateral branch development by up-regulating the transcription levels of auxin-related genes and leaf shape-related genes. The flowering of plants is affected by many factors, and many genes are involved in the flowering regulation of plants. For example, \textit{FLOWERING LOCUS T (FT)} can integrate signals from different pathways and transmit floral induction signals to downstream floral meristem recognition genes \textit{LEAFY (LFY)}, \textit{APETALA1 (AP1)}, and \textit{FRUITFULL (FUL)}(Amasino 2010; Fornara et al. 2010). The transcription levels of flowering-related genes (\textit{FT, AP1, LFY, FUL}) in transgenic \textit{Arabidopsis} were significantly lower than those in wild-type \textit{Arabidopsis} (Fig 6c), indicating that \textit{HcCUC1} may delay \textit{Arabidopsis} flowering by down-regulating the transcription levels of flowering-related genes. However, the specific regulatory mechanism of \textit{HcCUC1} in \textit{Arabidopsis} is still unclear, and the regulatory mechanism of kenaf may also be different from that of \textit{Arabidopsis}.

**Conclusions**

The six \textit{HcCUC} genes of kenaf are divided into two categories, which are homologous to \textit{CUC2} or \textit{CUC3} of other species. They are relatively conserved in structure and motifs, and tissue-time dependently expressed. Overexpression of \textit{HcCUC1} in \textit{Arabidopsis} resulted in longer cotyledons and missing petioles, significantly increased the number of rosette leaves and lateral branches, and delayed flowering time. The \textit{HcCUC1} might affect leaf or lateral branch development by up-regulating the expression of auxin-related genes (\textit{YUC2}, \textit{YUC4}, \textit{PIN1}, \textit{PIN3}, \textit{PIN4}) and leaf shape-related genes (\textit{KNAT2}, \textit{KNAT6}); In addition, overexpression \textit{HcCUC1} down-regulated the expression of flowering-related genes (\textit{FT, AP1, LFY, FUL}) caused the delay of flowering. This study has potential benefits for insight into the potential biological functions of the \textit{CUC} family of kenaf and other crops.
Declarations

Acknowledgements This research work was supported by the National Natural Science Foundation of China (Grant No. 31960368).

Author contributions QW: Data curation; Methodology, Formal analysis, Roles/Writing - original draft. CC; JY: Investigation, Formal analysis. SC; XL: Investigation, Data curation, Formal analysis. MW; HZ: Software, Methodology, Formal analysis. XW; CW; DL: Formal analysis, Validation. PC: Conceptualization, Methodology, Writing-review & editing, Funding acquisition, Project administration.

Data availability The data generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of interest The authors declare that they have no known competing financial interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References


**Figures**

**Figure 1**

Figure 2

Phylogenetic and collinear analysis. a: The neighbor-joining phylogenetic tree analysis of CUC protein sequences between kenaf and other species; b: The collinearity of HcCUCs and the collinearity of HcCUCs - AtCUCs; Collinear gene pairs between HcCUCs are connected by the red line in b, and collinear gene pairs between HcCUCs and AtCUCs are connected by the blue line.

Figure 3
Transcription activity analysis of *HcERF4*. The transformants with pGBK7 (up) and pGBK7-*HcERF4*(down) grow normally on SD-Trp medium, and only the transformants pGBK7-*HcERF4* (down) can grow normally on SD-Trp-His-Leu medium.

**Figure 4**

Over expression of *HcCUC1* regulated the growth and development of *Arabidopsis* leaves and lateral branches. 

*a*: *Arabidopsis* at 5 days old; 
*b*: *Arabidopsis* at 21 days old; 
*c*: The expression level of *HcCUC1* in wild-type and transgenic plants, data are presented as mean±SD(n=3), the asterisks indicate significant differences.
(Student's t test, P<0.01); d: Number of rosette leaves of wild type and transgenic plants at 21 days of age; e: Number of lateral rosette branches of wild type and transgenic plants at 31 days of age. Data in d and e showed mean ±SD (n=10), different letters represent significant differences (one-way ANOVA, Duncan's test, P < 0.05).

Figure 5

Overexpression of HcCUC1 delayed the flowering time of Arabidopsis. a: Arabidopsis at 23 days old; b: Arabidopsis at 31 days old; c: Arabidopsis at 56 days old; d: Bolting time of wild type and transgenic plants; e: Flowering time of wild type and transgenic plants; f: Height of wild type and transgenic plants at 56 days of
Data in d-f showed mean ±SD (n=10), different letters represent significant differences (one-way ANOVA, Duncan's test, P < 0.05).

Figure 6

*HcCUC1* affects the expression of genes related to auxin, leaf shape development and flowering regulation. a: Transcription levels of auxin-related genes in wild-type and transgenic *Arabidopsis*; b: Transcription level of leaf development-related genes in wild-type and transgenic *Arabidopsis*; c: Transcription levels of genes involved in flowering regulation in wild-type and transgenic *Arabidopsis*. Data showed mean ±SD (n=3), different letters represent significant differences (one-way ANOVA, Duncan's test, P < 0.05).

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