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Establishment and application of highly efficient regeneration, genetic transformation and genome editing system for cucurbitacins biosynthesis in *Hemsleya chinensis*

Juan Wang^{1,2,3}, Chao-Hui Li^{1,2}, Chun-Fan Xiang^{1,2}, Pin-Han Zhou^{1,2}, Le-Song Li^{1,2}, Xia Li^{1,2}, Sheng-Chao Yang^{1,3}, Guang-Hui Zhang^{1,2,3} and Yan Zhao^{1,2,3*}

Abstract

Background *Hemsleya Chinensis* is a perennial plant in the Cucurbitaceae family containing antibacterial and anti-inflammatory compounds. The lack of genetic transformation systems makes it difficult to verify the functions of genes controlling important traits and conduct molecular breeding in *H. chinensis*.

Results Highly efficient calli were induced on MS medium added 1.5 mg·L⁻¹ 6-benzylaminopurine (6-BA) and 0.02 mg·L⁻¹ 1-naphthylacetic acid (NAA) with high efficiency (> 95%). The frequency of shoot induction was increased to 90% with a plant growth regulator combination of 1.5 mg·L⁻¹ 6-BA and 0.1 mg·L⁻¹ NAA. Our results also showed that 100% of shoot regeneration was achieved in a shoot regeneration medium. Additionally, more than 92% of kanamycin-resistant plants were confirmed. Furthermore, we achieved 42% genome editing efficiency by applying this transformation method to *HcOSC6*, a gene that catalyzes the formation of cucurbitadienol. HPLC analysis showed OE-*HcOSC6* lines exhibited significantly higher cucurbitadienol levels than the genome-edited lines. Transcriptomic analysis revealed that some downstream genes related to cucurbitadienol biosynthesis, such as *HcCYP87D20*, *HcCYP81Q58*, and *HcSDR34*, were up-regulated in OE lines and down-regulated in mutants.

Conclusions Here, we established a process for regeneration, transformation, and genome editing of *H. chinensis* using stem segments. This provides valuable insight into the underlying molecular mechanisms of medicinal compound production. By combining high-efficiency tissue culture, transformation, and genome editing systems, we provide a powerful platform that supports functional research on molecular mechanisms of secondary metabolism.

Keywords Medicinal plants, *H. chinensis*, *HcOSC6*, Genetic transformation system, Genome editing

*Correspondence:

Yan Zhao
zhaoyankm@126.com

¹Key Laboratory of Medicinal Plant Biology of Yunnan Province, National & Local Joint Engineering Research Center on Germplasm Innovation

& Utilization of Chinese Medicinal Materials in Southwest China, Yunnan Agricultural University, Kunming 650201, China

²College of Agronomy & Biotechnology, Yunnan Agricultural University, Kunming 650201, China

³Yunnan Characteristic Plant Extraction Laboratory, Kunming, Yunnan 650106, China



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Background

Medicinal plants contain bioactive ingredients that could help prevent, treat and provide health care [1]. Further research in plant genetic transformation and medicinal plant breeding will be facilitated by high-efficiency regeneration systems. Plant genetic transformation technology has been used previously to study biosynthesis and breeding in medicinal plants such as *Salvia miltiorrhiza*, *Cannabis sativa*, and *Dendrobium officinale* [2–4]. Medicinal plants that have undergone genetic transformation can be precisely regulated in terms of their secondary metabolites, which can significantly increase their active ingredient content [5, 6]. In addition, it has the potential to enhance the stress resistance of medicinal plants, allowing them to maintain growth and efficacy even under more challenging environments [7]. Furthermore, plant genetic transformation technology significantly reduces breeding time. With the use of this technology, breeding years can be shortened significantly [8], allowing new varieties to be launched faster. The technology of plant genetic transformation not only improves the quality and yield of medicinal plants, but it also offers new opportunities for the development of traditional Chinese medicine.

Plants belonging to the Cucurbitaceae family are widely found in tropical and subtropical areas. They are mainly popular melon vegetables, including *Cucumis sativus*, *Citrullus lanatus*, *Cucumis melo*, *Cucurbita moschata*, and medicinal plants such as *H. chinensis* [9]. Most of these species do not yet have efficient genetic transformation systems. In cucurbit crops, genome editing tools and biotechnology advances have greatly improved the molecular characterization of functional genes, which will lead to the development of new breeding methods [10]. The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) and CRISPR-associated nuclease 9 (Cas9) systems are particularly effective for achieving this goal [10–13]. It has been reported that CRISPR/Cas9 has been successful in editing the genome of cucumber plants [14, 15]. The ‘optimal infection intensity’ strategy has proven to be effective for genetically transforming melons, pumpkins, and cucumbers in recent years. It has also enabled, for the first-time, heritable genome editing in melons and pumpkins [16]. Thus, most Cucurbitaceae, including *H. chinensis*, require a stable, versatile, and efficient genetic transformation system, which can provide a powerful platform for advancing fundamental research into the molecular mechanisms of secondary metabolism in *H. chinensis* and other important Cucurbitaceae plants, to accelerate the molecular breeding process.

Hemsleya chinensis is a perennial plant in the Cucurbitaceae family. Dried tubers are used for medicinal purposes [9]. *H. chinensis* mainly contains tetracyclic triterpenoids such as cucurbitacin IIA, cucurbitacin IIB and

oleanolic acid [9]. These compounds have clinical effects such as anti-biotic, anti-diabetic, anti-inflammatory, anti-cancer, and antiviral properties [17–25]. The demand for *H. chinensis* plants is increasing. However, plants of the genus *Hemsleya* grow slowly and have a long planting cycle; they require 5–6 years to be harvested under artificial cultivation [26]. The development and application of *H. chinensis* are severely hindered by this problem.

The cyclization of 2,3-oxidosqualene to cucurbitadienol, which further generates cucurbitacin, has been confirmed in previous studies [9, 27]. Among the enzymes involved in cucurbitacin F biosynthesis in *H. chinensis*, which is the key enzyme HcOSC6 for the synthesis of cucurbitadienol, and it has been proven in tobacco and yeast that HcOSC6 catalyzes 2,3-oxidosqualene to form cucurbitadienol [9, 28]. There has been only one reported research of *H. macrosperma*, which could accumulate bioactive compounds using suspension cell cultures under MeJA treatment [26]. Currently, the biosynthesis pathway of cucurbitacin F, the active ingredient in *H. chinensis*, is not fully understood. Moreover, a genetic transformation system for *H. chinensis* has not been investigated. Consequently, the development of a rapid and stable method of *Agrobacterium*-mediated genome editing and transformation will provide a powerful platform for studying the molecular mechanisms of secondary metabolism in traditional medicinal plant with significant medicinal value.

In this study, the best explants were selected for callus induction and regeneration by evaluating explants, and the HcOSC6 gene was targeted to establish an efficient *Agrobacterium*-mediated genetic transformation system for *H. chinensis*. Furthermore, we generated Hcosc6-edited plants using the CRISPR/Cas9 system. This study has made a breakthrough in the genetic transformation and genome editing of *H. chinensis*, which provides a deeper understanding of the gene function of *H. chinensis* in the future.

Materials and methods

Plant materials, strain, and plasmids

H. chinensis were cultivated in Yunnan Agricultural University in Kunming, Yunnan Province, China (E 102.95618, W 25.17884). In this study, *Agrobacterium tumefaciens* strain GV3101, plant expression vector pCambia1300-GFP and pYLcrispr/Cas9 vector were used. The sterile seedlings of *H. chinensis* were inoculated on MS medium (pH 5.8) supplemented with 30 g/L sucrose, 4 g/L agar, 0.5 mg/L indole-3-butyric acid (IBA), 2 mg/L 6-BA and 1 g/L activated carbon.

Callus induction

Different explants (roots, stem segments and leaves) of sterile seedlings from *H. chinensis* were selected for

further study. These explants were cut into 0.5–1 cm and placed on MS medium (pH 5.8) supplemented with 30 g/L sucrose, 7 g/L agar, 1.5 mg/L 6-BA, 0.02 mg/L NAA for callus induction. Each of the above explants was replicated 10 times, with 11 explants per replicate. To evaluate the cellular process of callus induction, the callus formation was analyzed at different culture periods (7 days and 14 days).

Scanning electron microscopy analysis

Conventional scanning electron microscopy (SEM) was used to examine the structural characteristics of the cell walls, following the established methods detailed by Sun et al. [29]. The stems were sectioned into 3–4 mm diameter transverse segments and immersed in a formaldehyde-acetic acid-ethanol (FAA) solution for a duration of 48 h to fix the cellular structures. Subsequently, the segments underwent a dehydration process using a series of ethanol solutions with increasing concentrations, ranging from 10 to 90% in 20% increments, each for a period of 30 min. This was followed by two further rounds of treatment with 100% ethanol for an additional 30 min to ensure complete dehydration. Once the samples were adequately dried, they were mounted on aluminum SEM stubs using conductive adhesive. To enhance the quality of the SEM images, a thin layer of gold was applied via the E-1010 ion sputtering instrument. Finally, the samples were examined and photographed using the Hitachi FlexSEM-1000 scanning electron microscope, providing high-resolution images of the cell wall structures.

Vector construction

The full coding sequence of the *HcOSC6* gene (2313 bp) from *H. chinensis* was cloned. The pCAMBIA-1300-*HcOSC6*-GFP (OE-*HcOSC6*) construct was generated by subcloning the fragment between the *Xba*I and *Kpn*I sites of pCAMBIA1300-GFP vector using primers listed in Supplementary Table 1. The CRISPR/Cas9 system [30] was used to verify the regulatory module of *HcOSC6*. The website CRISPR-GE (<http://skl.scau.edu.cn/>) [31] was used to design two sgRNA sequences that targeted *HcOSC6*. *A. thaliana* promoters AtU3d and AtU3b were used to ligate two target sites (Target 1: TTATCA GCCTCTACGTTACTGG; Target 2: TTCCATATCACG AAATTGACTGG) to the two sgRNA intermediate vectors using overlapping PCR. To complete the constructs, two sgRNA expression cassettes were cloned into pYL-CRISPR/Cas9p35S-N vectors using Golden Gate ligation [30, 32]. These vectors were transferred into *H. chinensis*, and genomic DNA was isolated from transgenic *H. chinensis* plants. Amplification of the primers that contained the two target sites was performed using PCR and then sequenced using the Sanger method. After analyzing the data with SnapGene, experiments were conducted to

identify base insertions or deletions near the target sites. The primers required to construct knockdown vectors are listed in Supplementary Table S2.

Stable transformation of *H. chinensis*

The recombinant construct, pCAMBIA-1300-*HcOSC6*-GFP and CRISPR-Cas9-*Hcosc6*, were introduced into the *A. tumefaciens* strain GV3101 by electroporation for *H. chinensis* stable transformation. The transgenic plants of *H. chinensis* were obtained as follows. *H. chinensis* calli were transferred into a resuspended *Agrobacterium* solution and soaked for 8 min. Sterile filter paper was then used to filter the mixture of bacteria and calli, and the calli were placed on MS solid medium for 2 days in the dark. After cultivation, the cells were transferred to MS solid medium (containing 100 mg/L kanamycin) for shoot induction. Antibiotic-resistant shoots were selected and collected from calli of stem segments and transferred to medium for root generation after 2 weeks. Seedlings grew on rooting medium containing 100 mg/L kanamycin, cultured for 6 weeks, and positive seedlings were detected and identified using PCR analysis.

RNA extraction and DNA extraction

Leaves from *H. chinensis* were ground using liquid nitrogen. The TRIzol reagent was used to extract total RNA. The TransScript II One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen) and Oligo (dT)18 primer were used to reverse-transcribe the first-strand cDNA in a 20 µL volume system.

RT-qPCR analysis

First-strand cDNA is synthesized using 1 µg of total RNA in a 20 µL reaction volume containing reverse transcriptase (TaKaRa). Make a 20 µL RT-qPCR reaction mixture using 1 µL cDNA, 8.2 µL distilled water, 10 µL SYBP qRFP Master Mix, and gene-specific primers. PCR was performed using the Step One Plus Real-Time PCR System (Applied Biosystems), which included one cycle (95 °C, 30 s), 40 cycles (95 °C, 10 s, 58 °C, 20 s), one cycle (95 °C, 30 s), one cycle (60 °C, 1 min) and one cycle (95 °C, 15 s). A total of three biological replicates were performed. The relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method. The primers used for RT-qPCR are included in Supplementary Table S2.

Determination of cucurbitadienol contents

The fresh *H. chinensis* sample should weigh approximately 0.1 g. To initiate the extraction, accurately weigh the sample and deposit it into a 2 mL centrifuge tube. Proceed by adding 1 mL ethyl acetate to the sample, then apply ultrasonication at 180 W, 40 kHz for 1 h. Once the sonication is complete, allow the mixture to cool at room

temperature. Then, all extracted liquid solutions were combined to obtain the concentrated extract.

RNA-seq and DEGs analysis

RNA was extracted from healthy leaves of wild-type, OE-*HcOSC6* and CRISPR-Cas9-*Hcosc6* plants in Yunnan Agricultural University using TRIzol reagent. The quality and concentration of RNA were determined using the NanoDrop 2000 (Thermo Fisher Scientific). Using Oligo dT-coated magnetic beads, nine samples were selected to enrich mRNAs with polyA tails. The fragments were reverse transcribed to synthesize cDNA. The library was constructed and sequenced using the Illumina Novaseq X Plus platform at Guangzhou Genedenovo Biotechnology Co., Ltd (Guangzhou, China). The expression level of the transcript was determined by the FPKM value (fragments per kilobase of exon per million mapped fragments). $P < 0.05$ and $|\log_2\text{FoldChange}| > 2$ were identified as differentially expressed genes (DEGs), RT-qPCR were performed to ensure the accuracy of RNA-seq data. The TBtools software package was used to perform heat map analysis on the gene expression in all comparison groups [33]. A hypergeometric distribution test was used to determine gene ontology (GO) functions and KEGG enrichment pathways, the Omicsmart online platform (<https://www.omicsmart.com>) was used to extract pertinent information. The sequenced raw reads generated in this study have been submitted to NGDC SRA database with BioProject ID: PRJCA027392 (<https://ngdc.cnbc.ac.cn/gsub/submit/bioproject/PRJCA027392>).

Statistical analysis

Statistical analysis was performed by one-way ANOVA analysis of variance using GraphPad Prism 10.3 software. Data were shown as mean $\pm$ standard deviation (SD) of three independent biological replicates.

Results

Developmental changes in the morphology of calli from *H. chinensis* explants

During three culture stages (0 day, 7 days, and 14 days), roots, stem segments, and leaves from *H. chinensis* were cultured simultaneously, and their phenotypic changes were recorded. Following a 7-day cultivation period, the roots displayed no significant alterations, with an absence of primary callus formation at the wound sites. Concurrently, the leaves initiated a transition to yellowing and exhibited curling, with no callus development around the wounds (Fig. 1).

After 14 days of culture, the roots remained unchanged; however, the leaves turned dark yellow and curled, with no primary callus forming around the wounds of the explants. However, induction rates were 95% for the entire stem segments, which was notably white and

extremely loose (Fig. 1, Supplementary Table S1). The cross-sectional profiles of stem explants during the initial phase of callus induction were examined using SEM to better understand the cellular mechanisms associated with callus induction. Callus cells began to form at the wound sites of the stem segments after 7 days of culture, resulting in tissue loosening. The tissue structure became even loosened at 14 days, with callus tissue uniformly enveloping the entire stem segments. Callus induction is most efficient in stem segments close to the incision edge, indicating that stem segments possess the most robust capability for inducing callus (Supplementary Figure S1). The callus derived from 14-days-old stem segments differentiated from is ready for subsequent transformations.

Establishment of *H. chinensis* genetic transformation system

To establish a genetic transformation system for *H. chinensis*, we have selected stem segments to initiate callus formation (Fig. 2A). Fresh stem segments of *H. chinensis* were harvested and placed on a callus induction medium and allowed to develop until they reached an optimal transformation state (Fig. 2B). 14-days-old stem segment callus were immersed in a suspension containing *Agrobacterium* strain GV3101/pCAMBIA1300-OE-*HcOSC6* for 8 min. Sterilized filter paper was used to dry the callus after rinsing with sterile water. These explants were then transferred to MS medium containing kanamycin after a two-day interval. From the kanamycin-resistant callus, adventitious buds began to appear two weeks after inoculation (Fig. 2C). The transformed cells carrying the GV3101/pCAMBIA1300-OE-*HcOSC6* construct displayed normal morphology and rapid growth. Transformants were then placed in rooting medium (RM) for rooting. After four weeks of cultivation on RM medium, the plants showed robust growth. The plants eventually became fully mature and complete plants (Fig. 2D-F).

Molecular analysis of transformed *H. chinensis* plants

The first step in identifying positive seedlings from transgenic materials was to conduct a phenotypic analysis. Compared to wild-type (WT) plants, the transgenic plants overexpressing OE-*HcOSC6* did not show any significant phenotypic changes (Fig. 3A). Total genomic RNA was extracted from the fresh leaves of WT and transgenic *H. chinensis* plants to validate the expression of the OE-*HcOSC6* gene. The target gene expression levels were assessed by RT-qPCR. Transgenic plants expressed significantly higher levels of *HcOSC6* than WT plants. Among the three transgenic lines, the OE1 line had the highest level of *HcOSC6* expression, surpassing both OE2 and OE3 (Fig. 3B). Therefore, the OE1 line was selected for further transcriptome sequencing. As a key step in producing cucurbitacin, *HcOSC6* is known to catalyze

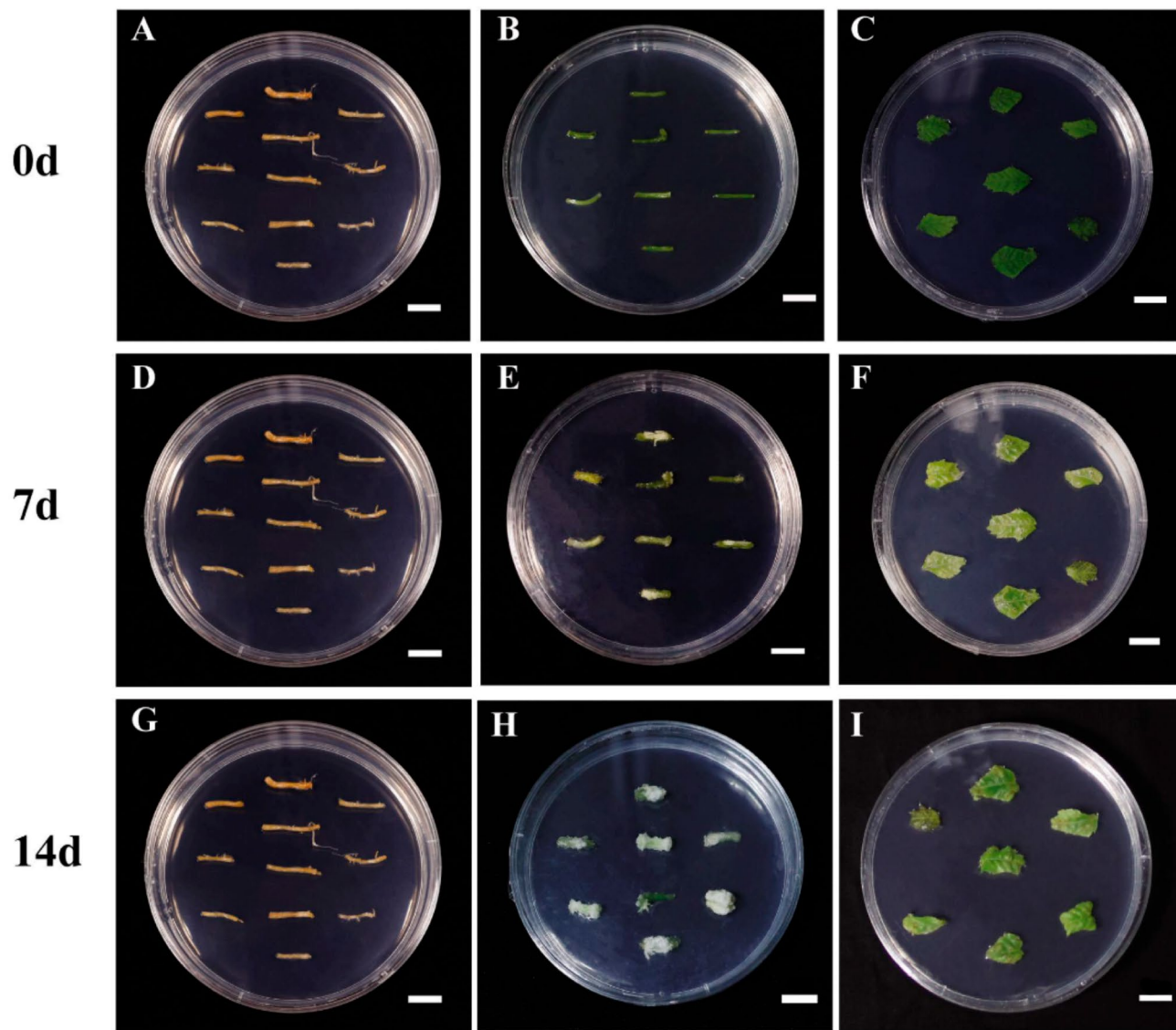


Fig. 1 Morphology of different explants during callus induction. (A) 0 day roots. (B) 0 day stems. (C) 0 day leaves. (D) 7 days roots. (E) 7 days stems. (F) 7 days leaves. (G) 14 days roots. (H) 14 days stems. (I) 14 days leaves. Bar = 1 cm

2,3-oxidosqualene to form cucurbitadienol. To investigate how *HcOSC6* overexpression impacts cucurbitacin production, HPLC was used to determine cucurbitadienol levels in both WT and OE-*HcOSC6* overexpressing plants. Cucurbitadienol content was significantly higher in OE-*HcOSC6* overexpressing plants than in WT plants, with a 2.12-fold increase (Fig. 3C). The collective results of these studies indicate that a genetic transformation system has been successfully established in *H. chinensis*, which helps to further research on the role of *HcOSC6* in cucurbitacin biosynthesis and its potential applications for improving medicinal value.

Applying the CRISPR-Cas9 system to establish an efficient genetic transformation system

CRISPR-Cas9 is a powerful molecular tool that has been used to edit plant genomes in a wide variety of species, utilizing *Agrobacterium*-mediated transformation as a reliable delivery mechanism, as recently demonstrated in several studies [34–37]. *H. chinensis*, a medicinal plant predominantly found in the southwest of China, is known for its tubers, which contain cucurbitacin, an important ingredient in the treatment of digestive and respiratory inflammatory conditions [38]. There has been no efficient and stable genome editing approach for *H. chinensis*, despite its medicinal significance. For exploring the application of the CRISPR-Cas9 system for precise and efficient editing in *H. chinensis*, we targeted the *HcOSC6*

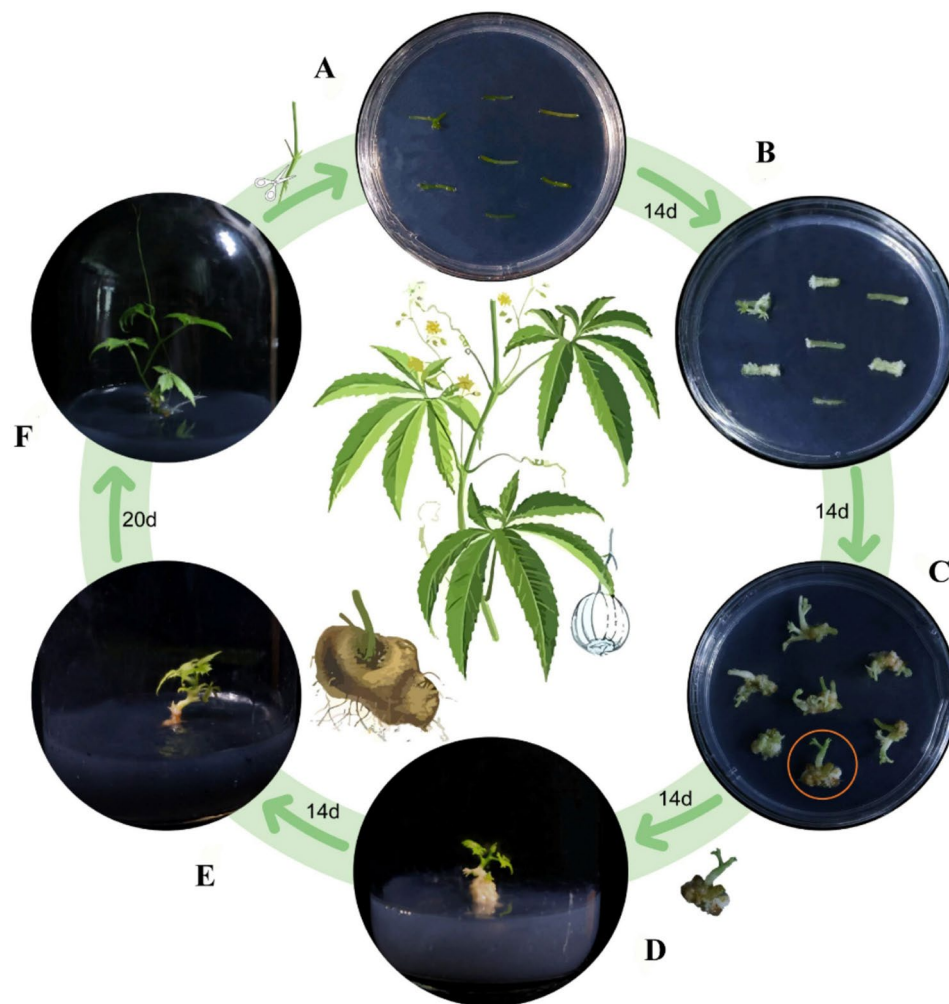


Fig. 2 Process of genetic transformation in *H. chinensis*. (A) 0 day *H. chinensis* callus. (B) 14 days callus induction: White, loose callus was observed. (C) Bud differentiation occurred 2 weeks after transformation. (D) *H. chinensis* seedlings 4 weeks after transformation. (E) *H. chinensis* seedlings 6 weeks after transformation. (F) The transformed explant eventually develops into a complete *H. chinensis* plant

gene, which is implicated in cucurbitacinol biosynthesis regulation. We successfully infected and transformed callus tissue derived from stem segments of *H. chinensis* using an *Agrobacterium*-mediated genetic transformation protocol, resulting in the development of complete plants (Fig. 4A). Compared to WT plants, CRISPR-Cas9-*Hcosc6* plants had a different phenotype. Eight regenerated plants were produced via genetic transformation. The leaves of the three knockdown plants exhibited a curling phenotype and grew at a faster rate than the WT. The mutant plants differentiated a greater number of buds than the WT, which displayed a single bud differentiation. In contrast, the mutant plants (ko1, ko3 and ko6) differentiated two or three buds. Additionally, the mutant plants exhibited greater height than the WT. The height of the ko1, ko3, and ko6 plants was 2.13, 2.82 and 2.11 cm, respectively, in comparison to 1.81 cm for the WT (Fig. 4A). After the plants reached 4–6 cm in height,

two leaves from each were harvested for DNA extraction, and transgenic-positive plants were identified. We found three plants that had been genetically modified, with their target site fragment amplified and their base mutation characterized. The *HcOSC6* gene target site showed two distinct mutation types (Fig. 4B). The expression levels of the target genes were assessed using RT-qPCR. CRISPR-Cas9-*Hcosc6* plants showed significantly lower expression levels than WT (Fig. 6C). The cucurbitadienol content of WT and CRISPR-Cas9-*Hcosc6* plants was measured using HPLC. A 1.41-fold decrease in cucurbitacinol content was observed in CRISPR-Cas9-*Hcosc6* transgenic plants as compared to WT plants. Further analysis of transcriptome data revealed significant increases in the expression of the *HcOSC6* gene following overexpression. Conversely, *HcOSC6* expression was substantially decreased in CRISPR-Cas9-*Hcosc6* transgenic plants (Fig. 4C, Supplementary Figure S2). CRISPR/

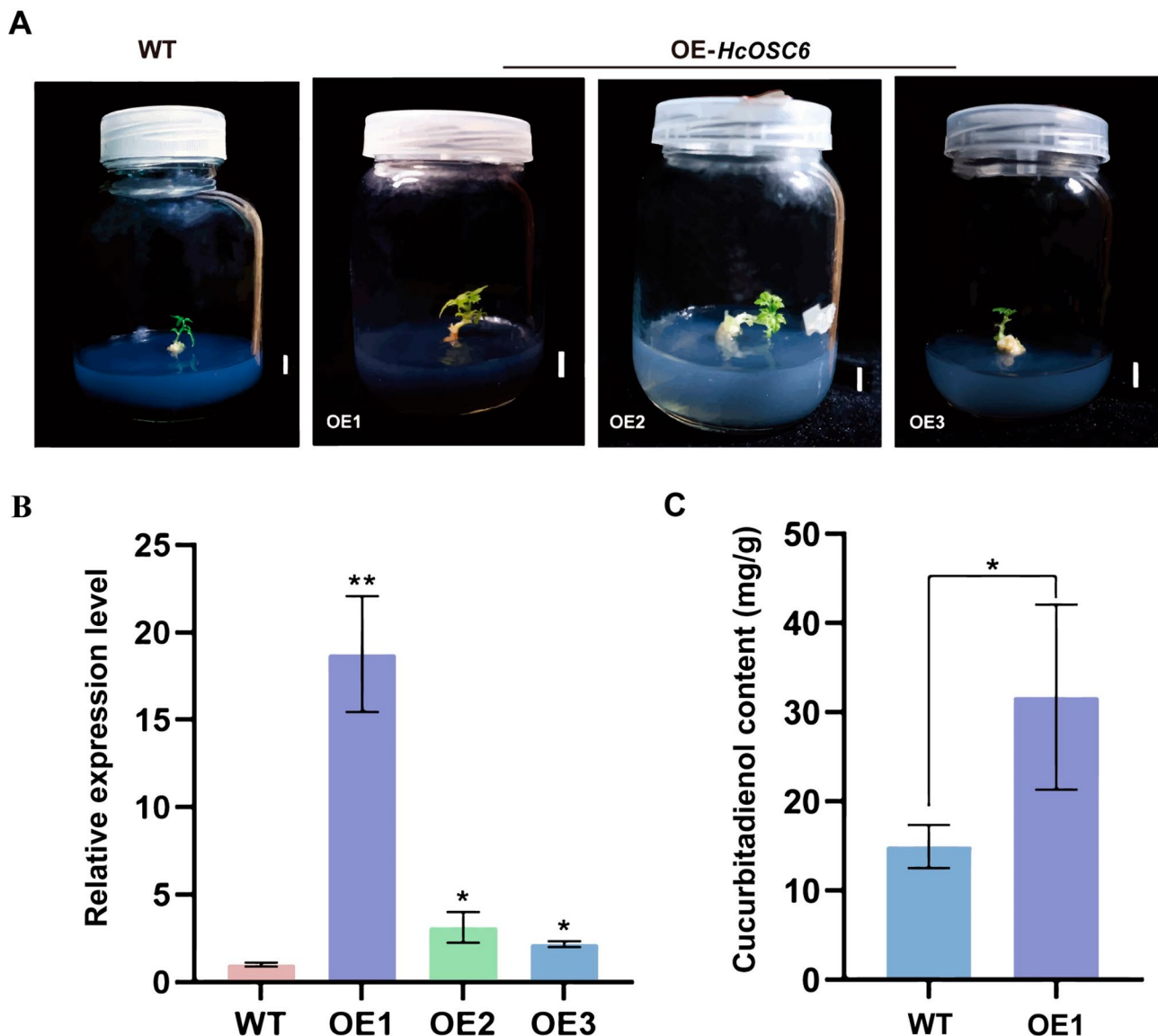


Fig. 3 Characterization of transgenic *H. chinensis* lines overexpressing *HcOSC6*. **(A)** Phenotype of transgenic *H. chinensis* lines and WT plants. WT: a wild-type (WT) *H. chinensis* plant; OE1, OE2, OE3: Three independent transgenic *H. chinensis* plants overexpression *HcOSC6*. **(B)** RT-qPCR assay of WT and transgenic lines OE1, OE2 and OE3. **(C)** Analysis of cucurbitadienol content in transgenic *H. chinensis* and WT plants. Data represent mean $\pm$ SD of three biological replicates. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$). Bar = 1 cm

Cas9-mediated gene editing has shown efficiency in *H. chinensis*, marking a significant advance in genetic manipulation of this medicinally useful species.

HcOSC6 is responsible for regulating the expression levels of genes related to cucurbitacins biosynthesis

Our aim was to determine whether *HcOSC6* influences expression patterns of downstream genes across the whole genome using RNA-seq. Transgenic lines with either overexpression of *HcOSC6* or knockdown of *HcOSC6* via CRISPR/Cas9 were compared with WT lines. Significant shifts in gene expression dynamics were observed in the comparative analysis. In the transgenic

line with *HcOSC6* overexpression, we identified 4,952 differentially expressed genes (DEGs), of which 2,480 were up-regulated and 2,472 were down-regulated. As a result of CRISPR/Cas9-*Hcosc6* knockdown, 4,463 DEGs were observed, including 2,515 up-regulated and 1,948 down-regulated genes. These findings demonstrated that both overexpression and knockdown of *HcOSC6* have profound effects on gene expression profiles (Fig. 5A, Supplementary Table S3, S4, S5).

KEGG analysis of these DEGs showed significant enrichment in pathways related to plant secondary metabolites compared to genome editing lines. Furthermore, in the comparison between the WT and the

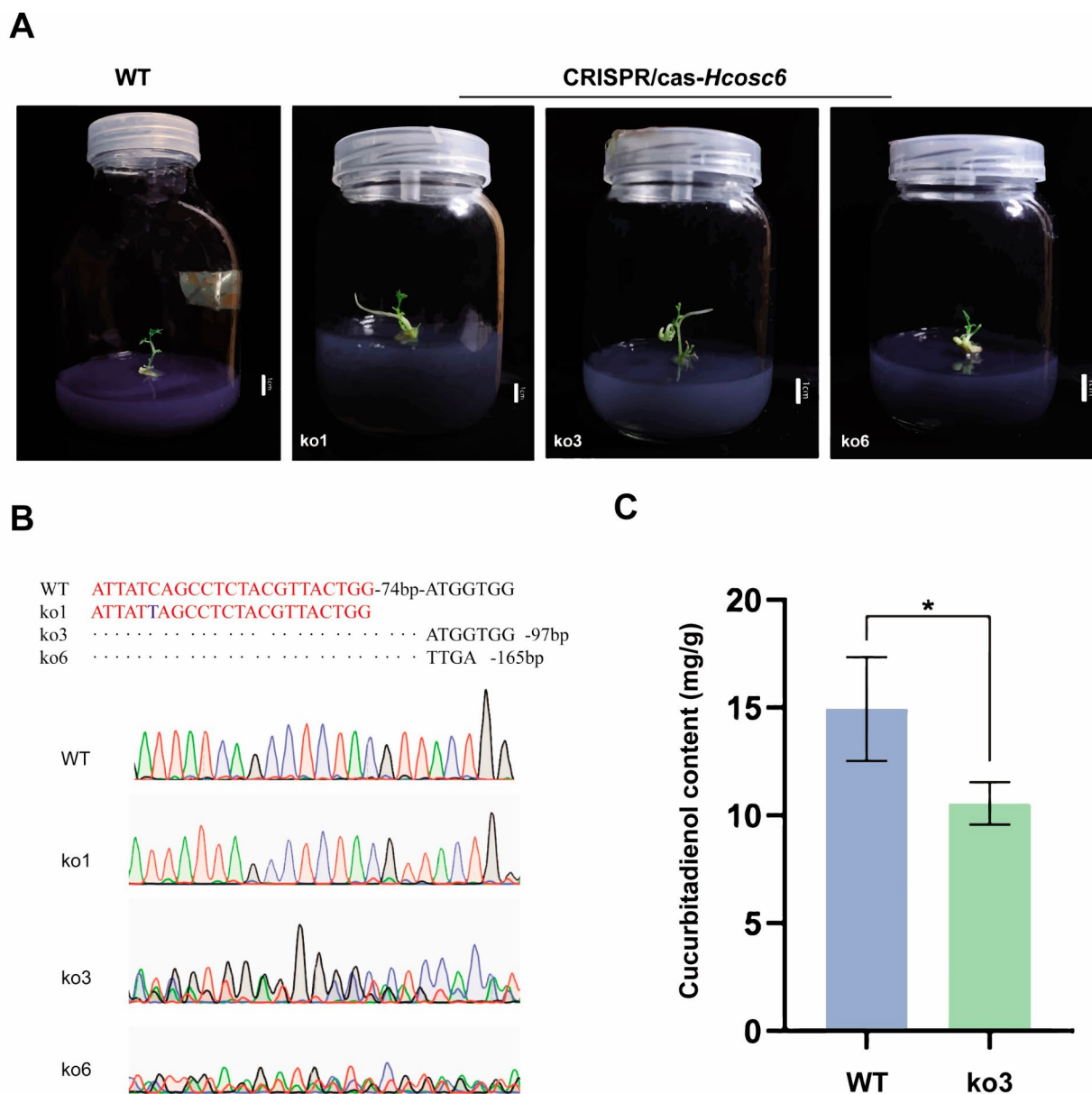


Fig. 4 Mutagenesis of the *HcOSC6* gene using the CRISPR-Cas9 system. **(A)** Phenotype of transgenic *H. chinensis* lines and WT plants. WT: a wild-type (WT) *H. chinensis* plant; ko1, ko3, ko6: Three independent genome-edited plants. **(B)** DNA sequencing peak of the *HcOSC6* gene showing mutations at the target1 position. The normal sequence is depicted at the top, with sequence alterations indicated for each panel (Purple font, replace; -, deletion). **(C)** Analysis of cucurbitadienol content in transgenic and WT *H. chinensis* plants

OE-*HcOSC6* line, DEGs were predominantly enriched in pathways associated with secondary metabolite biosynthesis, mitogen-activated protein kinase (MAPK) signaling pathways, metabolic pathways, and pathogen interactions (Fig. 5B). These DEGs were further characterized by Gene Ontology (GO) analysis. Compared with WT, the DEGs of ko3 line were mainly associated with catalytic activity, oxidoreductase activity, plastid DNA binding transcription factor activity, and nuclease

activity. The DEGs were primarily involved in oxidoreductase activity, transmembrane transport protein activity, transcriptional regulatory activity, and DNA-binding transcription factor activity when comparing the WT with the OE-*HcOSC6* line (Supplementary Figure S3). Many of the up- and down-regulated genes are predicted to be involved in cucurbitacin F biosynthesis downstream of *HcOSC6*, including *HcCYP87D20*, *HcCYP87D19*, and *HcSDR34* (Fig. 6). To validate the RNA-seq data,

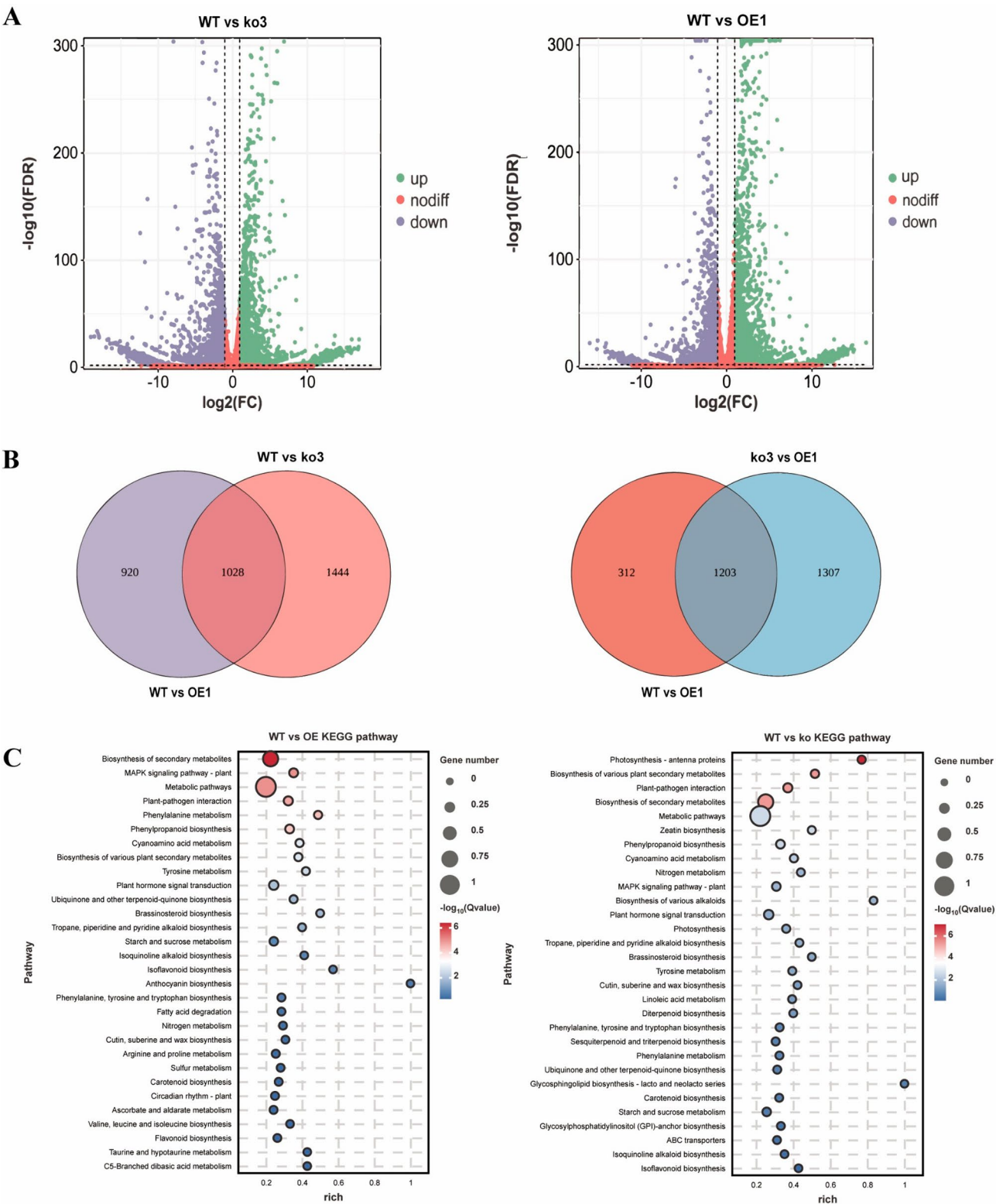
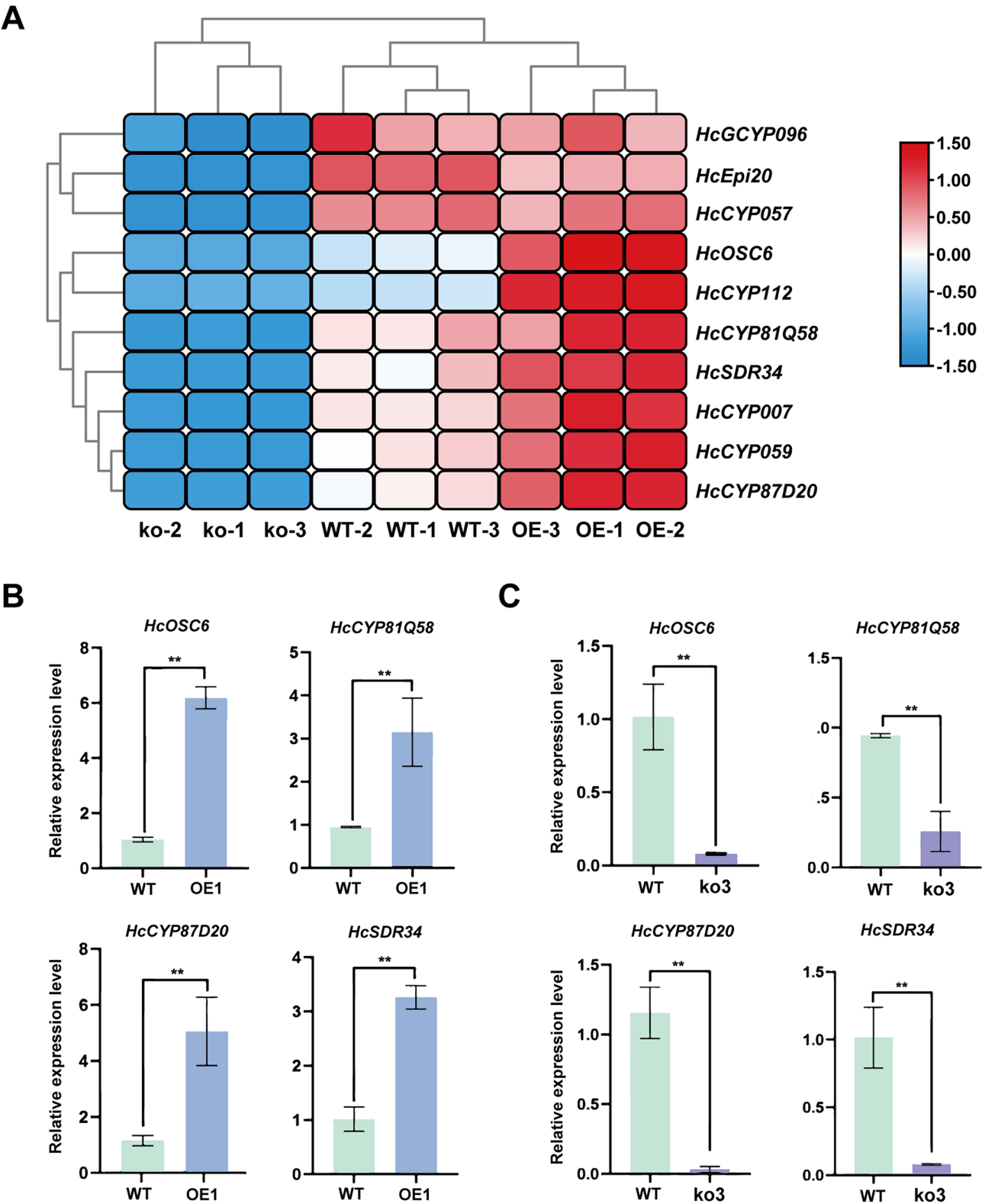


Fig. 5 Gene expression levels and enrichment profiles of differentially expressed genes (DEGs). **(A)** Volcano plots showing the number of DEGs among various regulatory models. **(B)** Venn diagrams illustrating the relationship between select genes downstream of *HcOSC6* involved in regulating the biosynthesis of cucurbitacin F. **(C)** Enrichment map depicting KEGG pathways under different regulatory models



RT-qPCR was used to determine the gene expression levels in the OE-*HcOSC6*, CRISPR-Cas9-*HcOSC6*, and WT lines. Transgenic OE-*HcOSC6* plants exhibited significantly higher transcription levels than WT plants, whereas CRISPR/Cas9-*HcOSC6* plants exhibited significantly reduced transcription levels (Fig. 6). Our successful development of an efficient genetic transformation system for *H. chinensis* has allowed us to understand the regulatory role of *HcOSC6* in gene expression and its downstream impact on the biosynthesis of cucurbitacin.

Discussion

Regeneration and genetic transformation of plants are crucial for plant biotechnology. These systems play a key role in elucidating the molecular mechanisms for plant growth and development, as well as accelerating the improvement and breeding of medicinal plants [39–42]. Previous study has showed that bioactive compounds from endangered species can be produced sustainably by callus cultures [43]. However, differentiation (organogenesis) has an important effect on the production of secondary metabolites in vitro [44], and regenerative capacity varies significantly between plant species and tissues, making development of transformation systems challenging [45]. We evaluated the regenerative efficiency of root, stem, and leaf explants of *H. chinensis* in this study. Roots and leaves showed the lowest regenerative potential, while stem segments had the highest. Furthermore, plant tissue culture offers significant advantages over traditional propagation methods by allowing aseptic, scalable, and efficient preservation of plant tissue with low environmental impact, lower maintenance costs, and pest-free plants [46]. Using stem segments as explants, we developed a genetic transformation system that achieved an efficient transformation. Furthermore, this highlights the potential of stem segments in genetic transformation and provides a novel strategy to overcome regenerative hurdles previously encountered in medicinal plants.

Hormones play a key role in establishing plant regeneration systems, coordinating cell proliferation, differentiation, and callus formation [47]. The successful application of these methods is essential to the regeneration of whole plants from transformed cells and to the success of plant tissue culture [48, 49]. This study examined the effects of hormone combinations on callus induction and seedling differentiation. To induce callus tissue, we combined 6-BA with NAA, which synergistically promoted cell division. Following the differentiation of buds into seedlings, we applied a mixture of IBA and 6-BA to facilitate the transition from undifferentiated callus to shoots. Hormone application is essential for the development of callus tissue, bud differentiation, and plant regeneration. The cytokinin 6-BA stimulates cell division and promotes

differentiation, whereas NAA, an auxin, induces callus formation [50, 51]. Thus, a robust regeneration system in medicinal plants requires precise regulation of hormone types and concentrations. Efficient plant regeneration can be achieved by optimizing hormonal balance, providing a reliable platform for genetic transformation and functional genomics in medicinal plants. Plant biotechnology can be advanced through fine-tuned hormonal manipulation. Previous studies successfully established an effective in vitro approach to enhance plant regeneration for *Fritillaria cirrhosa* [1], *Phyllostachys edulis* [52], Lily (*Lilium* spp.) [11].

A growing number of medicinal plants have been successfully edited with the CRISPR/Cas9 system, including *Salvia miltiorrhiza* [53] and *Macleaya cordata* [5]. This technology has been used to modulate the biosynthesis of key medicinal compounds, demonstrating its potential for precise genetic modification [54]. In our study, we used CRISPR/Cas9 to edit the specific *HcOSC6* gene, resulting in genetically modified plants. A total of seven positive seedlings were obtained, of which three exhibited deletions and substitutions, achieving an editing efficiency of 42%. Genome-edited plants exhibited a reduction in core compounds and a downregulation of downstream genes, providing valuable insight into molecular mechanisms involved in the production of medicinal compounds. Through targeted gene modification, complex metabolic pathways can be elucidated, and plant medicinal traits can be improved [4, 30, 55].

We created genetically edited plants using CRISPR/Cas9 technology, and transcriptomic analysis revealed that downstream structural genes showed upregulation in expression levels. By fine-tuning secondary metabolite production, new avenues for plant medicinal properties can be explored. On the other hand, our transcriptomic analysis of overexpression lines demonstrated that key regulatory genes were overexpressed to increase downstream structural gene expression. In the overexpressing plants, RT-qPCR validation confirmed the transcriptomic findings, demonstrating consistent increases in gene expression and secondary metabolite content. CRISPR/Cas9 was effective in downregulating specific biosynthetic pathways, as evidenced by the decreased levels of metabolites in the genetically edited plants. To advance future work in medicinal plant breeding and quality formation, both overexpression and knockdown systems are essential. Besides facilitating the development of new cultivars with enhanced medicinal properties, these systems also provide insights into the complex regulatory networks governing secondary metabolism. The use of these tools enables researchers to achieve consistent and high-quality production of bioactive compounds for pharmaceutical applications.

Conclusion

Here, we developed a rapidly and highly effective system for plant regeneration, transformation, and genome editing in *H. chinensis*. Additionally, we utilized this method for *HcOSC6* genome-editing, which is crucial for cucurbitacins biosynthesis in *H. chinensis*, achieving a high efficiency rate. These methods, characterized by their speed and efficiency, will facilitate fundamental research into gene function, the molecular underpinnings of secondary metabolic processes.

Abbreviations

6-BA	6-Benzylaminopurine
NAA	1-Naphthylacetic acid
IBA	Indole-3-butyric acid
SEM	Scanning electron microscopy
RM	Rooting medium
MS	Murashige and Skoog
WT	Wild-type
TC	Tissue culture

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12870-024-05717-x>.

Supplementary Material 1: Additional file 1: Table S1. Comparison of callus induction rate among different types of explants.

Supplementary Material 2: Additional file 1: Figure S1. Morphological changes in stem explants during the process of callus induction. Figure S2. HPLC analysis of cucurbitadienol contents in both transgenic *H. chinensis* and WT plants. Figure S3. Gene Ontology (GO) analysis of differentially expressed genes among different groups.

Supplementary Material 3: Additional file 1: Table S2. List of primers used in this study. Table S3. Differentially expressed genes between WT and OE-*HcOSC6* lines. Table S4. Differentially expressed genes between WT and CRISPR-Cas9-*HcOSC6* lines. Table S5. Differentially expressed genes between OE-*HcOSC6* and CRISPR-Cas9-*HcOSC6* lines.

Acknowledgements

We are grateful to Prof. Yaoguang Liu (South China Agricultural University) for providing the CRISPR/Cas9 plant expression vectors.

Author contributions

YZ conceived and designed the experiments. YZ, SY and GZ guided experiment. JW, CL, XL and CX performed the experiments and data analysis. JW, PZ and LL participate in mapping. JW, CL and YZ wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Natural Science Foundation of China (81960691), Yunnan Characteristic Plant Extraction Laboratory (2022YKZY001), Yunnan Province Youth Talent Support Program (XDYC-QNRC-2022-0219).

Data availability

All sequencing data generated are available at the NGDC SRA database under accession number PRJCA027392 (<https://ngdc.cnbc.ac.cn/bioproject/browse/PRJCA027392>).

Declarations

Ethics approval and consent to participate

All local, national or international guidelines and legislation were adhered to in the production of this study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 7 August 2024 / Accepted: 16 October 2024

Published online: 07 November 2024

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