A novel gene silencing strategy based on tobacco rattle virus in *Hibiscus mutabilis*

Shiye Sang, Yiqiong Liu, Xiu Li, Jiao Ma, Xiaoli Liu and Yuanzhao Yang

Chengdu Botanical Garden, Chengdu, Sichuan, China

ABSTRACT

Background: *Hibiscus mutabilis* L. is a popular regional characteristic plant in China, cultivated for its attractive flower colors, extended bloom time, and medicinal properties. To enhance molecular breeding and gene function studies, we conducted transcriptome analysis and identified valuable genes in previous research. Nonetheless, the current inefficient and labor-intensive transformation techniques have hindered their applications. Virus-induced gene silencing (VIGS) provides a precise and effective strategy for post-transcriptional down-regulation of endogenous gene expression.

Methods: We investigated the performance of tobacco rattle virus (TRV) as a tool for targeting and silencing the gene encoding the protein involved in chloroplast development, cloroplastos alterados 1 (altered chloroplast; *CLA1*), of *H. mutabilis* through *Agrobacterium tumefaciens*-mediated infiltration.

Results: By effectively suppressing the *CLA1* gene associated with chloroplast development in *H. mutabilis via* the TRV-VIGS system, we have illustrated the inaugural implementation of VIGS in this species. Quantitative RT-PCR proved that *HmCLA1* expression in agro-infiltrated plants was lower than in the mock-infiltrated (mock) and the control (CK) plants. Phenotypic observations corroborated the albino phenotype in leaves following successful *HmCLA1* silencing.

Conclusions: Our study showcases TRV-VIGS as a potential gene silencing tool for *H. mutabilis*, facilitating functional genomics studies and molecular breeding efforts in this species.

INTRODUCTION

Hibiscus mutabilis, also known as cotton rose, is a flowering shrub belonging to the genus *Hibiscus* in the family Malvaceae. It stands out as one of the most popular regional characteristic plants in China. *H. mutabilis* has been cultivated south of the Yangtze River for over 2,000 years and holds significant importance as the city flower of Chengdu. It is widely planted in public gardens and urban greenbelts due to its diverse flower colors, extended flowering duration, and floral development and morphogenesis (*Yang et al., 2022*). Besides, the leaves of cotton rose serve as an ingredient of traditional herbal remedies due to their blood cooling, detoxification, anti-inflammatory, and analgesic properties (*Liu et al., 2015a*, *2015b*). To facilitate research on molecular breeding and gene

function, we have identified numerous excellent genes through transcriptome analysis. However, the hard and inept plant transformation methods hamper the application of these findings to various species. It is extremely important that more suitable techniques be successfully employed to facilitate the investigation of plant gene roles (*Wang et al., 2019*).

Virus-induced gene silencing (VIGS) is an effective genetic method used to analyze the functions of a gene. It uses plant viruses engineered as tools or vectors to specifically decrease the endogenous expression of a gene *via* post-transcriptional gene silencing (PTGS) (*Caplan & Dinesh-Kumar, 2006*; *Purkayastha & Dasgupta, 2009*; *Becker & Lange, 2010*; *Ramegowda, Mysore & Senthil-Kumar, 2014*; *Krishnan et al., 2015*; *Ashfaq et al., 2020*). To begin with, VIGS was first employed in *Nicotiana benthamiana* to silence the gene encoding phytoene desaturase (PDS) (*Kumagai et al., 1995*). Subsequently, it has been adopted in several angiosperms, namely, *Arabidopsis thaliana* (*Burch-Smith et al., 2006*), cotton (*Qu et al., 2012*), tomato (*Orzaez et al., 2009*), wolfberry (*Qu et al., 2021*), and some woody plants (*Jiang et al., 2014*; *Liu et al., 2014*; *Wang et al., 2019*). The availability of numerous vector systems for VIGS in eudicot plants provides an improved possibility of constructing one for *H. mutabilis*. Double-stranded RNA (dsRNA) is a key factor in VIGS. Typically, the RNA interference (RNAi) pathway starts with the cleavage of dsRNA by DICER into 21- to 25-nucleotide-long short interfering RNAs (siRNAs) with two strands, the guide strand and the passenger strand (*Lu et al., 2003*; *Burch-Smith et al., 2004*; *Jiang et al., 2014*). The RNA-induced silencing complex (RISC) further identifies these siRNAs and causes the degradation of the passenger strand while using the guide strand to identify, incorporate, and degrade complementary single-stranded RNA in the cell (*Mustafa et al., 2016*). Consequently, the gene gets silenced, leading to the generation of substantial quantities of siRNAs (*Fuchs, Damm-Welk & Borkhardt, 2004*).

Tobacco rattle virus (TRV), belonging to the *Tobravirus* genus (Virgaviridae family), is a vector system suitable for VIGS (*Jiang et al., 2014*). Researchers have developed and applied TRV-mediated VIGS using Agrobacterium-based transformation in cotton (*Gao et al., 2011*; *Mustafa et al., 2016*; *Ge et al., 2016*; *Shi et al., 2021*). The TRV genome consists of RNA1 and RNA2, two positive-sense single-stranded RNAs. Among these, RNA1 (TRV1) encodes proteins associated with movement and replication, while RNA2 (TRV2) encodes the coat protein and a few nonessential structural proteins. And these nonessential structural proteins can be deleted to insert foreign sequences (*Hayward, Padmanabhan & Dinesh-Kumar, 2011*; *Senthil-Kumar & Mysore, 2014*; *Mustafa et al., 2016*). Besides, *CLA1* is a highly conserved gene involved in chloroplast development across various plants, such as *Arabidopsis* (*Mandel et al., 1996*), and its mutant albino phenotype makes it a valuable marker for assessing silencing efficiency (*Cheng et al., 2023*).

Therefore, the present study employed the TRV-based system to silence the *CLA1* gene in *H. mutabilis*. We developed the TRV vector system for gene targeting and infiltrated leaves of *H. mutabilis*, using TRV alone as the mock. Furthermore, we analyzed the phenotype and *HmCLA1* expression in the leaves of the treated, mock, and control plants. The study's findings will demonstrate the efficacy of the TRV system in silencing *H. mutabilis* genes *via* the VIGS approach. Importantly, this study represents the first

successful use of VIGS in *H. mutabilis*, filling a crucial gap in gene silencing techniques and advancing the screening of functional genes and molecular breeding in *H. mutabilis*.

MATERIALS AND METHODS

Plant material and growth conditions

The *H. mutabilis* cultivar 'single-petal white' was used. Seeds were collected from the Chengdu Botanical Garden. The seeds were subjected to a 15-min treatment with concentrated sulfuric acid and then washed thoroughly with sterile water. These seeds were then sown in pots filled with a peat and garden soil (v:v; 1:1) and maintained in an incubator at 25 °C controlled temperature, lux light, and a photoperiod of 14 h light/10 h dark. This study was performed in nursery of Chengdu Botanical Garden from June 2023 to May 2024.

Analysis of *HmCLA1* sequence and construction of phylogenetic tree

Utilizing the *HmCLA1* nucleotide sequence (GenBase accession no. C_AA041596.1) as a foundation, the inferred protein sequence was compared with the *CLA1s* from diverse species using the ClustalX2 software (*Larkin et al., 2007*). Furthermore, the amino acid sequences obtained from NCBI (https://www.ncbi.nlm.nih.gov/) were utilized to build a phylogenetic tree in MEGA 11. The Neighbor-Joining (NJ) method was used with 1,000 standard bootstrap replicates (*Koichiro, Glen & Sudhir, 2021*).

Construction of the VIGS vector

Total RNA was isolated from the *H. mutabilis* 'single-petal white' leaves using RNA-prep Pure (TIANGEN, Beijing, China) and reverse transcribed to cDNA using the HiScript II 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme, Nanjing, China). Further, PCR was performed using this cDNA with the *HmCLA1*-F and *HmCLA1*-R primers (Table 1) to amplify a partial fragment of *HmCLA1*. These primers were designed by the DNAman 9.0 software for the conserved domain, with the *EcoR* I and *BamHI* sites incorporated into the upstream and downstream primers, respectively. PCR was conducted under the following thermal cycling conditions: 32 amplification cycles, including denaturation for 15 s at 95 °C, annealing for 15 s at 60 °C, and extension for 1 min at 72 °C. The reaction mixture, with a final volume of 50 μL, consisted of 2 × Phanta Max Master Mix (25 μL), the dNTP mix (1 μL; 10 mM each), primer (2 μL; 10 μM), cDNA (4 μL), and Phanta Max Super-Fidelity DNA Polymerase (1 μL; 1 U/μL). The pTRV1 and pTRV2 vectors were employed for vector construction (*Ratcliff, Martin-Hernandez & Baulcombe, 2001*). The amplicons were ligated into the *EcoR* I and *BamHI* double-digested pTRV2 vector with the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China) to obtain the pTRV2-*HmCLA1* construct.

Agroinfiltration

In the context of *H. mutabilis*, the pTRV2-*HmCLA1* vector was initially introduced into *Agrobacterium tumefaciens* 'GV3101' cells by the freeze-thaw method (*Höfgen & Willmitzer, 1988*). Subsequently, a single colony confirmed by PCR was inoculated into

Table 1 Primer used in TRV-VIGS system of *H. mutabilis*. Lowercase letters representing the flanking sequences.

Primer name	Primer sequence
TRV-HmCLA1-F	gctcggtaccggatccCACAACATCGATGATTTAG
TRV-HmCLA1-R	ttaaggttaccgaattcATGATGAGTAGATTGCACA
qRT-HmCLA1-F	ATCGGCAAAGGAAGGGTACT
qRT-HmCLA1-R	CGTGGGATTCCAATAACGAG
qRT-HmActin-F	GATGAAGATTCTTACTGAGAG
qRT-HmActin-R	CAGAAGAGCTGCTCTTGGCAG

3 mL of Yeast Extract Peptone Broth medium containing rifampicin (25 mg/L), kanamycin (50 mg/L), and gentamicin (50 mg/L) and allowed to multiply in a shaker at 200 rpm and 28 °C for 12 h. Additionally, *A. tumefaciens* cells carrying the empty vector (pTRV1 or pTRV2) were cultured in 3 mL of the same media under identical conditions. These starter cultures were subcultured into 50 mL of fresh media and maintained at 28 °C for almost 16 h. For the ensuing assay, the Agrobacterium cultures were centrifuged at $6,000 \times g$ for 10 min. The obtained pellets were then dissolved in a buffer (150 μM acetosyringone (AS), 10 mM MES, 10 mM $MgCl_2$ (pH = 5.6)) to an optical density (OD_{600}) of 2.0 and left undisturbed for 3 h at room temperature. Finally, the pTRV2-*HmCLA1* and pTRV1 Agrobacterium cultures were mixed at a 1:1 ratio and infiltrated into the fully opened *H. mutabilis* cotyledons with a 1 mL needleless syringe. A mixture of Agrobacterium cells carrying pTRV1 and pTRV2 constructs in a 1:1 ratio was infiltrated into the cotyledons and used as mock plants. This procedure followed a previously established protocol (*Gao et al., 2011*).

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was carried out with the qRT-*HmCLA1*-F/qRT-*HmCLA1*-R primers (Table 1) to quantify the *HmCLA1* transcripts (relative abundance) in the gene-silenced leaves with visible phenotypes, with the control group and mock group as references. *HmActin* and *HmAFD2* were used as the reference gene for the assay. Three biological replicates were analyzed per group. Each biological replicate was subjected to three technical replicates. Total RNA extraction and reverse transcription refer to the method description provided earlier. The cDNA was then used to assess the transcript abundance performing qPCR with the AceQ qPCR SYBR Green Master Mix (Jizhenshengwu, Shanghai, China) on a CFX96 System (Bio-Rad, Hercules, CA, USA). PCR was conducted under the following thermal cycling conditions: one cycle of pre-denaturation for 5 min at 95 °C, 44 cycles of denaturation for 10 s at 95 °C, and annealing and extension for 30 s at 60 °C. The reaction mixture, with a final volume of 10 μL, consisted of 5 μl of 2xSYBR Green Mix, 0.5 μL of each Primer F+R (each 10 uM), and 2 μL of cDNA. Finally, the relative expression level of *HmCLA1* was determined following the $2^{-\Delta\Delta Ct}$ method (*Livak & Schmittgen, 2001*; *Gu et al., 2018*). We use standard deviation

to measure the repeatability within groups, and we use a T-test to assess whether there is a significant difference between groups.

Statistical analysis

One-way analysis of variance (ANOVA) and Duncan's multiple range test ($P < 0.05$) were performed using R (Version 4.3.2).

RESULTS

Sequence analysis of *HmCLA1* in *H. mutabilis*

Our research shows that the full-length *HmCLA1* CDS is 2,166 bp, with the longest ORF encoding 721 amino acids. The predicted gene products of *HmCLA1* were 77.23 KDa proteins with pI of 6.8 (https://web.expasy.org/protparam/). The forward primer used for vector construction spans bases 997 to 1,015, while the reverse primer spans bases 1,417 to 1,399. For qPCR detection of *HmCLA1* expression, the forward primer spans bases 1,723 to 1,742, and the reverse primer spans bases 1,828 to 1,809 (Fig. S1). Additionally, we also performed CDS sequence alignments of *CAL1* among the five species (Fig. S3).

By performing multiple sequence alignments of *CAL1* proteins, we identified a significant similarity between *HmCLA1* and *CLA1* from other plant species (Fig. 1 and Table S1). *HmCLA1* and *HhCLA1* formed a clade on the phylogenetic tree. This observation indicated that *HmCLA1* shared a high level of similarity with *HhCLA1* found in *H. hamabo*, followed by *GhCLA1* and *GbCLA1* from *G. hirsutum* and *G. barbadense* in the Malvaceae family (Fig. 2).

HmCLA1 silencing using the VIGS approach

In this study, we inoculated seventy-one plants with *A. tumefaciens* 'GV3101' harboring pTRV2-*HmCLA1*, 9–12 days after the emergence of the two cotyledons. These plants showed no symptoms of infection in the initial 2 weeks. However, 18 days post-infection, white streaks appeared on the emerging leaves of the pTRV2-*HmCLA1* agro-infiltrated plants. Ultimately, 74.65% of these plants displayed white-streak leaf symptoms resembling the photobleached phenotype (Table 2 and Fig. 3B). In contrast, no white streaks were found on the leaves of the mock and CK plants (Figs. 3A and 3C). Individual leaves of all infiltrated plants are shown in Fig. 3D. The observed phenotypic characteristics of the leaves suggested that plants infiltrated with pTRV2-*HmCLA1* might experience suppression of *HmCLA1* gene expression compared with mock and CK plants.

Subsequently, qPCR was employed to assess the levels of *CLA1* mRNA in the infiltrated plants exhibiting white-streaked leaf symptoms. The expression of the *CLA1* gene displayed comparable levels between the CK and Mock plants. Conversely, the expression levels of the *CLA1* gene were notably reduced by 81.46–92.12% in plants agroinfiltrated with pTRV2-*HmCLA1* compared to CK plants (Figs. 4, S2 and Table S2). This expression pattern of *HmCLA1* consistently matched the phenotypic features, indicating a notable reduction in *HmCLA1* expression *via* TRV-VIGS in *H. mutabilis*, ultimately resulting in an albino leaf phenotype.
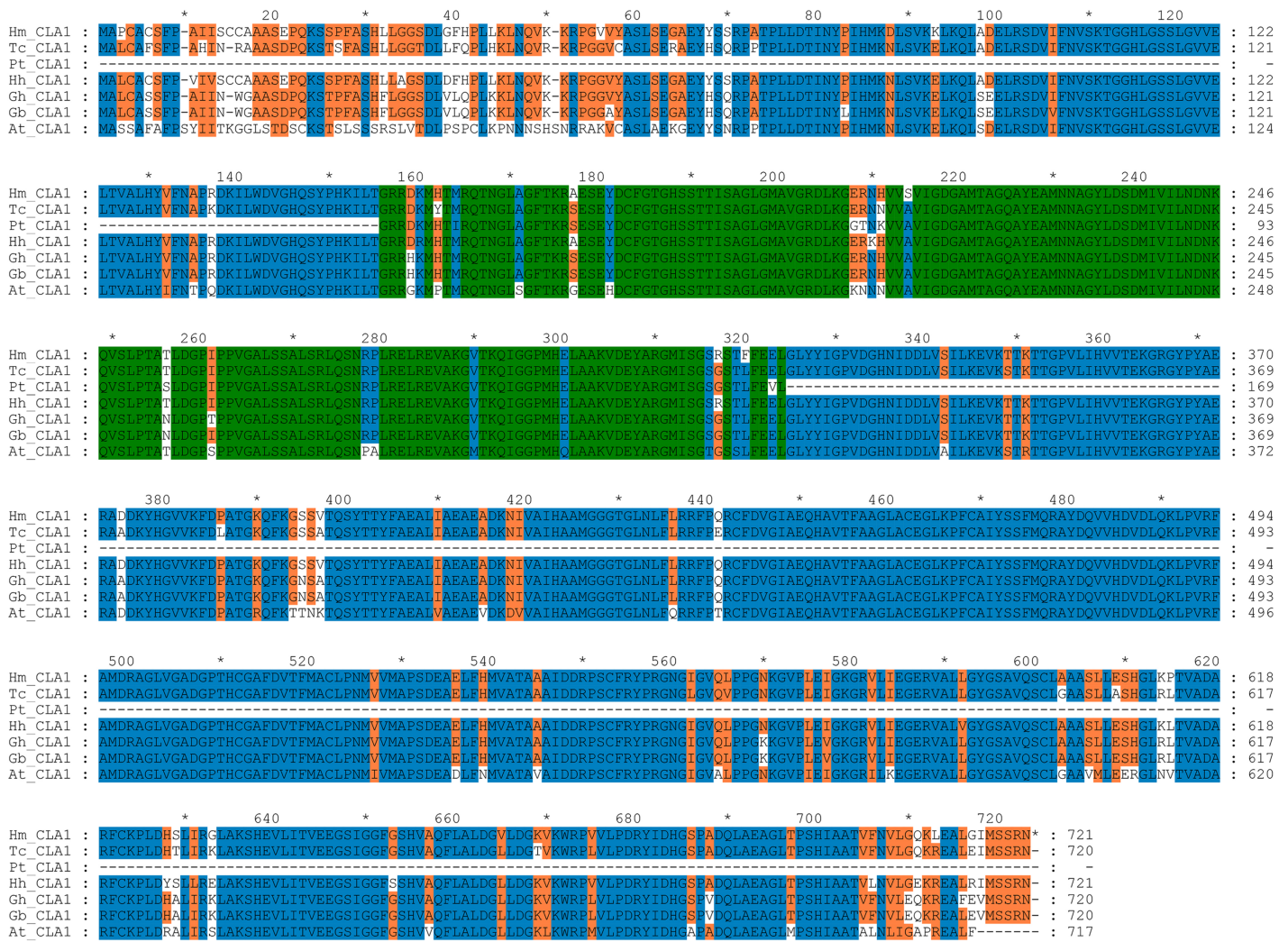
Figure 1 Multiple alignment of the HmCLA1 amino acid sequence with CLA1 amino acid sequences from different species using the ClustalW program. Multiple alignment of protein sequences of the CLA1 gene in *Hibiscus mutabilis*, *Hibiscus hamabo* (MK229167), *Gossypium barbadense* (ABN13970.1), *Gossypium hirsutum* (NP_001314056.1), *Theobroma cacao* (EOY06359.1), *Populus tomentosa* (AGT02336.1), and *Arabidopsis thaliana* (NP_193291.1).

Full-size 🖼 DOI: 10.7717/peerj.18211/fig-1

DISCUSSION

Woody plants, encompassing trees, shrubs, and semi-shrubs, serve as essential resources for human sustenance and economic production. However, their extended growth cycles, significant heterozygosity, and limited regenerative capacity present challenges in genetic transformation. The development of cost-effective and efficient techniques, like the VIGS system, is crucial for rapid gene function validation. Presently, only a few vectors have been adopted for VIGS in woody plants. Among the members of the *Hibiscus* genus, TRV-mediated VIGS systems have been successfully established for *H. hamabo* and *Hibiscus cannabinus* (*H. cannabinus*) (*Wang et al., 2019*; *Yang et al., 2023*). Research has also shown that the gene count in the plants of the *Hibiscus* genus far exceeds that of other
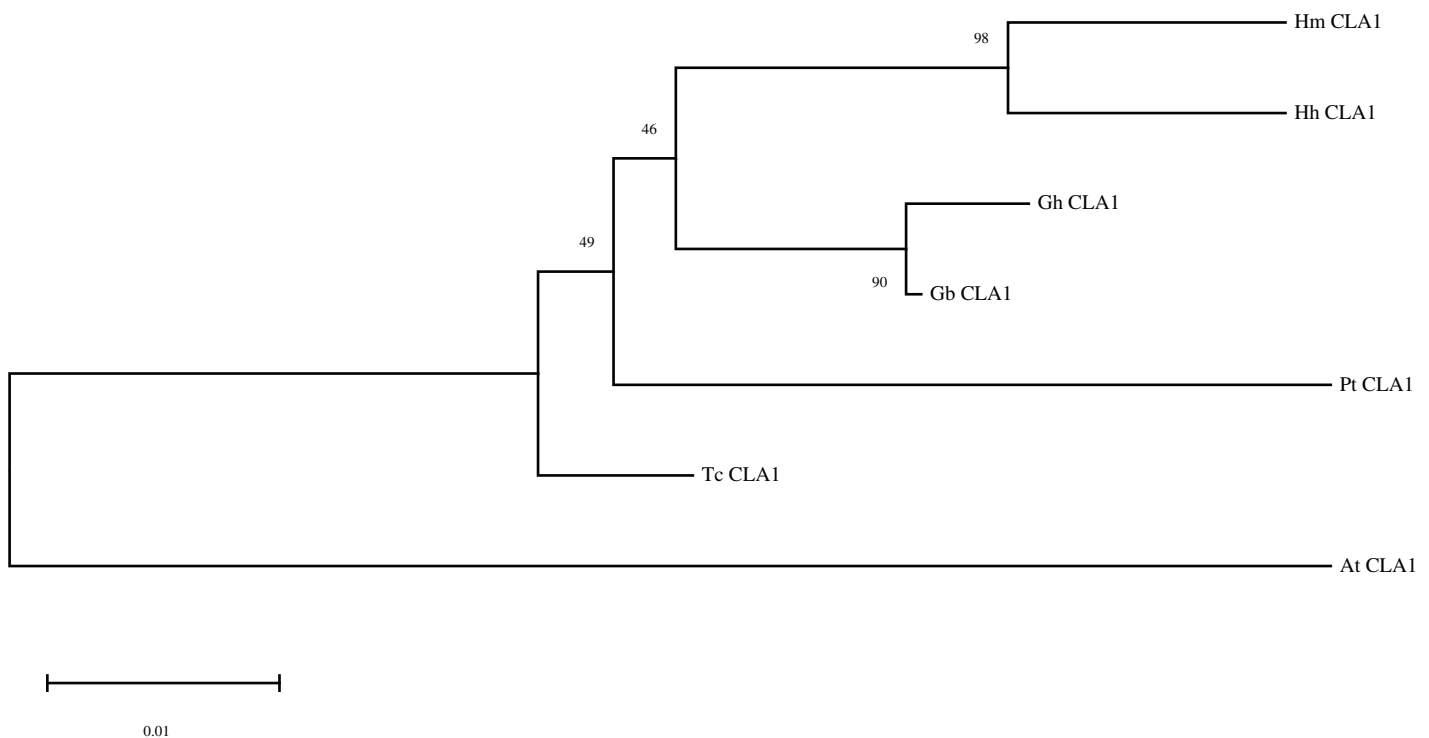
Sang et al. (2024), *PeerJ*, DOI 10.7717/peerj.18211

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Figure 2 Phylogenetic analysis of the protein of HmCLA1. Phylogenetic analysis of CLA1 proteins in different species.

Full-size ☒ DOI: 10.7717/peerj.18211/fig-2

Table 2 Efficiency of *HmCLA1* gene silencing in *H. mutabilis* using TRV-VIGS system at 18-days post agroinfiltration.

Treatment	Number of plants assayed	Silencing efficiency*
pTRV2-*HmCLA1*	71	53/71 (74.65%)
Mock	10	0/10 (0%)
Ck	10	0/10 (0%)

Note:
* Silencing efficiency indicates the number of plants showing silencing phenotypes/number of plants treated by TRV-VIGS system.

plants in the Malvaceae family (*Yang et al., 2022*). Therefore, the construction of VIGS vectors for other *Hibiscus* species is of great significance for studying functional genes.

Our study first demonstrated the potential of TRV-VIGS as an effective gene silencing tool in down-regulating endogenous gene expression levels in *H. mutabilis*. Moreover, the complete genome sequences of *H. mutabilis* have recently become publicly available (*Yang et al., 2022*). Knowledge about the complete genome sequence and the availability of TRV-VIGS together could help determine gene functions in *H. mutabilis*. Thus, the TRV system presented in this study will enhance functional genomic research based on VIGS in *H. mutabilis*. Further, our research aims to employ the vector system to examine the effects of biotic and abiotic stresses, such as diseases, pests, and low temperatures, on the growth and development of *H. mutabilis*, to breed more excellent cultivars with strong resistance.
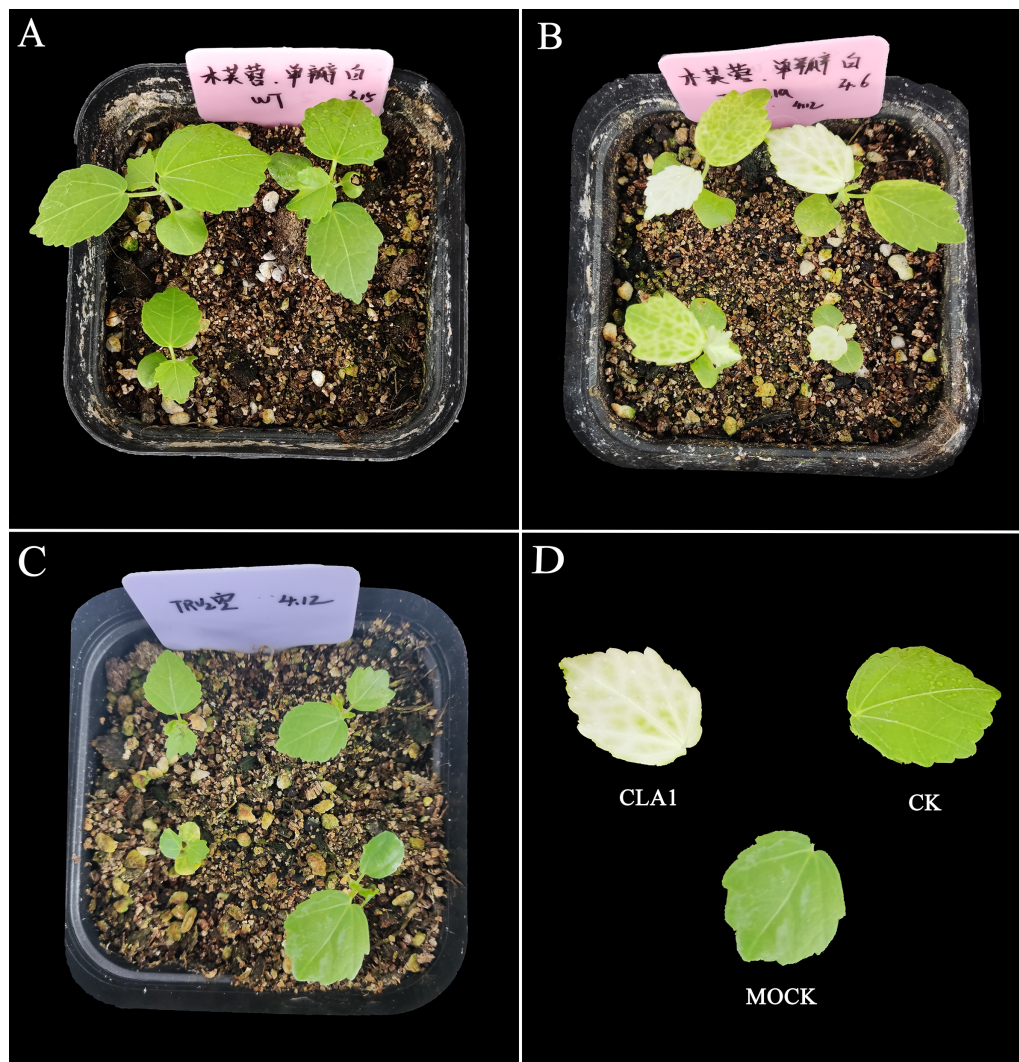
Figure 3 TRV-induced HmCLA1 silencing in *H. mutabilis*. (A) Control plants (CK). (B) Newly formed leaves of *H. mutabilis* plants infiltrated with pTRV2-HmCLA1 (CLA1) showing white-streaked leaf symptoms after 18 days. (C) Empty vector infiltrated plants (Mock) with the normal phenotype. (D) The leaf phenotypes of the treatments. The leaf on the upper left in Fig. 3D are from plants infiltrated with pTRV2-HmCLA1 (CLA1), the leaf on the upper right is from a control plant (CK) and the bottom one is from an empty vector infiltrated plant (Mock).　　Full-size 🖼 DOI: 10.7717/peerj.18211/fig-3

Researches have shown that VIGS outperforms previously developed gene silencing techniques in plants (*Beyaz, 2023*). Given the current benefits of VIGS technology, numerous VIGS applications in modern biology appear promising. Researchers can utilize the genetic outcomes of VIGS (virus-induced gene silencing) to extensively screen and identify important and astonishing traits in plants (*Unver & Budak, 2009*). The TRV (Tobacco rattle virus) vector is one of the tools used in VIGS (virus-induced gene silencing) technology, characterized by its ability to overcome host (plant) range and meristem exclusion constraints (*Liu, Schiff & Dinesh-Kumar, 2002*). The TRV infects a wide range of species, spreads rapidly, and results in relatively mild symptoms in infected
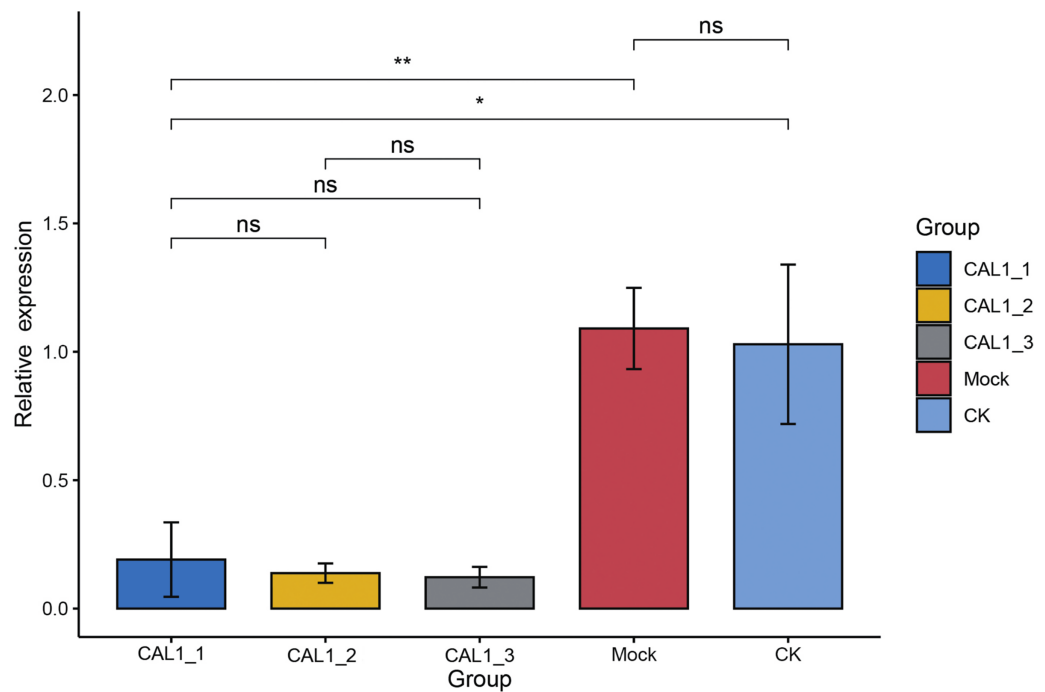
Figure 4 Relative expression levels of HmCLA1 transcripts in control (CK), empty vector-infected (Mock) and pTRV-HmCLA1-infected plants (pTRV2-HmCLA1). Error bars indicate the standard errors, and * denotes significant differences at the $P < 0.05$ level, two asterisks (**) indicate significant differences at the $P < 0.01$ level, while "ns" indicates no significant difference at the $P < 0.05$ level.

Full-size ◱ DOI: 10.7717/peerj.18211/fig-4

plants. Moreover, it can spread within plant meristems, leading to specific target gene degradation. Therefore, it is an ideal vector for analyzing plant gene function (*Ratcliff, Martin-Hernandez & Baulcombe, 2001*; *Shi et al., 2021*). Although the range of plants suitable for TRV vectors is relatively wide compared to other VIGS vectors, their efficiency varies from plant to plant and among materials of the same species or genus (*Zhang et al., 2016*). *Brigneti et al. (2004)* found that the TRV vector failed to induce effective gene silencing in the potato cultivar *Solanum tuberosum* cv. Cara. The efficacy with which TRV vector infects woody plants has also been examined. TRV-induced VIGS demonstrated effectiveness in *Vernicia fordii*, displayed limited potency in *Populus tomentosa* Carr., and yielded no significant outcomes in *Camellia oleifera* (*Jiang et al., 2014*). For *Hibiscus*, *Wang et al. (2019)* and *Yang et al. (2023)* individually applied the TRV-VIGS system to *H. hamabo* and *H. cannabinus*, and found that the TRV-VIGS system exhibited remarkable silencing efficiency, resulting in 87% of *H. hamabo* and 90–100% of *H. cannabinus* agro-infected plants displaying a white-streaked leaf phenotype. Our study also successfully silenced the *CLA1* gene in *H. mutabilis* cultivar 'single-petal white' *via* VIGS using the TRV system. However, due to the numerous varieties of *H. mutabilis*, the general applicability of this system requires further research.

  CLA1 is a protein that regulates the development of chloroplast and serves as a visual and valuable indicator of the TRV-VIGS system. Multiple sequence alignment of *CLA1* from various species in this study revealed that *HmCLA1* is highly homologous to the

HhCLA1, followed by *GhCLA1* protein and *GbCLA1* protein. The phylogenetic relationship of the *CLA1* protein is generally consistent with that of the family and genus to which it belongs, with one exception being *PtCLA1*. This discrepancy in *PtCLA1*'s placement may be attributed to the incomplete sequence data available for *PtCLA1*.

CONCLUSIONS

To conclude, our study demonstrated the efficacy of TRV-mediated VIGS in gene silencing within *H. mutabilis*, further enriching the roster of woody species amenable to VIGS investigations. The utilization of this loss-of-function assay presents promising prospects for advancing our understanding of the intricate molecular mechanisms governing *H. mutabilis* growth, development, and response to various stimuli. By silencing specific genes that influence key traits, we can unlock valuable insights into enhancing the species' overall fitness. For example, we could combine VIGS with next-generation sequencing technology to efficiently analyze and validate the functions of crucial genes in *Hibiscus mutabilis* in the future.

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ADDITIONAL INFORMATION AND DECLARATIONS

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Shiye Sang conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Yiqiong Liu performed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Xiu Li performed the experiments, prepared figures and/or tables, and approved the final draft.
- Jiao Ma performed the experiments, prepared figures and/or tables, and approved the final draft.

- Xiaoli Liu conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Yuanzhao Yang conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

The *HmCLA1* sequence described here is accessible *via* GenBase, accession no. C_AA041596.1.

Data Availability

The following information was supplied regarding data availability:

The raw measurements are available in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.18211#supplemental-information.

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