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Efficient virus-induced gene silencing in *Hibiscus hamabo* Sieb. et Zucc. using *Tobacco rattle virus*

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**Background.** *Hibiscus hamabo* Sieb. et Zucc. is a semi-mangrove plant used for the ecological restoration of saline-alkali land, coastal afforestation and urban landscaping. Because the genetic transformation of *H. hamabo* is difficult, molecular breeding and gene functional studies have been severely restricted. In plants, virus-induced gene silencing provides a pathway to rapidly and effectively create targeted gene knockouts for gene functional studies. **Methods.** In this study, we tested the efficiency of a *Tobacco rattle virus* vector in silencing the cloroplastos alterados 1 (*CLA1*) gene through agroinfiltration. **Results.** The leaves of *H. hamabo* showed white streaks typical of *CLA1* gene silencing three weeks after agroinfiltration. In agroinfiltrated *H. hamabo* plants, the *CLA1* expression levels in leaves with white streaks were all significantly lower than in those of mock-infected and control plants. **Conclusions.** Thus, this virus-induced gene silencing system was efficient in *H. hamabo* and may be a powerful tool for large-scale reverse-genetic analyses of gene functions in *H. hamabo*. 
Efficient virus-induced gene silencing in *Hibiscus hamabo* Sieb. et Zucc. using *Tobacco rattle virus*

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Abstract

Background. *Hibiscus hamabo* Sieb. et Zucc. is a semi-mangrove plant used for the ecological restoration of saline-alkali land, coastal afforestation and urban landscaping. Because the genetic transformation of *H. hamabo* is difficult, molecular breeding and gene functional studies have been severely restricted. In plants, virus-induced gene silencing provides a pathway to rapidly and effectively create targeted gene knockouts for gene functional studies.

Methods. In this study, we tested the efficiency of a *Tobacco rattle virus* vector in silencing the cloroplastos alterados 1 (*CLA1*) gene through agroinfiltration.

Results. The leaves of *H. hamabo* showed white streaks typical of *CLA1* gene silencing three weeks after agroinfiltration. In agroinfiltrated *H. hamabo* plants, the *CLA1* expression levels in leaves with white streaks were all significantly lower than in those of mock-infected and control plants.

Conclusions. Thus, this virus-induced gene silencing system was efficient in *H. hamabo* and may be a powerful tool for large-scale reverse-genetic analyses of gene functions in *H. hamabo*.

Introduction

*Hibiscus hamabo* Sieb. et Zucc., which is a shrub plant in the genus *Hibiscus*, family Malvaceae, is an important semi-mangrove plant (Nakanishi 1979). Because of its excellent salt tolerance and morphological characteristics, *H. hamabo* is widely used in public parks, waysides and coastal sands near sea level (Fowler 2017; Li et al. 2012; YANG et al. 2008). In addition, *H. hamabo* is a good plant material for exploring the salt-stress response mechanisms of woody plants (Li et al. 2012). The desire to determine gene functions and regulatory mechanisms in *H. hamabo* has led to the urgent need to manipulate genes. However, to date, the inefficient and laborious transformation procedures used have impeded this research. Additionally,
transcriptome analyses have mined many excellent genes that are awaiting functional identification. Appropriate techniques need to be applied successfully to allow the study of gene functions in this plant.

Virus-induced gene silencing (VIGS) is a powerful technology that uses engineered viruses to specifically silence host gene expression through post-transcriptional gene silencing (Becker & Lange 2010; Krishnan et al. 2015; Purkayastha & Dasgupta 2009). VIGS is an effective method for the large-scale analysis of genes and their functions, and it has been successfully performed in many plants, including tobacco, Arabidopsis, tomato, cotton, wheat, and many woody plants (Burch-Smith et al. 2006; Jiang et al. 2014; Kumagai et al. 1995; Orzaez et al. 2009; Scofield et al. 2005). VIGS works via a mechanism that is similar to that of RNA interference (Baulcombe 1999; Waterhouse et al. 2001). Double-stranded (ds) RNA is the key to the VIGS process; the dsRNA is cleaved into short interfering (si) RNAs of 21 to 25 nucleotides (Jiang et al. 2014). Two strands can be obtained from the siRNAs, the guide and passenger strands. The RNA-induced silencing complex incorporates the guide strand to degrade the specific single-stranded RNA that is complementary to the guide RNA, and then, the passenger strand is degraded (Mustafa et al. 2016). As a result, the target gene is silenced and large amounts of siRNAs are produced (Fuchs et al. 2004).

Tobacco rattle virus (TRV), belonging to genus Tobravirus (family Virgaviridae), is a suitable virus vector system for VIGS (Jiang et al. 2014). A positive sense single-stranded RNA genome exists in TRV, consisting of two components, RNA 1 and RNA 2 (Mustafa et al. 2016). RNA 1 encodes genes with viral replication and movement functions, while RNA 2 encodes the coat protein and some nonessential structural proteins that can be replaced by foreign sequences (Hayward et al. 2011). The TRV vector has been used in Gossypium spp., Arabidopsis and Vernicia fordii to silence the chloroplastos alterados 1 (CLA1) gene, which is involved in chloroplast development (Jiang et al. 2014; Manhães et al. 2015; Mustafa et al. 2016). Silencing CLA1 is a useful marker for determining the silencing efficiency because of the bleached phenotype (Mustafa et al. 2016).

In this study, we tested the feasibility of using the TRV-VIGS system in H. hamabo using the HhCLA1 gene as a reporter. The agroinfiltrated leaves of H. hamabo showed white streaks typical of HhCLA1 silencing at three weeks after infection, and the expression levels of the HhCLA1 gene in leaves having white streaks were significantly lower than in leaves from mock-infected and control plants. Thus, the TRV–VIGS system was efficient in H. hamabo. To our knowledge, this is the first report of the successful application of VIGS in H. hamabo.

Materials & Methods

Plant materials and growth conditions. Seeds of H. hamabo were collected from Nanjing’s Sun Yat-Sen Memorial Botanical Garden. The seeds were then treated with concentrated sulfuric acid for 15 min and rinsed thoroughly with sterile water. Next, the pretreated seeds were sown into flowerpots containing a mixture of
peat and vermiculite (1: 1, v: v) in an illuminated incubator with day/night temperatures of 26°C
/22°C and a 16/8 h photoperiod.

Sequence analysis.
Based on the HhCLA1 sequence (GenBank accession no. MK229167), the deduced protein
sequence was analyzed with those of other 20 species by ClustalX. The amino acid sequences
were obtained from NCBI (https://www.ncbi.nlm.nih.gov/). Then, the sequences were used to
construct a phylogenetic tree, which was drawn with MEGA 7.0 using the Neighbor-Joining (NJ)
method and 1,000 bootstrap replicates.

VIGS vector construction.
Total RNA was extracted from the leaves of H. hamabo using a Plant RNeasy Mini Kit (Qiagen,
Hilden, Germany). The first-strand cDNA was synthesized using a SuperScript II reverse
transcriptase kit (TaKaRa, Dalian, China). The primer pair HhCLA1-F and HhCLA1-R (Table 1)
was designed using Oligo 6.0 software (Molecular Biology Insights, Inc., Cascade, CO, USA)
based on the conserved domain of HhCLA1. To amplify partial fragments of the HhCLA1 gene,
these primers, synthesized cDNA as the template and PrimeSTAR™ HS DNA polymerase
(TaKaRa) were used. EcoRI enzyme cleavage sites were added to the upstream primers and SacI
enzyme cleavage sites were added to the downstream primers. The pTRV1 and pTRV2 vectors
were used in this study as described previously (Gao et al. 2011; Liu et al. 2002). The PCR
products were ligated into pTRV2 double-digested with EcoRI and SacI enzymes using a
ClonExpress® II One Step Cloning Kit (Vazyme, Nanjing, China). The resulting vector was
designated as pTRV2-HhCLA1.

Agroinfiltration.

pTRV2-HhCLA1 was transformed into Agrobacterium tumefaciens strain GV3101 using the
freeze–thawing method (Höfgen & Willmitzer 1988). PCR-confirmed single colonies were then
selected and independently inoculated into 3 mL of Luria-Bertani medium containing 25 mg/L
rifampicin and 50 mg/L kanamycin and grown overnight in a 28°C shaker. For the VIGS assay,
3-mL cultures of A. tumefaciens strain GV3101 independently containing pTRV1 and pTRV2
were grown overnight in the same culture conditions. These overnight starter cultures were
subsequently used to inoculate 50-mL cultures that were grown overnight at 28°C.

Agrobacterium cultures were harvested by centrifugation at 4,000 × g for 10 min, and the pellets
were resuspended in an infiltration buffer (10 mM MES (2- (N-morpholino) ethanesulfonic
acid), 10 mM MgCl₂ and 200 µM acetosyringone, pH 5.6) at an optical density of 2.0 at 600 nm
and incubated at room temperature for 3 h without shaking. Agrobacterium cultures containing
mixtures of pTRV1 and pTRV2-HhCLA1 (1: 1 ratio) were infiltrated with needleless 1-mL
syringes into the backs of cotyledons of 2-week-old H. hamabo seedlings, following a protocol
described previously (Gao et al. 2011). To determine whether the TRV vector can directly infect
H. hamabo, a mixture of Agrobacterium cultures containing pTRV1 and pTRV2 constructs in a
1: 1 ratio was infiltrated into the backs of cotyledons of eight 2-week-old H. hamaboto serve as
the mock. Experimental and non-injected control plants were transferred to a growth chamber
and maintained under set conditions.
Quantitative real-time PCR (qPCR).
To determine the relative levels of the endogenous \textit{HhCLA1} transcripts in infected leaves exhibiting visible silencing phenotypes, qPCR was performed using the primer pair q\textit{HhCLA1}-F/q\textit{HhCLA1}-R (Table 1). For the experiments, leaves from plants with significant white streak symptoms were analyzed in comparison with leaves of the mock and control plants three weeks after agroinfiltration. Total RNA was extracted from these leaves using a Plant RNeasy Mini Kit (Qiagen) and treated with DNase I to remove residual DNA. The first-strand cDNA was synthesized using a SuperScript II reverse transcriptase kit (TaKaRa). The qPCR assays were performed using the SYBR Green PCR Master Mix (Bimake, Houston, TX, USA) and a StepOne™ System (ABI, USA). The transcript level of \textit{18S rRNA} served as internal standards. All experiments were repeated three times. The relative gene expression level was calculated using the $2^{-\Delta\Delta C_t}$ method (Gu et al. 2018).

Statistical analysis.
Results were expressed as means ± standard errors. Statistical procedures were conducted with IBM SPSS (Version 21). Data of qPCR was analyzed with one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test ($P < 0.05$).

Results
Characterization of the \textit{HhCLAI} gene.
The amino acid sequence alignment indicated that the \textit{HhCLAI} protein showed high homology to known CLA1 proteins from \textit{Gossypium barbadense}, \textit{Theobroma cacao} and other species (Fig. 1). The phylogenetic analysis showed that \textit{HhCLAI} clustered with \textit{G. barbadense} in a clade and is closely related to CLA1 proteins from \textit{T. cacao}, \textit{Bixa orellana} and \textit{Aquilaria sinensis} (Fig. 2).

Silencing of the \textit{HhCLAI} gene in \textit{H. hamabo} using the VIGS system.
In total, fifty-two \textit{H. hamabo} plants were inoculated with \textit{A. tumefaciens} ‘GV3101’ harboring pTRV2-\textit{HhCLAI}. Two weeks after agroinfiltration, white streaks began to appear in the emerging leaves of partially agroinoculated plants. At three weeks post infiltration, 87% of the \textit{H. hamabo} plants showed white-streak leaf symptoms (Table 2; Fig. 3A). At three weeks after agroinfiltration, plants inoculated with pTRV1 and pTRV2 (mock) showed no obvious differences in leaf morphology compared with the control (Fig. 3B and 3C). This suggested that \textit{HhCLAI} gene expression maybe silenced in these treated plants.

qPCR.
The gene silencing efficiency was analyzed at the molecular level by monitoring \textit{HhCLAI} mRNA levels in plants showing white-streak leaf symptoms. \textit{HhCLAI} gene expression levels were unchanged in mock-injected plants, while the \textit{HhCLAI} expression levels were 62.6%-76.4% lower in the pTRV2-\textit{HhCLAI} agroinfiltrated plants than in the non-infiltrated plants (control) (Fig. 4). These results clearly indicated that the expression of \textit{HhCLAI} was significantly down-regulated through TRV-VIGS in \textit{H. hamabo}. 
**Discussion**

In this study, we demonstrated for the first time that TRV-VIGS can effectively down-regulate endogenous gene expression levels in the salt-tolerant species *H. hamabo*. The genetic transformation of this species is laborious, time-consuming and technically challenging. To resolve these problems, effective and low-cost techniques need to be developed to enable the rapid validation of gene functions. In future studies, stress-responsive genes isolated in *H. hamabo* could be silenced in loss-of-function screens using the TRV-VIGS system.

The CLA1 gene is involved in chloroplast development and is a useful marker in the TRV-VIGS system (Mustafa et al. 2016). In this research, multiple sequence alignments indicated that HhCLA1 was similar to those from other species. Additionally, the phylogenetic analysis indicated that HhCLA1 was highly similar to other CLA1 proteins in *G. barbadense*, *T. cacao*, *B. orellana* and *A. sinensis*.

The most cost-efficient and effective method of inoculating plants with virus-based vectors is agroinfection (Grimsley et al. 1986), but its efficiency varies among plants (Zhang et al. 2016). In turf grass, the silencing efficiency of the RTBV-VIGS system in *Cynodon dactylon* is such that 65.8-72.5% of the agroinfected plants developed symptoms typical for phytoene desaturase gene silencing, while the silencing efficiency in *Zoysia japonica* is much weaker, with only 52.7%-55% of agroinfected plants developing the phenotype (Zhang et al. 2016). The ability of the TRV vector to directly infect woody plant species has been tested, and TRV-mediated VIGS was effective in *Vernicia fordii*, weak in *Populus tomentosa* Carr., and ineffective in *Camellia oleifera* (Jiang et al. 2014). In this study, the silencing efficiency of the TRV-VIGS system in *H. hamabo* was high, with 87% of agroinfected plants developing a white-streak leaf phenotype, indicating *HhCLAI* gene silencing. The *HhCLAI* mRNA level was also down-regulated by TRV-VIGS in *H. hamabo*.

**Conclusions**

In conclusion, we demonstrated that TRV-mediated VIGS is effective in *H. hamabo*, which adds to the increasing list of wood species for which VIGS-mediated studies can be used. The loss-of-function assay using TRV-mediated VIGS developed in this study provides an alternative tool for functional genomics studies of *H. hamabo*.

**Acknowledgements**

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**References**


Burch-Smith TM, Schiff M, Liu Y, and Dinesh-Kumar SP. 2006. Efficient virus-induced gene

Fowler E. 2017. Assessment and Characterization of Microbial Communities in Salt Affected Soils on Galveston Island.


Multiple alignment of the HhCLA1 amino acid sequence with sequences from different species.

Gossypium barbadense (ABN13970.1), Theobroma cacao (EOY06359.1), Ricinus communis (EEF45457.1), Bixa orellana (AMJ39459.1), Hevea brasiliensis (BAF98288.1), Trema orientale (POO02371.1), Parasponia andersonii (PON80259.1), Hedera helix (APY22341.1), Siraitia grosvenorii (AEM42997.1), Catharanthus roseus (AGL40532.1), Actinidia chinensis var. chinensis (PSS33932.1), Osmanthus fragrans (AOT86855.1), Withania somnifera (AOX15279.1), Cucurbita maxima (XP_023000608.1), Cestrum nocturnum (ATN96999.1), Pogostemon cablin (AHK06499.1), Lycium ruthenicum (AIX87515.1), Plectranthus barbatus (AOZ60044.1), Aquilaria sinensis (AFU75321.1) and Pueraria montana var. lobata (AAQ84169.1)
Figure 2 (on next page)

Phylogenetic analysis of HhCLA1. The tree was constructed with the Neighbor-joining method using the MEGA program, version 7.
Figure 3 (on next page)

TRV-induced *HhCL1* silencing in *Hibiscus hamabo*.

A. Newly formed leaves of *H. hamabo* plants infiltrated with pTRV2-*HhCL1* (*CL1*) showing white-streaked leaf symptoms after three weeks. B. Empty vector infiltrated plants (Mock) with the normal phenotype. C. Control plants (CK). D. The leaf phenotypes of the treatments.
Relative expression levels of *HhCLA1* transcripts in control (CK), empty vector-infected (Mock) and pTRV-*HhCLA1*-infected plants (pTRV2-*HhCLA1*) *Hibiscus hamabo* plants.

Error bars represent standard errors, and any two samples with a common letter are not significantly different at the $P < 0.05$ level.
Table 1 (on next page)

Primers used in this study.

Note: Underlines indicate restriction enzyme cleavage sites.
Table 1 Primers used in this study

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>HhCLAI</em>-F</td>
<td>CTGIGAGTAAGGTTACCGAATTTCATGGTTCGACTGAGAAAGG</td>
</tr>
<tr>
<td><em>HhCLAI</em>-R</td>
<td>CTGGAGACGCGTGAGCTCCATAGCAATCTTTACAGGCAG</td>
</tr>
<tr>
<td><em>qHhCLAI</em>-F</td>
<td>CGCCAGGGAAACAAGGGGTT</td>
</tr>
<tr>
<td><em>qHhCLAI</em>-R</td>
<td>AATCGTGATCCGCAGAGT</td>
</tr>
<tr>
<td><em>18S rRNA</em>-F</td>
<td>GGTCGGATTGGAAACGGCGA</td>
</tr>
<tr>
<td><em>18S rRNA</em>-R</td>
<td>CTCCACGGGC GTATCGAGG</td>
</tr>
</tbody>
</table>

Note: Underlines indicate restriction enzyme cleavage sites.
Table 2 (on next page)

Efficiency of *HhCLA1* gene silencing in *Hibiscus hamabo* using TRV-VIGS system at three weeks post agroinfiltration.

*Silencing efficiency indicates the number of plants showing silencing phenotypes/number of plants assayed.*
Table 2 Efficiency of *HhCLA1* gene silencing in *Hibiscus hamabo* using TRV-VIGS system at three weeks post agroinfiltration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of plants assayed</th>
<th>Silencing efficiency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTRV2-<em>HhCLA1</em></td>
<td>52</td>
<td>45/52 (87%)</td>
</tr>
<tr>
<td>Mock</td>
<td>8</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>0/8 (0%)</td>
</tr>
</tbody>
</table>

*Silencing efficiency indicates the number of plants showing silencing phenotypes/number of plants assayed.*