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. The genetic transformation *H. hamabo* is currently inefficient and laborious, restricting gene functional studies on this species. 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 those in leaves from mock-infected and control plants.

Conclusions. The system presented here can efficiently silence genes 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). Gene manipulation technologies can be used to determine the gene functions and regulatory mechanisms in *H. hamabo*. However, to date, the inefficient and laborious genetic transformation procedures used have impeded such 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 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; Baulcombe 2004; Burch-Smith et al. 2004; Lu et al. 2003; Waterhouse et al. 2001). Double-stranded (ds) RNA is the key to the VIGS process; the dsRNA can be cleaved into short interfering (si) RNAs of 21 to 25 nucleotides (Burch-Smith et al. 2004; Jiang et al. 2014; Lu et al. 2003). 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). Previous studies have reported the detailed protocol for agrobacterium-mediated VIGS system in cotton, based on tobacco rattle virus (TRV), and have optimized the conditions for VIGS in three cultivated cotton species, indicating that TRV is a useful vector for VIGS in *Gossypium* species (Gao et al. 2011; Ge et al. 2016).

T~~The t~~obacco 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 *G.* spp., *Arabidopsis* and *Vernicia fordii* ~~in silencing the~~to silence cloroplastos alterados 1 (*CLA1*) gene, which is involved in chloroplast development (Jiang et al. 2014; Manhães et al. 2015; Mustafa et al. 2016). The *CLA1* gene is highly conserved in various plant species (Jiang et al. 2014). The silencing phenotypes of albino leaves were observed in *Vernicia fordii* two weeks after inoculation using a heterologous TRV-based VIGS system, in which *CLA1* was isolated from *Populus tomentosa* Carr. (Jiang et al. 2014). ~~Silencing~~ The silenced *CLA1* is a useful marker for determining silencing efficiency because of the bleached phenotype (Mustafa et al. 2016).

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

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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, t~~The pretreated seeds were sown into flowerpots containing a mixture of peat and vermiculite (1: 1, v: v) in an illuminated incubator with controlled temperatures of 26 °C/22 °C under a 16 h/8 h (day/night) photoperiod.

Sequence analysis.

Based on the *HhCLA1* sequence (GenBank accession no. MK229167), the deduced protein sequence was analyzed with CLA1 proteins of other species using ClustalX (Liu et al. 2015). 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 *HhCLA1*, the primer pair, cDNA and PrimeSTAR^{TM} HS DNA polymerase (TaKaRa) were used. *Eco*RI enzyme cleavage sites were added to the upstream primers and *Sac*I enzyme cleavage sites were added to the downstream primers. PCR product were generated with the following reaction program: 30 cycles of 98°C for 10 s, 60°C for 5 s and 72°C for 1 min. The reactions final volume was 50 µL, containing 25 µL of 2 × PrimeSTAR^{TM} GC Buffer, 4 µL dNTP m~~M~~ixture (2.5 mM), 0.2 µM of each primer (final), 100 ng of cDNA and 0.5 µL of PrimeSTAR^{TM} HS DNA Polymerase (2.5 U/µL). 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 (Supplementary Fig. S1) (double-digested with *EcoR*I and *Sac*I enzymes) using a ClonExpress® IIOne 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 shaker at 28°C. For the VIGS assay, 3-mL cultures of *A. tumefaciens* strain GV3101 independently containing either pTRV1 or pTRV2 was 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- (4- Morpholino) Ethanesulfonic Acid), 10 mM $MgCl_2$ 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 1-mL 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. hamabo* to 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 *HhCLA1* transcripts in infected leaves exhibiting visible silencing phenotypes, qPCR was performed using the primer pair q*HhCLA1*-F/q*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 after three weeks of 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 *18S rRNA* served as the internal controls. All experiments were repeated three times. The relative gene expression level was calculated using the $2^{-\Delta\Delta Ct}$ method (Gu et al. 2018; Liao et al. 2016).

Statistical analysis.

~~Results were expressed as means ± standard errors. Statistical procedures were conducted with IBM SPSS (Version 21). qPCR data was analyzed with o~~One-way analysis of variance (ANOVA) ~~followed by~~and Duncan's multiple range test ($P < 0.05$) were performed using IBM SPSS (Version 21).

Results

Characterization of the *HhCLA1* gene in *H. hamabo*.

The amino acid sequence alignment indicated that the HhCLA1 protein showed high homology to known CLA1 proteins from *G. barbadense*, *G. hirsutum* and other species (Fig. 1 and Supplementary Table S1). To understand the evolutionary relationship of CLA1 proteins from different species, the amino acid sequence of HhCLA1 was aligned with other CLA1 proteins, and an un-rooted neighbor-joining (NJ) phylogenetic tree was constructed using MEGA 7.0 software. The phylogenetic analysis showed that HhCLA1 clustered with *G. barbadense* and *G. hirsutum* in a clade (Fig. 2).

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Silencing efficiency of the *HhCLA1* gene in *H. hamabo* using the VIGS system.

In total, fifty-two *H. hamabo* plants were inoculated with *A. tumefaciens* 'GV3101' harboring pTRV2-*HhCLA1*. 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 *H. hamabo* plants showed white-streak leaf symptoms similar to the photobleached phenotype (Table 2; Fig.

3A and Supplementary Fig. S2). 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, 3C and Supplementary Fig. S2). The three leaves on the left in Fig. 3D are from plants infiltrated with pTRV2-*HhCLA1* (*CLA1*), the leaf in the middle is from an empty vector infiltrated plant (Mock) and the right one is from a control plant (CK). Leaf phenotypic characteristics suggested that the *HhCLA1* gene expression ~~maybe~~ might be ~~silenced~~ suppressed in ~~treated~~ plants infiltrated with pTRV2-*HhCLA1* compared with mock and CK plants.

q-PCR analysis of the knockdown levels of *HhCLA1*.

The efficiency of gene silencing was analyzed ~~at molecular level~~ by monitoring expression levels of *HhCLA1* in plants showing white-streak leaf symptoms. Four groups of plants with significant white streak symptoms, one control group, and one mock group, in order to analyze the test results more accurately, were further analyzed in this experiment. Each group contained three biological replicates. ~~The r~~Results showed that *HhCLA1* gene expression levels were unchanged in mock-injected plants, while the *HhCLA1* expression levels were 62.6%-76.4% lower in the pTRV2-*HhCLA1* agroinfiltrated plants than in the non-infiltrated plants (control) (Fig. 4 and Table S2). The phenotypic characteristics were consistent with the expression characteristics of *HhCLA1*. This clearly indicates that the expression of *HhCLA1* was significantly down-regulated through TRV-VIGS in *H. hamabo*, and TRV-VIGS led to an albino phenotype on true leaves.

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 currently 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 CLA1 proteins of other species. Additionally, the phylogenetic analysis indicated that HhCLA1 was highly similar to CLA1 proteins in Malvaceae, including *G. barbadense* and *G. hirsutum*.

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* was 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* was much ~~weaker~~lower, 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*

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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 the *HhCLA1* gene was silenced~~. The *HhCLA1* mRNA level was also down-regulated by TRV-VIGS in *H. hamabo*.

Conclusions

In conclusion, we demonstrated that TRV-mediated VIGS can effectively silence genes 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 genes studies of *H. hamabo*.

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