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Efficient Virus-Induced Gene Silencing in *Ilex dabieshanensis* Using Tobacco Rattle Virus

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Abstract: *Ilex dabieshanensis* is not only an important ornamental plant, but can also be used to produce Kuding tea, owing to its lipid-lowering and anti-inflammatory medicinal properties. The genetic transformation of *I. dabieshanensis* is currently difficult, which restricts functional gene studies and molecular breeding research on this species. Virus-induced gene silencing (VIGS) is a powerful tool for determining gene functions in plants. The present study reports the first application of VIGS mediated by a tobacco rattle virus (TRV) vector in *I. dabieshanensis*. We tested the efficiency of the VIGS system to silence *Mg-chelatase H subunit (ChlH)* gene through agroinfiltration. The agroinfiltrated leaves of *I. dabieshanensis* exhibited a typical yellow-leaf phenotype of *ChlH* gene silencing at 21 days post infiltration. Endogenous *ChlH* expression levels in the leaves of yellow-leaf phenotype plants were all significantly lower than that in the leaves of mock-infected and control plants. Overall, our results indicated that the TRV-based VIGS system can efficiently silence genes in *I. dabieshanensis*, and this system will contribute to efficient functional genomics research in *I. dabieshanensis*.

Keywords: *Ilex dabieshanensis*; virus-induced gene silencing; *Mg-chelatase H subunit* (*ChlH*); tobacco rattle virus

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1. Introduction

Ilex L. (Aquifoliaceae) is the largest woody dioecious angiosperm genus with approximately 700 species of forest trees and shrubs [1,2]. Most *Ilex* species have economic, ecological, and horticultural value [3]. The genus is widely distributed in mesic habitats, but the global diversity centers are in East Asia and South America, with only a few species growing in tropical Africa, northern Australia, and Europe [3,4]. About 200 *Ilex* species are known in China, mainly distributed in the humid and temperate regions of south and southwest China [5,6]. Among them, *Ilex dabieshanensis* K. Yao &M.B. Deng has been widely utilized as an ornamental tree because of its bright red drupes and dense evergreen foliage. Additionally, it can be used for making a bitter-tasting Kuding tea owing to its nutritional and medicinal value [7]. Recently, the next generation sequencing-based transcriptome sequencing has greatly facilitated the acquisition of abundant sequence data. However, due to the long childhood period of forest trees, such as *Ilex dabieshanensis*, and the inefficient and laborious genetic transformation procedures, research on *Ilex* gene functions is limited. Thus, to improve functional genomic studies and the molecular breeding of *Ilex* plants, developing appropriate genetic tools is necessary.

Virus-induced gene silencing (VIGS), a transcript suppression technique to identify the functions of plant genes, has been developed based on the defense mechanism of plants against viral infections [8]. When the virus infects plant tissues and spreads systemically, endogenous gene transcripts, which are homologous to the engineered sequence in the viral vector (VIGS vector), are degraded by post-transcriptional gene silencing [9].

Compared with traditional transgenic technology, VIGS has advantages of efficiency, simplicity, and short cycle time [10]. VIGS has been widely used to identify gene functions involved in plant development, secondary metabolism, stress response, and plant–pathogen interactions [11–13].

Since the last few years, many plant viruses have been developed into VIGS vectors to assess plant gene functions; particularly, among these viruses, the tobacco rattle virus (TRV) has been reported to have the broadest host range until now [11]. TRV consists of two positive-sense single-stranded RNA components: RNA1 and RNA2. RNA1 functions in viral replication and movement, whereas RNA2 encodes the viral coat protein and some unnecessary structural proteins that can be replaced by foreign sequences [14,15]. Previously, TRV-based VIGS vectors have been successfully used to silence genes in tomato [16], cotton [17], *Arabidopsis* [18], rose [19], petunia [20], etc. However, TRV-based VIGS has not yet been applied to unravel gene functions in *I. dabieshanensis*. The *Mg-chelatase H subunit* (*ChlH*) gene, which is involved in chlorophyll biosynthesis, has been widely and successfully used as a reporter gene because of its visual silencing phenotype in the VIGS tests of many species [13,21].

In this study, we developed a TRV-based VIGS system in *I. dabieshanensis* by silencing the *ChlH* gene. *I. dabieshanensis* leaves exhibited a yellow-leaf phenotype at 21 days after *Agrobacterium tumefaciens* infection, and the transcripts of the *IdChlH* gene in yellow leaves were significantly lower than those in the leaves of mock-infected and control plants. The results indicated that the TRV-based VIGS system could effectively silence genes in *I. dabieshanensis*. The VIGS system presented here will pave an important way for identifying gene functions in *I. dabieshanensis*.

2. Materials and Methods

2.1. Plant Materials and Growing Conditions

I. dabieshanensis was acquired from the Nanjing Botanical Garden, Mem. Sun Yat-sen (118°49′55″ E, 32°3′32″ N) (Figure S1), Nanjing, Jiangsu, China. Uniformly sized cuttings (current-year semi-lignified shoots, 5–8 cm in length) were rooted in a 1:2 mixture of perlite and peat, grown in a greenhouse under natural light, and then transferred into a 1:1 mixture of soil and vermiculite. The rooted cuttings were cultivated in a greenhouse under a 16 h light/8 h dark photoperiod, and a day/night temperature of 25/22 °C with a relative humidity of 70%. The plants were watered and fertilized throughout the experiment.

2.2. Sequence Analysis of IdChlH

According to the *IdChlH* gene sequence (GenBank accession number: OP820080), the deduced amino acid sequence was analyzed with that of other plant homologs using BLAST and DNAman software. Phylogenetic analysis was performed using the neighbor-joining method with 1000 bootstrap replicates implemented in MEGA 5.0 software [22]. The amino acid sequences of *ChlH* homologs were obtained from the NCBI database.

2.3. pTRV2-IdChlH Vector Construction

Total RNA was extracted from snap-frozen *I. dabieshanensis* leaves using the Quick RNA Isolation Kit (Huayueyang, Beijing, China) according to the manufacturer's protocol. First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Based on the *IdChlH* sequence, the primer pair *IdChlH*-F/R was designed using Primer 5.0 software to amplify the partially conserved *IdChlH* fragment (Table 1). The upstream primers contained the *BamHI* restriction site, whereas the downstream primers contained the *SacI* restriction site. The PCR reactions were performed in a final 50 µL volume with 25 µL 2 × PCR buffer for KOD FX, 10 µL dNTPs (2 mM), 1.5 µL each of the forward and reverse primers (10 pM), 1 µL KOD FX DNA polymerase, 1 µL cDNA, and 10 µL ddH_2O. Thermal cycling consisted of 94 °C for 2 min, followed by 35 cycles of 98 °C for 10 s, 55 °C for 30 s, and 68 °C for 30 s, and a final extension at 68 °C for 7 min. Further, TRV-VIGS vectors (pTRV1 and pTRV2) were used in this study

as reported previously [16]. The *IdChlH* fragment was assembled into the pTRV2 vector (double-digested with *BamH* I and *Sac* I restriction enzymes) using the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China) to generate a pTRV2-*IdChlH* vector.

Table 1. List of primers used in this study.

Prime Name	Primer Sequence (5′ to 3′)
IdChlH-F	GAAGGCCTCCATGGGGATCCGCGCTGGATCCACAATCTATTC
IdChlH-R	CGAGACGCGTGAGCTCCTCTAACCTCAACGCCAAGCGC
IdChlH-qd-F	TTCTTCGTGGAATGATGAGA
IdChlH-qd-R	CCGTGAGTGAGATTTCTGA
Actin-F	CCACCTACAACTCCATCAT
Actin-R	TTCCTTGCTCATACGATCA

Note: Underlines indicate restriction enzyme cleavage sites and partial homologous sequences of the pTRV2 vector used in this TRV-VIGS system.

2.4. Agroinfiltration of the Ilex Plant

pTRV1, pTRV2, and pTRV2-*IdChlH* were introduced into *Agrobacterium strain* GV3101 using the freeze-thaw method [23], separately. PCR-verified single *Agrobacterium* cells were independently inoculated into 2 mL LB medium containing 50 μg/mL kanamycin and 50 μg/mL rifampicin, and cultured overnight at 28 °C in a shaker. These overnight starters were subsequently inoculated into 50 mL cultures (containing 50 μg/mL kanamycin, 50 μg/mL rifampicin, 10 mM MES, and 20 μM acetosyringone), and shaken overnight at 28 °C. After that, *Agrobac*terium cultures were centrifuged at 6000 rpm for 10 min, resuspended in an infiltration buffer (10 mM MES, 10 mM $MgCl_2$, and 200 μM acetosyringone, pH 5.6), adjusted to an OD600 of 1.8 and incubated at room temperature for 3–4 h in the dark. *Agrobacterium* cultures containing pTRV1 and pTRV2 or pTRV2-*IdChlH* were mixed in a 1:1 ratio before infiltration [10]. *Ilex* plants were inoculated using the leaf syringe-infiltration method [24]. The underside of the *I. dabieshanensis* leaves were pierced gently using a needle and then infiltrated with the mixed bacterial solution using a 1 mL needleless syringe. Subsequently, infiltrated and non-injected control plants were grown in a growth chamber under a 16 h light/8 h dark cycle at 25/23 °C and 70% relative humidity.

2.5. Expression Analysis by qRT-PCR

To determine the effect of the VIGS system on endogenous *IdChlH* gene expression in *I. dabieshanensis*, qRT-PCR was performed using the primer pair q*IdChlH*-F/R listed in Table 1. For the experiments, leaves from plants exhibiting the visible silencing phenotype were collected after three weeks of VIGS treatment; additionally, leaves were collected from the TRV2 empty vector-infected plants (mock plants) and untreated plants (control plants), separately. Four plant groups (exhibiting a significant yellow-leaf phenotype), one mock group, and one control group, were used for qRT-PCR analysis. Each group included three independent biological replicates of three different plants. Total RNA was extracted from the leaf samples using the Quick RNA Isolation Kit (Huayueyang, Beijing China), and treated with RNase-free DNase I to remove genomic DNA. A 1μg amount of total RNA was reverse transcribed into ss cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Transcript abundance was assessed with an ABI 7500 Fast System using SYBR Premix Ex Taq™ (TaKaRa, Nojihigashi, Japan). To prevent interference with the inserted plant gene sequence, the specific primer pair *IdChlH*-qd-F/R was designed to amplify a region of the targeted transcript outside of the fragment cloned into the VIGS vector (Table 1). The actin gene was used as an endogenous control to normalize the results. All experiments were repeated three times. The relative transcript abundance was calculated using the $2^{-\Delta\Delta Ct}$ method [25].

2.6. Statistical Analysis

The experimental data were analyzed by one-way analysis of variance and Duncan's multiple range test ($p < 0.01$) using SPSS Statistics v. 25.0.

3. Results

3.1. IdChlH Sequence Characteristics

The amino acid sequence alignment showed that IdChlH was highly homologous to other known ChlH proteins, with a similarity of 92.62% to CsChlH (AEI83420.1) from *Camellia sinensis*, 92.25% to CiChlH (XP_042945153.1) from *Carya illinoinensis*, 92.11% to VvChlH (NP_001268078.1) from *Vitis vinifera*, and 85.9% to AtChlH (AT5G13630) from *Arabidopsis thaliana* (Figure 1). The phylogenetic analysis indicated that IdChlH was most closely related to CsChlH (Figure 2).

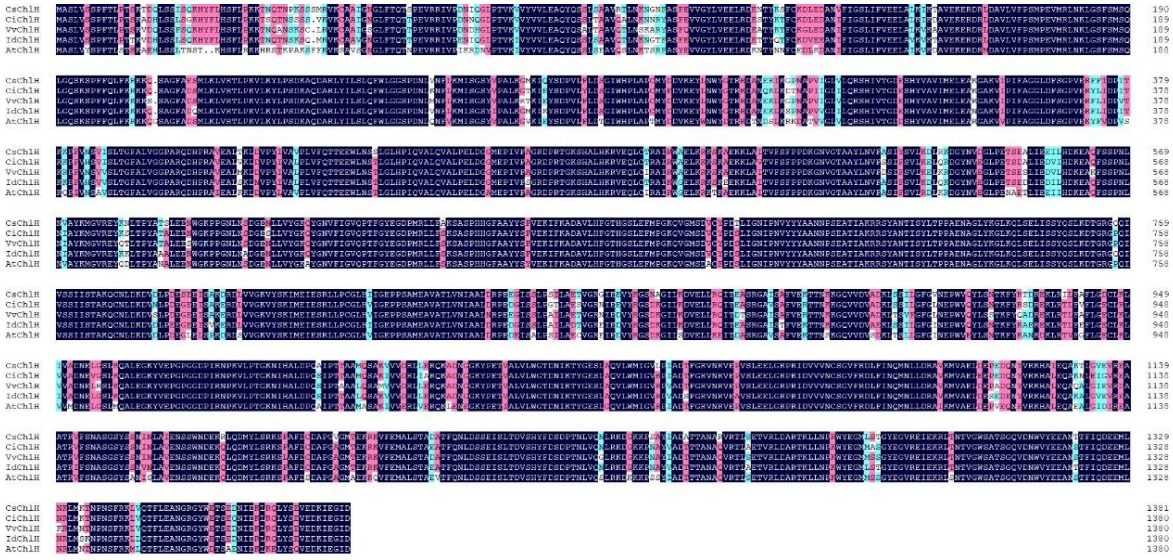

Figure 1. Alignment of the putative amino acid sequence of IdChlH with homologous proteins.

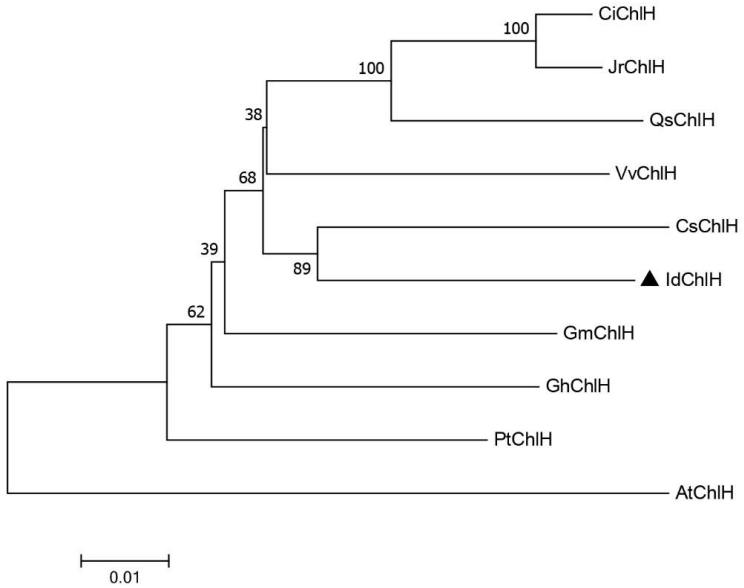

Figure 2. Phylogenetic analysis of IdChlH (black triangle) and ChlH proteins from other species. The accession numbers of the proteins are CsChlH (AEI83420.1), CiChlH (XP_042945153.1), VvChlH (NP_001268078.1), PtChlH (XP_024459042.1), GmChlH (XP_003535922.1), JrChlH (XP_018816348.1), GhChlH (XP_040932511.1), QsChlH (XP_023922908.1), and AtChlH(AT5G13630).

3.2. Construction of the pTRV2-IdChlH Vector

To generate the pTRV2-*IdChlH* vector, a 442 bp fragment of *IdChlH* with *BamHI* and *SacI* restriction sites was amplified (Figure 3) and subsequently ligated into the pTRV2 vector (Figure 4). PCR verification was performed using pTRV2-*IdChlH* as the template to investigate the accuracy of pTRV2-*IdChlH* construction (Figure S2A). In addition, the pTRV2-*IdChlH* plasmid was sequenced to further verify the result. Alignment of the sequencing result of pTRV2-*IdChlH* and the inserted *IdChlH* fragment showed that the two sequences shared 100% similarity (Figure S2B). Consequently, the results indicated that the pTRV2-*IdChlH* vectors were constructed accurately.

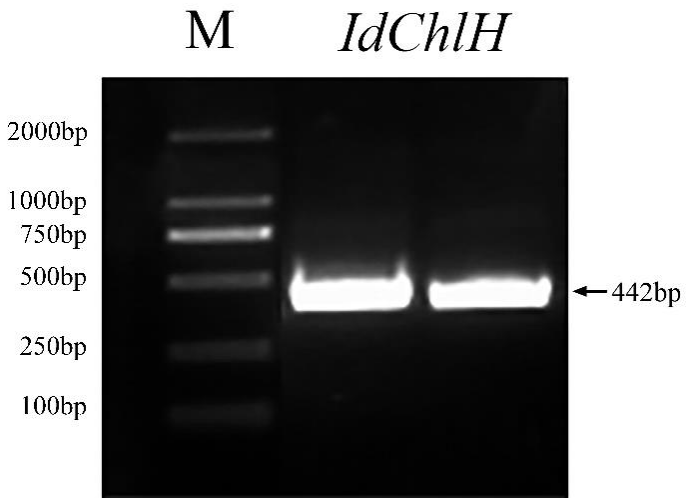

Figure 3. Cloning of *IdChlH* gene specific fragment in *I. dabieshanensis*. M: 2000 DNA marker.

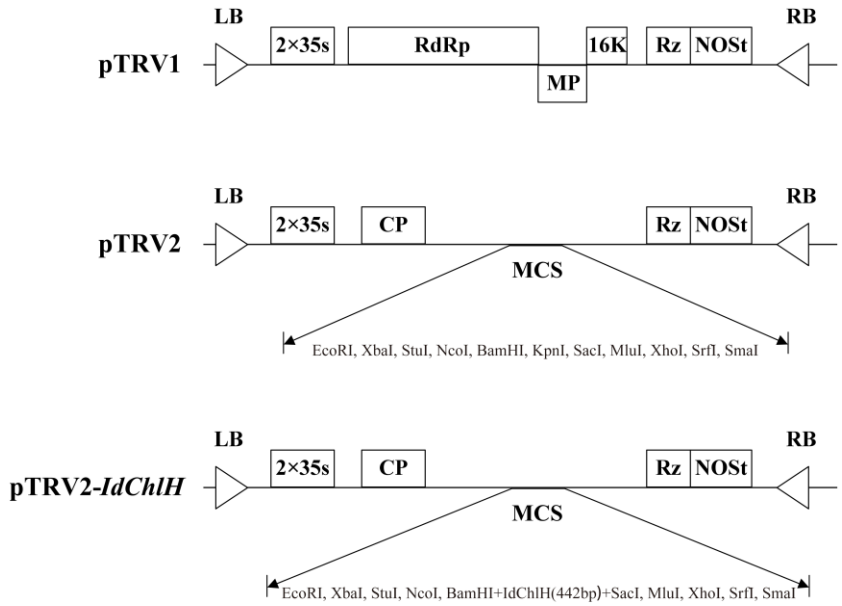

Figure 4. TRV-based VIGS vectors and construction. pTRV2-*IdChLH* were constructed to analyze the ability of TRV vectors to silence endogenous *IdChLH* gene in *I. dabieshanensis*.

3.3. VIGS-Mediated Silencing of the IdChlH Gene in I. dabieshanensis

In this study, forty-six uniformly-sized *I. dabieshanensis* plants were inoculated with *Agrobacterium strain* GV3101 containing the pTRV2-*ChlH* vector. All treated plants survived, suggesting that this method was suitable for the tested plants. Approximately 14 days post infiltration (dpi), the yellow-leaf phenotype started appearing in the newly developed

leaves of the plants partially treated with pTRV2-*ChlH* vector. At 21 dpi, 84.8% of the treated *I. dabieshanensis* plants showed a yellow-leaf phenotype in all newly developed leaves (Table 2; Figure 5A). Additionally, compared with the control plants, the plants infiltrated with pTRV1 and pTRV2 (Mock) showed no significant differences in leaf phenotype at 21 dpi (Figure 5B,C). Figure 5D shows the leaves collected from plants inoculated with the pTRV2-*ChlH* vector, empty vector-infected plant (Mock), and the control plant (CK), separately. These results suggest that yellow-leaf phenotypes might be induced by the silencing of the endogenous *IdChlH* gene.

Table 2. Silencing efficiency of *IdChlH* in *I. dabieshanensis* using TRV-based VIGS system at 21 dpi.

Table	Number of Treated Plants	Silencing Efficiency [a]
pTRV2-*IdChlH*	46	39/46 (84.8%)
Mock	9	0/9 (0%)
Control	9	0/9 (0%)

[a] Number of plants exhibiting silencing phenotypes/number of treated plants.

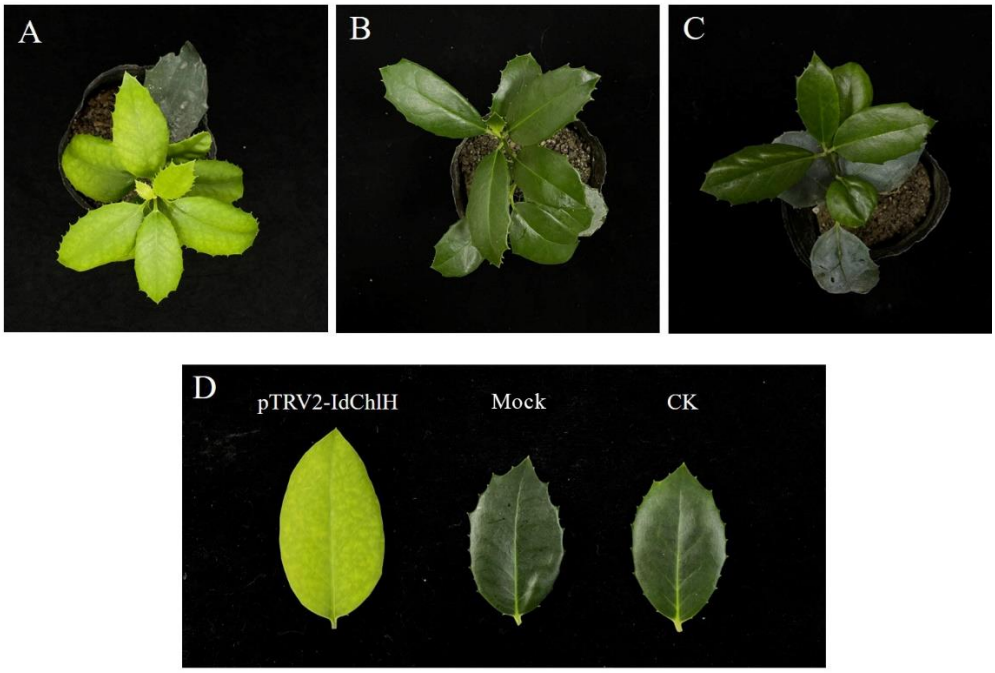

Figure 5. TRV-mediated *IdChlH* gene silencing in *I. dabieshanensis*. (**A**) *I. dabieshanensis* plants infected with pTRV2-*IdChlH* exhibiting yellow-leaf phenotype in newly developed leaves at 21 days post infiltration (dpi). (**B**) Empty vector-infected plants (Mock) with the normal phenotype. (**C**) Control plants (CK). (**D**) Leaf phenotypes after various VIGS treatments. Photographs were taken at 21 dpi.

3.4. qRT-PCR Analysis

To further investigate the efficiency of gene silencing, the transcript levels of *IdChlH* in plants inoculated with pTRV2-*IdChlH* were detected using qRT-PCR. The *IdChlH* transcript levels reduced by 77.2%–83.8% in the silenced plant leaves compared to control plant leaves (Figure 6). Furthermore, the *IdChlH* transcript levels in the control and mock-infected leaves were similar (Figure 6). The phenotypic characteristics were consistent with the expression profile of *IdChlH*. These results indicated that pTRV2-*IdChlH* could induce the yellow-leaf phenotype by silencing endogenous *IdChlH* in *I. dabieshanensis*.

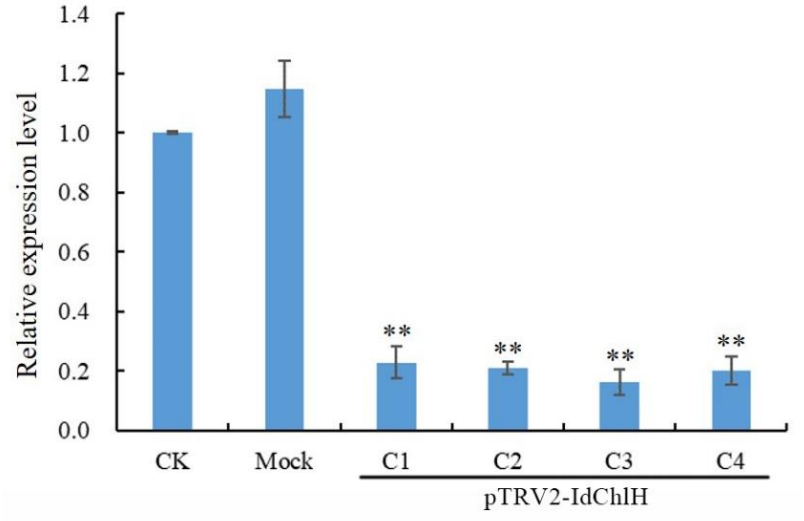

Figure 6. Relative expression levels of *IdChlH* in the leaves of plants exhibiting the silenced phenotype (pTRV2-*IdChlH*), empty vector-infected plants (Mock), and control plants (CK). The error bars represent the ± standard error of three replicates. Asterisks indicate statistically significant differences according to Duncan's multiple range test (** $p < 0.01$).

4. Discussion

Gene function studies have promoted an in-depth understanding of the molecular mechanisms in plants. However, identifying gene functions in woody plants is challenging and time-consuming using traditional genetic transformation. VIGS is an effective tool for characterizing gene functions in plenty of herbaceous plant species [16,18,20,26]. Recently, the application of VIGS has been successfully extended to some woody plants, such as *Vitis vinifera* [27], *Vernicia fordii* [28], and Rosaceae fruit trees [29]. However, the VIGS has rarely been investigated in many other woody plants, due to the lack of a compatible VIGS vector [28,30]. To date, there are only a few VIGS-inducing virus vectors which have been tested in woody plants, including apple latent spherical virus (ALSV) [29], grapevine leafroll-associated virus-2 (GLRaV-2) [27], poplar mosaic virus (PopMV) [31], plum pox virus (PPV) [32], and TRV vector [28]. Among them, TRV is the most widely used VIGS vector because of its wide host range and relatively mild symptoms of infection [28,33]. However, it is unknown whether TRV-based VIGS can be used to dissect gene functions in *I. dabieshanensis*. The present study reported the first application of a TRV-based VIGS system in *I. dabieshanensis*. This tree species has a long growth cycle, and its genetic transformation process is laborious and technically challenging. Therefore, developing a rapid and effective transformation system is important for gene function analysis in *I. dabieshanensis*. We herein described a TRV-based VIGS system for efficient silencing of endogenous genes in *I. dabieshanensis*. In the future, this system has the potential to provide a powerful tool for the large-scale reverse-genetic analysis of gene functions in *I. dabieshanensis*.

The *ChlH* gene encodes the H subunit of magnesium chelatase, which is involved in chlorophyll biosynthesis, and has been widely used as an indicator gene of VIGS because its silencing generates a yellow-leaf phenotype [13,24,34]. In this study, multiple sequence alignment showed that IdChlH was considerably similar with the ChlH proteins of other plant species. Furthermore, a phylogenetic tree showed that IdChlH was highly similar to CsChlH acquired from *Camellia sinensis*. Here, we adapted a TRV-based VIGS system that could reveal the function of the *ChlH* gene in *I. dabieshanensis*. Our results showed that newly developed leaves exhibited the silencing phenotype after VIGS treatment, suggesting the establishment of systemic TRV viral infection in *I. dabieshanensis*.

Previous studies have shown that the most effective and cost-effective way to inoculate plants with viral vectors is agroinfection [35], but its efficiency varies among different plant

species. In rice, 77% of plants agroinfected with pRTBV-MVIGS-PDS showed a white streak leaf phenotype; moreover, the silencing efficiency of RTBV-VIGS in *Cynodon dactylon* was compared with that of rice, wherein 65.8%–72.5% of the agroinoculated seedlings showed symptoms typical of *PDS* gene silencing, while the silencing efficiency in *Zoysia japonica* was much lower, with only 52.7%–55% agroinoculated seedlings showing white streak leaf symptoms [36]. Further, Jiang et al. [28] tested the feasibility of the TRV vector to directly infect woody plant species, and found that TRV-mediated VIGS was effectively elicited in *Vernicia fordii*, weakly in white poplar hybrid, but not in *Camellia oleifera*. Mustafa et al. [14] showed that TRV-mediated silencing was complete in *Gossypium hirsutum* and *G. arboretum*, but the silencing efficiency differed for each *G. hirsutum* variety. In our study, 84.8% of the agroinoculated plants developed a yellow-leaf phenotype, indicating that the silencing efficiency of TRV-based VIGS in the *Ilex* plant was high. Furthermore, qRT-PCR showed that the *IdChlH* transcripts were significantly decreased in the silenced plant leaves. However, the efficiency of TRV-based VIGS and the subsequent recovery varied between woody species [14,28]. Therefore, specific VIGS treatment conditions need to be screened and optimized for other holly species of interest in the future. Overall, our VIGS system described here will pave an important way for gene function studies in *I. dabieshanensis*.

5. Conclusions

In conclusion, we established a TRV-based VIGS system that could effectively silence genes in *I. dabieshanensis*, which increases the growing list of woody plant species that can be used for VIGS-mediated research. This established system provides a powerful tool for functional genomic studies on *I. dabieshanensis*. In future, the TRV-based VIGS system will be used to determine the functions of genes related to fruit development, synthesis of medicinal components, and stress resistance in *I. dabieshanensis*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14030488/s1, Figure S1. Location of the *I. dabieshanensis* sample. Figure S2. Verification of the construction of the pTRV2-*IdChlH* vector. (A). Detection of pTRV2-*IdChlH* plasmid. M: 2000 DNA marker; 1–7: PCR verification of pTRV2-*IdChlH* monoclonal bacterial solution. (B) Alignment of the sequencing result of pTRV2-*IdChlH* and the inserted *IdChlH* fragment. C-A: sequencing result of pTRV2-*IdChlH* plasmid; C-B: sequencing result of insert *IdChlH* fragment.

Author Contributions: X.C. and H.C. conceived and designed the project. Y.W., X.X. and F.Z. performed the experiments. C.W., Y.Z. and T.Z. prepared the figures and/or tables. X.C. wrote the manuscript. Y.L., X.L. and H.C. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data are available from the corresponding author upon request.

Conflicts of Interest: The authors declare no competing interests.

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