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Establishment of a Virus-Induced Gene-Silencing (VIGS) System in *Passiflora edulis* Sims and Its Use in the Functional Analysis of *PechlH*

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Abstract: Passion fruit is a very important tropical and subtropical fruit that not only has a good edible flavour, but also has high ornamental value. However, the functions of important trait genes in passion fruit have been difficult to elucidate due to the difficulty of stable genetic transmission in passion fruit. The VIGS system for the tobacco rattle virus (TRV) has been widely used in a number of crops, but no success has been reported in passion fruit. In this study, we used the *PePDS* marker gene to unravel the passion fruit VIGS system. The use of albino phenotype observation and quantitative PCR to detect gene expression showed that the PDS albino phenotype was successfully induced in the young leaves of passion fruit seedlings after 7 days, while a significant down-regulation of expression occurred in the phenotypic plants. The albino phenotype can be maintained for 14–16 d. In addition, we chose the chlorophyll-synthesis-related gene *PechlH* for the VIGS silencing of passion fruit leaves. When the *PechlH* gene was knocked out, the leaves of the passion fruit lost their green colour and showed yellowing symptoms. This revealed that the *PechlH* gene was involved in the chloroplast synthesis pathway. Finally, using different OD_{600} gradients in *PePDS*, we demonstrated that the silencing efficiency reached 46.70% at an OD_{600} of 0.8. Thus, an effective and stable VIGS system was achieved in passion fruit. This will help to accelerate the study of gene function in passion fruit in the future.

Keywords: *Passiflora edulis*; virus-induced gene silencing (VIGS); tobacco rattle virus (TRV); *PePDS*; *PechlH*

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1. Introduction

Passion fruit (*Passiflora edulis* Sims), is found in tropical and subtropical regions around the world. It is popular for its balanced nutrition and good taste [1]. Passion fruit (*Passiflora* spp.) fruit are typically available in three different colours: purple, red, and yellow [2]. The differences between the yellow and purple fruit are mainly concentrated in the higher accumulation of flavonoids and carbonyl glycosides in yellow fruit, and higher flavonols, anthocyanin glycosides, and flavanols in purple fruit [3]. The majority of *Passiflora* spp. are diploid, but they vary in the number of chromosomes [4]. Genetic engineering has shown great potential for the genetic improvement of certain varieties, compensating well for the limitations of traditional crossbreeding. In 1994, the transformation of Passiflora leaves and stems with the pMON200 plasmid using *Agrobacterium tumefaciens* was achieved for the first time [5]; however, the efficiency of transformation with *Agrobacterium* was very low [6], at only 0.67% [7]. Our laboratory has developed a method for the in situ conversion of passion fruit that can achieve up to 26% conversion [8]. For this reason, it is important to identify a more efficient method for the transformation of *Agrobacterium* in passion fruit.

Virus-induced gene silencing (VIGS) is an essential method for analysing gene function in many species that are not amenable to stable genetic transformation [9]. In 1995, Kumagai et al. inserted a fragment of the coding sequence of the PDS cDNA into a viral vector and transfected it into *Nicotiana benthamiana* leaves, observing the down-regulation of PDS expression in combination with the white leaf phenotype [10]. Virus-induced gene silencing (VIGS) is an RNA-mediated reverse genetics technology that is now widely used in arable crops, horticultural crops, and forest trees [11]. The VIGS system showed the silencing of genes for odour, flower colour, and flower structure in roses, with the corresponding phenotypes observed 5–6 weeks post infiltration [12]. In olives, using the plant's post-transcriptional gene-silencing mechanism to induce *GOI* silencing [13], a fragment of the endogenous gene of interest (*GOI*) was introduced using the VIGS system. The VIGS system was also successfully applied to tea tree, where silencing the *CsTCS1* (caffeine synthase) gene reduced caffeine levels by approximately 6.26 times compared to the wild type [14]. In this study, the TRV-mediated VIGS system of passion fruit was constructed using the reporter gene *PDS*. This system was used to knock out the *PechlH* gene, and chloroplast yellowing occurred. The establishment of this system in passion fruit will contribute to the study of functional genes in passion fruit and accelerate the process of passion fruit genome research, as well as improving the breeding efficiency of passion fruit and creating new varieties with better agronomic traits and stress tolerance.

2. Materials and Methods

2.1. Plant Material and Growing Conditions

Mature passion fruit seeds of *Passiflora edulis* Sims were used. The pericarp was cut to remove the seeds, which were washed and dried naturally. Healthy seeds were selected, washed with water, and sown in disposable seedling pots containing a mixture of substrate soil, vermiculite, and perlite (2:1:1) and transferred to an artificial climate chamber for cultivation with culture conditions of 25 °C, 70% relative humidity, and a photoperiod of 16 h light/8 h dark.

2.2. Gene Cloning and Vector Construction

The passion fruit PDS gene (ZX.08G0011290), named *PePDS*, was screened in the passion fruit genome using bioinformatics comparison analysis. The sequences containing only the PDS domain were amplified using high-fidelity enzymes (Vazyme, Nanjing, China, P501) with passion fruit cDNA as a template and primers designed by Premier5.0, and then inserted into the pGXT vector. The restriction endonucleases *Eco*RI and *Xho*I (Takara, Kusatsu City, Japan) were selected for the double digestion of the pTRV2 vector. A specific fragment of 283 bp in the *PePDS* open reading frame was selected and amplified with primers, and the recombinant plasmid pTRV2-*PePDS* was constructed using a seamless cloning kit (Vazyme, Nanjing, China, C113). The plasmid was introduced into *Agrobacterium tumefaciens* GV3101 and the bacterial solution was stored at −80 °C. The pTRV1 and pTRV2 vectors were provided by Jian Wu's team at the Academy of Horticulture, China Agricultural University [15]. In addition, for the pTRV2-*PeCHIH* gene of the passion fruit chlorophyll synthesis gene *PechlH*, the vector construction and bacterial preservation method were consistent with the above steps. All primers are shown in Table S3.

2.3. Agrobacterium Infestation

We selected passion fruit seedlings for *Agrobacterium* infection. The seedlings were grown from seed to two cotyledons in about 20 days. Single colonies of *Agrobacterium* carrying the target plasmid were picked and inoculated into 1 mL of LB liquid medium containing 50 mol·L kanamycin and 50 mol·L rifampicin and cultured for 24 h at 28 °C with 250 rpm shaking. The cultured bacterial solution was centrifuged at 8000 rpm for 6 min, and the supernatant was discarded and resuspended with the infiltration solution (10 mmol MES, 200 μmol AS, 10 mmol $MgCl_2$, pH 5.6) to the appropriate concentration. The bacterial fluids containing pTRV1 were mixed 1:1 (*v*/*v*) with the bacterial fluids of

pTRV2 and pTRV2-*PePDS*, respectively, and allowed to stand for 3–4 h at room temperature in the dark for infestation. Each replicate in all treatments was injected into 30 plants and each treatment was replicated three times.

The infestation methods used were vacuum infiltration and foliar injection. Vacuum infiltration was carried out by gently making wounds in the cotyledons of the passion fruit with a 1 mL needle, placing them in the bacterial solution, and soaking them at an air pressure of 0.8 KPA and vacuum infiltration time of 10 min, before planting them in nutrient soil. Foliar injection was performed by gently making a wound in the passion fruit cotyledon with a 1 mL needle and then injecting the fungal solution from the back of the leaf throughout the cotyledon using a 1 mL de-needled syringe. After injection, the seedlings were placed in a 27 °C dark culture room for 1–2 days and then transferred to a 27 °C light culture room for normal growth culture.

2.4. Fluorescence Quantitative PCR Analysis

TRV-specific primers were designed to detect virus invasion in passion fruit plants by PCR. Non-injected plants were used as a blank control and plants injected with an empty vector were used as a negative control. The relative expression of the *PePDS* and *PechlH* genes was determined using RT-qPCR using primers with 60S as the internal reference gene.

2.5. Data Analysis

Different letters indicate significant differences at $p < 0.05$ and highly significant differences at $p < 0.01$. The qRT-PCR data were analysed using SPSS v28.0.1.1 for analysis of significant differences ($p < 0.05$).

3. Results

3.1. TRV-Mediated VIGS System Successfully Applied to Passion Fruit

3.1.1. Homologous Cloning of *PePDS* and *PechlH* Genes in Passion Leaf

We used the annotated gene information in the passion fruit genome to perform the homologous cloning of two genes: *PePDS* and *PechlH* (Figure 1A,B). The partial structural domain sequence of the *PePDS* cDNA was amplified to 1695 bp, whereas the full-length cDNA of the *PechlH* was amplified to 4146 bp. A 283 bp and a 315 bp fragment were selected for recloning to construct the TRV2 silencing vector (Figure 1C,D). Moreover, evolutionary tree and amino acid sequence analyses showed that the *PechlH* gene in passion fruit is not highly homologous to other species (Figure S1).

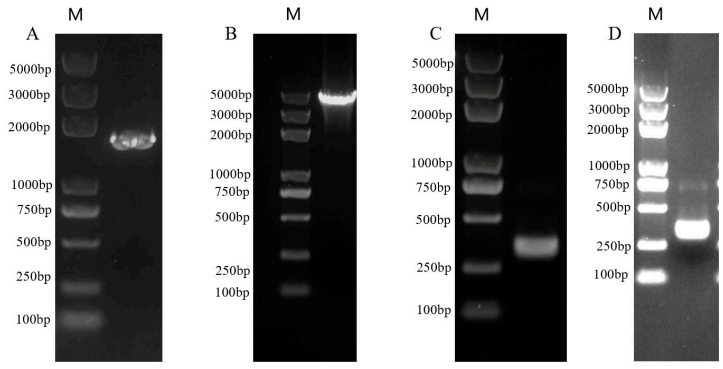

Figure 1. Full-length gene and TRV-silencing-fragment amplification. (**A**) The sequences containing only the *PePDS* domain, (**B**) the full-length gene amplification of *PechlH*, (**C**) the TRV-silencing-fragment amplification of *PePDS*, and (**D**) the TRV-silencing-fragment amplification of *PechlH*. M: DL2000 marker.

3.1.2. TRV-Mediated System Successfully Validated *PePDS* Gene

To test whether the TRV-based vector was effective in silencing and suppressing endogenous genes in passion fruit seedlings, we chose *PePDS* as a marker gene. The *PePDS* gene is one of the key genes in leaf green biosynthesis, and the inhibition of the expression

of this gene results in the photodegradation of chlorophyll or the loss of greenness of the plant leaves. Passion fruit seedlings at the cotyledon stage were infected by mixing equal volumes of *Agrobacterium* containing the pTRV1 vector and *Agrobacterium* containing the pTRV2-*PePDS*-silencing expression vector using a syringe. Passion fruit seedlings at the cotyledon stage were infected with an equal volume of the empty vectors pTRV1 and pTRV2 as a control. After 7 d of infestation, the new-growth leaves of the passion fruit seedlings began to show the photobleaching phenomenon (Figure 2A). The main manifestation was a phenotype of vein whitening or whitening of new shoots followed by the appearance of large areas of leaf whitening. The results showed that the albino phenotype could be maintained for 14–35 d. The newborn leaves of the control maintained normal growth, indicating that the silencing phenotype of the *PePDS* gene using the VIGS system in passion fruit seedlings was successful.

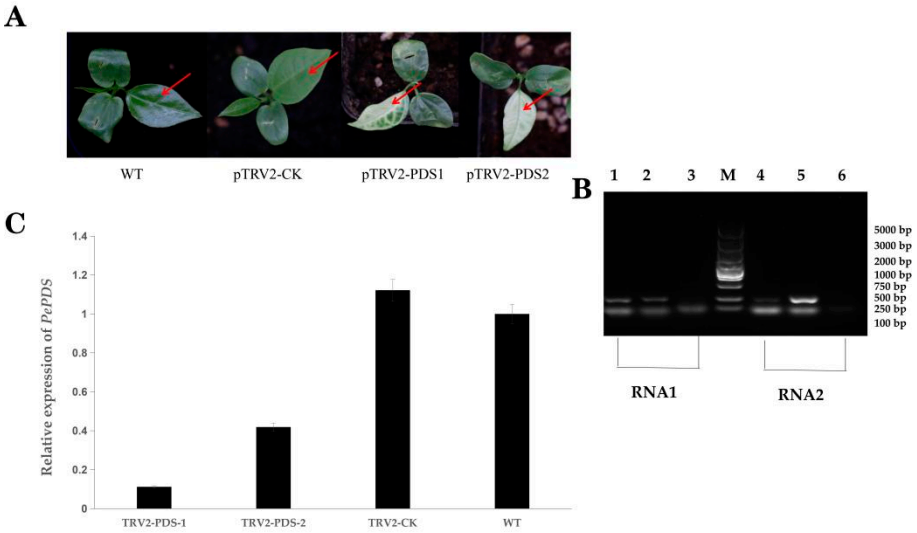

Figure 2. (**A**) TRV-mediated VIGS infestation of the *PePDS* gene in young passion fruit leaves (WT, wild type passion seedlings; pTRV2-CK, seedlings infected with pTRV1 + pTRV2 *Agrobacterium*; pTRV2-*PePDS*1 and pTRV2-*PePDS*2, seedlings infected with pTRV1 + pTRV2-*PePDS Agrobacterium*). (**B**) RT-PCR detection of RNA1 and RNA2 of TRV1 and TRV2 in the seedlings of passion fruit. Experiments 1–3 detected the strand of viral pTRV1, and the remainder detected the strand of viral pTRV2 (1, seedlings infected with pTRV1 + pTRV2; 2, seedlings infected with pTRV1 + pTRV2-*PePDS*; 3, uninfected leaves; 4, seedlings infected with pTRV1 + pTRV2; 5, seedlings infected with pTRV1 + pTRV2-*PePDS*; 6, uninfected leaves). M: DL5000 marker. (**C**) Quantitative gene expression assay between VIGS and wild type. The error bars represent the mean ± SE of three independent experiments. SE, standard error; TRV, tobacco rattle virus; WT, wild type. Red heads indicate an albino phenotype on the leaves. RNA1 refers to mobile proteins (MPs), while RNA2 refers to coat proteins (CPs).

Using PCR detection, specific bands of viral pTRV1 and pTRV2 were detected in negative controls transfected with the empty vector and in the leaves of pTRV2-*PePDS*-silenced plants (Figure 2B). The corresponding bands were not detected in blank plants without infested passion fruit seedling leaves. The RT-qPCR results revealed that the expression of *PePDS* was reduced by 90%, 58%, and 89% in the phenotypic leaves, phenotypic upper leaves, and injected leaves of pTRV2-*PePDS* plants compared with the empty-vector control (Figure 2C).

3.1.3. TRV-Mediated System Successfully Validated *PechlH*

To further validate the effect of VIGS in passion fruit, the VIGS system was again validated using the chlorophyll-synthesis-related gene *PechlH*. Foliar injection was used with a bacterial solution concentration of 0.8 OD and an incubation temperature of 25 °C. The results showed that plants silenced with *PechlH* showed yellowing symptoms in new

leaves and the negative control grew normally (Figure 3A). In the yellowing-phenotype leaves, expression fragments of MP and CP were detected in TRV1 and TRV2 (Figure 3B). The efficiency of silencing was 68.18%. The results of the quantitative expression levels indicated that the relative expression of *PechlH* was significantly down-regulated in all leaves of passion fruit seedlings with pTRV2-*PechlH* compared to the control (Figure 3C).

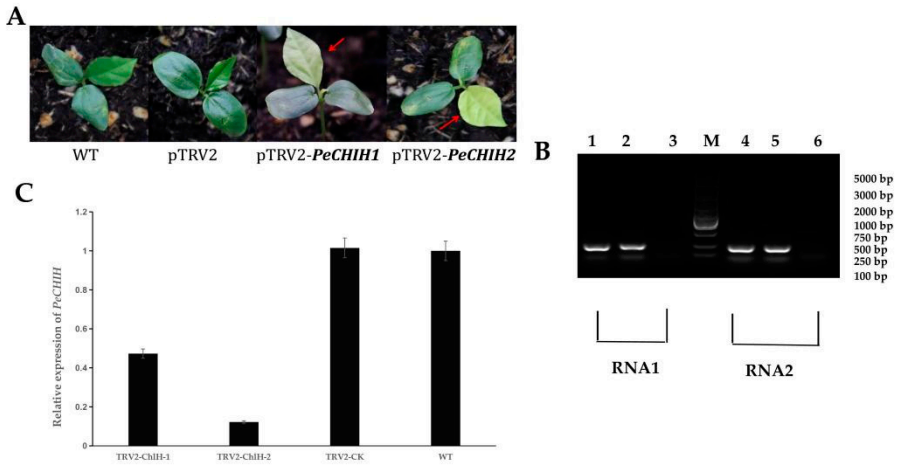

Figure 3. (**A**) TRV-mediated VIGS infestation of the *PechlH* gene in young passion fruit leaves (WT, wild type passion seedlings; pTRV2, seedlings infected with pTRV1 + pTRV2 *Agrobacterium*; pTRV2-*PechlH*1 and pTRV2-*PechlH*2, seedlings infected with pTRV1 + pTRV2-*PeCHLH Agrobacterium)*. (**B**) RT-PCR detection of RNA1 and RNA2 of TRV1 and TRV2 in the seedlings of passion. Experiments 1–3 detected the strand of viral pTRV1, and the remainder detected the strand of viral pTRV2 (1, seedlings infected with pTRV1 + pTRV2; 2, seedlings infected with pTRV1 + pTRV2-*PechlH*; 3, uninfected leaves; 4, seedlings infected with pTRV1 + pTRV2; 5, seedlings infected with pTRV1 + pTRV2-*PechlH*; 6, uninfected leaves). M: DL5000 marker. (**C**) Quantitative gene expression assay between VIGS and wild type. The error bars represent the mean ± SE of three independent experiments. SE, standard error; TRV, tobacco rattle virus; WT, wild type. Red heads indicate a yellowing phenotype on the leaves.

3.2. Optimised VIGS System in Passion Fruit

Based on the previous successful infestation of passion fruit seedlings using VIGS for the *PePDS* gene, the system was further optimised using a gradient of *Agrobacterium* concentrations. The pump pressure was maintained at 0.8 kPa. The OD_{600} of the *Agrobacterium* concentrations was selected in three gradients: 0.8, 1.0, and 1.2. The results showed that the silencing efficiency was highest for OD_{600} below 1.0 at 46.70% (Table S1).

4. Discussion

VIGS has been used in a number of important agricultural and forestry species, including sunflower, peanut, and rose, as well as other tree species [16]. In addition, VIGS's ease of use and speed of application help in the interpretation of unknown genes, especially in some species whose genomes have not yet been published [17]. Currently, the genetic transformation research regarding passion fruit mainly involves the *Agrobacterium* infestation of stem segments and in situ transformation techniques, which have the drawbacks of complicated operation and a long cycle time. In this study, we found that VIGS based on TRV could be used to reveal the function of the *PDS* and *chlH* genes in *Passiflora edulis*. Our results clearly show that VIGS-infected leaves exhibited a silent phenotype indicative of TRV virus infection (Figures 2A and 3A). To date, this study is the first to show that the VIGS of the TRV can be successfully applied to the functional identification of genes in passion fruit.

The first of two difficult steps in setting up the VIGS system for passion fruit was to decide which seedling period was crucial. Mature passion fruit leaves are thicker and heavier than cotyledon-stage leaves, and foliar injections tend to cause leaf breakage. This

seedling age refers to seedlings at the stage of unfolding of the cotyledon, which is when seedlings are prone to infestation. Secondly, the parameters that affect *Agrobacterium* infection also include infection time, vacuum pump pressure, *Agrobacterium* strain, and growth temperature [18]. In the present study on passion fruit, the highest infestation efficiency of 83.33% was observed at OD_{600} and under a vacuum pressure of 0.8 Kpa (Table S1). In this study, it was found that the leaf injection method had a higher infiltration efficiency than the vacuum injection method. It was hypothesised that due to the fact that passion fruit leaves have a thicker waxy layer compared to tobacco leaves, and therefore their leaves are less permeable, the vacuum injection method was likely not applicable to passion fruit and the foliar injection inoculation method was subsequently selected for the operation. Meanwhile, the infestation rate varied considerably between varieties, with guava-flavoured varieties being the highest at 35.6%, compared to Tainong, purple passion fruit, and Qin Mi No.9 (Table S2). In addition, the culture temperature, infiltration solution concentration, selection of specific fragments, and different Agrobacterium strains can all affect gene silencing efficiency. Therefore, the passion fruit VIGS system can be continuously optimised for different factors.

To improve the reliability of the results, we chose the classic reporter genes *PDS* and *chlH* for the successful establishment of passion fruit VIGS [19–21]. A gene related to chlorophyll synthesis, *PechlH*, was screened from the passion fruit genome and was hypothesised to be the catalytic subunit of magnesium chelatase, thus ensuring its activity, which may play an important regulatory role in chlorophyll synthesis [22]. Bioinformatic analysis showed that its CDS region is 4146 bp long and encodes 1382 amino acids, with 164 amino acids differing from the sequence in the ZX passion fruit genome (ZX.01G0052410) (Figure S1A); it is speculated that these amino acid differences may lead to differences in the function of *PechlH* proteins from different species. The protein was also predicted to belong to the PLN03069 superfamily structural domain, which is the same as the *chlH* gene of other species. In particular, for the *PechlH* gene, the clustering results indicate that *Passiflora edulis* is related to *Turnera subulate*, whose amino acid sequence differs significantly from that of other species (Figure S1). The pTRV2-*PechlH* silencing vector was used to infect passion fruit seedlings and it was found that the infection could cause the newborn passion fruit leaves to lose their green colour and turn yellow. This finding is in line with those of previous studies [23–25]. In addition, the gene expression of the silenced gene group was significantly lower than that of the control group, as shown by qRT-PCR and other assays, and the expression of the *PechlH* gene was significantly suppressed. For the quantitative PCR analysis, we chose the more stable endogenous *60S*, as opposed to the traditional endogenous genes (*ACTIN*, *GAPDH*, and *UBQ*) [26–28].

In conclusion, when the genetic transformation of passion fruit became difficult, we effectively established its transient expression system, which could advance the research of passion fruit functional genes, especially regarding the acceleration of the resolution of genes at the seedling stage.

5. Conclusions

Passion fruit does not currently have a very effective means of stable transformation, so we used the VIGS system to identify a transient expression method suitable for virus-infected and phenotypically stable passion fruit, which solved the problem of the difficulty of studying the function of passion fruit genes and constructed a practical research pathway in the field of passion fruit genome research. Using the VIGS system, we also showed, for the first time, that *PechlH*, a key enzyme gene, is involved in chlorophyll synthesis in passion fruit leaves, causing severe yellowing of the leaves.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10040422/s1.

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tion, Z.L.; writing—review and editing, M.M.A., L.Z., X.Y., S.W., Z.L. and F.C.; visualization, M.M.A., X.Y., S.W. and L.Z.; supervision, Z.L. and F.C.; project administration, Z.L.; funding acquisition, Z.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data is contained within the article or Supplementary Materials.

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Conflicts of Interest: The authors declare no conflicts of interest.

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