Tropical Plants

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https://doi.org/10.48130/tp-0024-0025

*Tropical Plants* **2024**, in press

# Construction and application of virus-induced gene silencing system in taro

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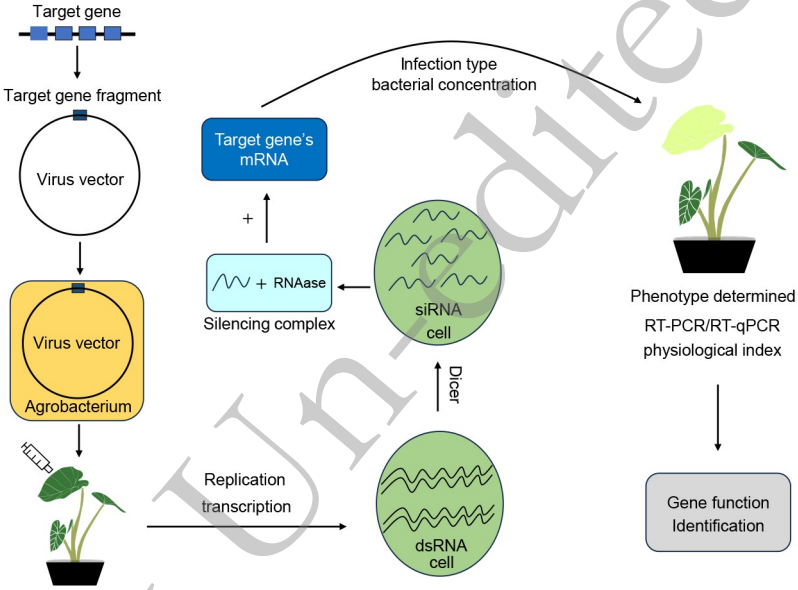
## In Brief

Taro is a tropical and subtropical crop with high economic benefits, which is considered as orphan crop and lack gene function verification system. In this study, we used CePDS as indictor gene to construct VIGS system in taro, then further optimized bacterium concentration to increase the silencing plant rate, and verified the function of CeTCP14 preliminarily during corm expansion to prove the robustness of this system. Gene function verification would promote germplasm resources utilization and taro breeding improvement.

## Graphical abstract




## Highlights

- *CePDS* was used as indictor gene to constructed VIGS system in taro.
- CePDS was used as indictor gene to constructed VIGS system in taro.
- *CeTCP14* was selected to further verify the robustness of this system.



**Citation:** Gui Y, Li B, Zhu Q, He Y, Zhang Y, et al. 2024. Construction and application of virus-induced gene silencing system in taro. *Tropical Plants* https://doi.org/10.48130/tp-0024-0025

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# Construction and application of virus-induced gene silencing system in taro

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## Abstract

Virus-induced gene silencing (VIGS) technique is an important means for rapid identification of plant gene function, and a stable VIGS system in taro can lay a technical support for rapid and efficient gene function verification. We constructed VIGS system with tobacco rattle virus (TRV)-based vector, and phytoene desaturase (*CePDS*) as indicator gene, then silence *CeTCP14* to further verify the robustness of this system. First, we used Ganyu No.1 as material to constructing taro VIGS system by leaf injection method with *CePDS* at $OD_{600}$ = 0.6, and the silencing plant rate was 12.23%, the expression level of *CePDS* was about 59.34%−77.18% compared to the control, and the chlorophyll content decreased 37.80%−56.11% in *CePDS* silencing plants. The silencing plant rate increased significantly by leaf injection at $OD_{600}$ = 1.0, reached to 27.77%, but there was no significant difference in silencing plant rate between leaf injection method and bulb vacuum treatment. We further silenced *CeTCP14* with bacteria solution $OD_{600}$ = 1.0 and bulb vacuum treatment in Ganyu No.2, the silencing plant rate was 20%, and the expression level of *CeTCP14* was 43.94%−63.34% of the control. Meanwhile, the starch content in the corms decreased significantly to 70.88%−80.61% compared to the control. In conclusion, the results indicated that TRV-based VIGS system is effective in taro. The concentration of bacterial solution is a key factor affecting VIGS system, *CeTCP14* can affect starch accumulation in taro bulbs. The robust VIGS system establishment in taro could lay a good foundation for the subsequent rapid gene function verification.

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## Introduction

Taro (*Colocasia esculenta* (L.) Schott), from Araceae family, is a tropical and subtropical crop, which is common used as food, vegetable and industrial processing raw material with high economic benefits. It ranks 5th among root crops and 14th among vegetable crops in the world[1], and world annual production of taro in 2022 was estimated at 19.67 million tons (FAOSTAT 2024). Taro is typically propagated from corms with long life cycle (harvested usually around 6 to 12 months after planting) that lead scientists to largely overlook it.

For it is rich in mucus protein, polysaccharide, vitamins, mineral elements and many other physiological active substances, which have been applied to alleviate a variety of human sub-health symptoms[2]. It can be predicted that taro will be a promising crop. In recent years, taro and its products are favored by consumers because of their unique nutritional value and healthcare functions, higher requirements are also put forward for new varieties, and clear gene function will lay the foundation for high-quality new varieties breeding to further meet consumer needs.

As research continues, the elucidation of gene function is urgent, but the genetic transformation system is still unstable in taro, which hinders the gene functional verification and greatly limits the utilization of excellent taro germplasm resources. It is urgent to build an effective gene function verification system. Due to virus-induced gene silencing (VIGS) advantages of stability, high efficiency, simplicity, low cost, high throughput and short cycle, it has been widely applied in the gene function research of vegetable crops[3]. The robust VIGS system establishment in taro can not only verify gene function, but also greatly promote the utilization of germplasm resources, which has an important role in promoting the improvement and breeding of taro.

At present, VIGS technology has been widely applied in the study of gene function related to growth and development, stress, substance synthesis and metabolic regulation of vegetable crops[3−5]. However, the silencing efficiency induced by viral vectors is host-depended. Based on previous studies, several viral vectors have been successfully applied in VIGS, including tobacco mosaic virus (TMV), potato virus X (PVX), tobacco rattle virus (TRV), tomato golden mosaic virus (TGMV), cabbage leaf curl virus (CbLCV)[6]. TRV virus vector has been widely used in gene silencing experiments because of its high silencing efficiency, long silencing duration and mild virus symptoms[7,8]. During the VIGS system establishment process, phytoene desaturase (*PDS*) is often selected as target gene to indicate the success of gene silencing.

TCP TFs named after the first known members (TB1, CYC, and PCFs) that share a highly conserved TCP domain, which harbors a non-canonical basic-helix-loop-helix (bHLH) structural motif[9,10]. They are classified into two divergent groups; TCP class I and TCP class II, the later is further divided into two clades, CIN and CYC/TB1[9,11]. As a plant-specific transcription

factor, they play a crucial role in plant growth and development, including regulating flower organ development, leaf morphology and lateral branch growth[11]. Currently available evidence has shown that most TCP class I genes are activators of plant development, whereas TCP class II members often function as a repressor of various growth and development pathways[12–14]. But the function of TCP TFs in taro have not yet been reported.

Although VIGS technology has been widely used to verify plant gene function, there are no reports of VIGS technology in taro. The establishment of taro VIGS technology system would lay a foundation for rapid verification of gene function, and clear gene function that control important agronomic traits in taro, which would promote germplasm resources utilization and taro breeding improvement.

## Materials and methods

### Experimental material

Ganyu No. 1 and Ganyu No. 2 were used for VIGS system construction, the little corms with 1cm in diameter were used as test materials. pTRV1 and pTRV2 were selected as VIGS vectors and stored in our laboratory (Fig. 1a and b). Escherichia coli *DH5α* and Agrobacterium GV3101 receptor cells were purchased from Huayueyang Biotechnology (Beijing) Co., LTD.

### Vector construction for VIGS

First, the protein sequence of SlPDS (*Solyc03g123760*) from tomato and AtPDS (*AT4G14210*) from Arabidopsis are used to blast in taro reference genome Taro_JAAS_v1.0 (BioProject: PRJNA587719)[15]. Then, obtained sequence were validated in the transcriptome of Ganyu No. 1, which has been published in our laboratory[16]. At last, the *CePDS* (*EVM0008568.1*) sequence with 1743bp was obtained and verified, *CeTCP14* (*EVM0000825.2*) was screened out based on RNA-seq analysis, the gene sequences were shown in Supplement information. The mRNA from young leaves was reverse transcribed into cDNA, and then used as template, the primers used in this study list in Table 1, pTRV2-CePDS was constructed by restriction enzymes (EcoRI and BamHI), pTRV2-CeTCP14 was constructed by homologous recombination, and the vector construction was verified by sanger sequencing (Fig. 1c and d).

### Vector transformation

Plasmids pTRV1, pTRV2, TRV2-CePDS and pTRV2-CeTCP14 were transferred into agrobacteria GV3101 by freeze-thaw method[17]. First, 5 ml YEB solution containing rifampicin (25 mg/L) and kanamycin (50 mg/L) was used to culture bacteria by shaken, and colony PCR detection was performed with specific primers to obtain positive monoclones (Table 1). Then 500 μL bacteria solution was transferred to 50 mL of YEB solution containing MES (10 mM), acetosyringone (20 μM) and corre-

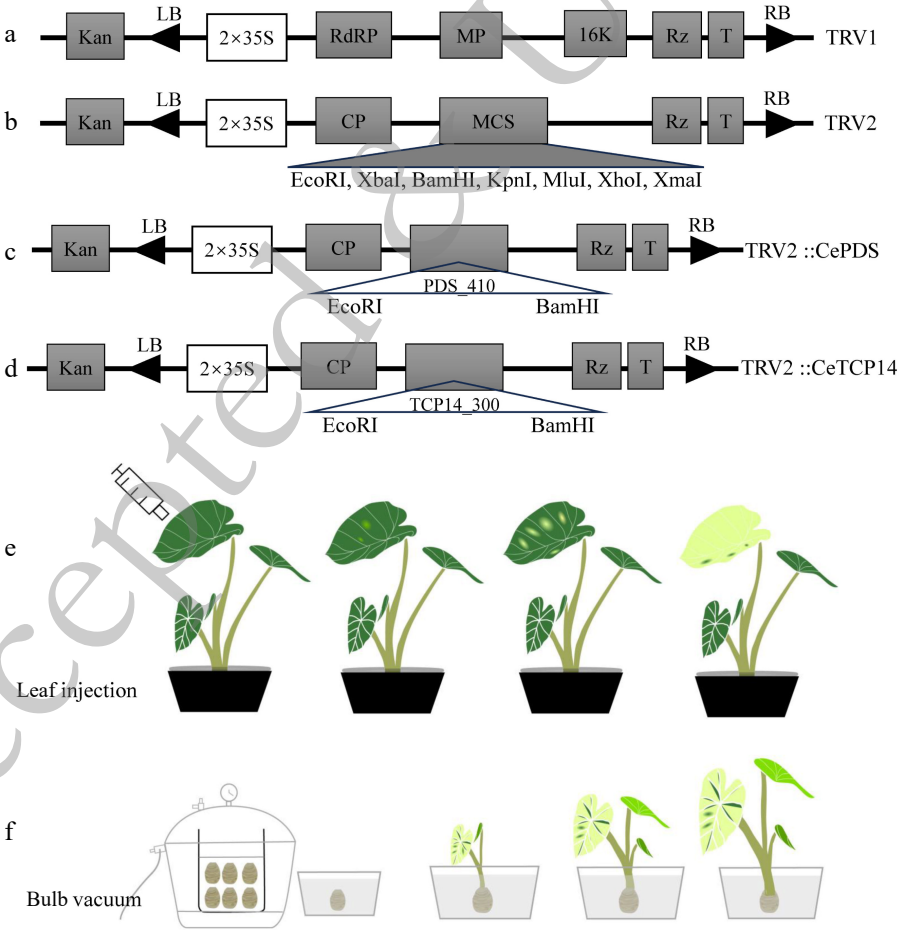


**Fig. 1** Vectors used and infection flow chart in this study. The plasmid profile of pTRV1 (A), pTRV2 (B), pTRV2::PDS410 (C), pTRV2::CeTCP14 (D). Color change of leaves by leaf injection (E) and bulb vacuum injection methods (F).

**Table 1.** Primers used in this study

| prime name | Primer sequence (5 '- 3') | Length | purpose |
|---|---|---|---|
| CePDS-EcoRI-F | **G**GAATTCATGGGCTTTACCAGTTCTCTTTCGG | 410 bp | used for fragments amplified of CePDS and CeTCP14 inserted in TRV2 vector |
| CePDS-BamHI-R | **CG**GGATCCTCCAGCAATATAGGCTTATGACCTG | | |
| TRV2-TCP14_F | GTGAGTAAGGTTACCGAATTCATGGGGGAGAGCCACCAG | 300 bp | |
| TRV2-TCP14_R | CGTGAGCTCGGTACCGGATCCATCGACGGCCTTGCTGGG | | |
| qCePDS-F | GGTCGTTGGGGAGGAAGC | 140 bp | Used for the qRT-PCR of CePDS |
| qCePDS-R | TCTAGTCGGGCGTGGTGA | | |
| qCeTCP14_F | CCACACCGCCATCCAGTT | 110 bp | Used for the qRT-PCR of CeTCP14 |
| qP_TCP14_R | CGAGCTCGTCTATGGCGG | | |
| CeActinF | CTAGTGGTCGCACAACAGGT | 191 bp | Used for the qRT-PCR of reference genes |
| CeActinR | TTCACGCTCAGCAGTGGTAG | | |
| TRV1F1 | CGTGTTGCATTTCGATGAA | 525 bp | Used for the detection of RdRp in TRV1 |
| TRV1R1 | GACAACGCCACGATTAAGT | | |
| TRV2F1 | GTTGAAGAAGTTACACAGCA | 407 bp | Used for the detection of coat protein in TRV2 |
| TRV2R1 | TCTTCAACTCCATGTTCTCT | | |
| pTRV2_F | TGTCAACAAAGATGGACATTGTTAC | 198bp/480bp | Used for the detection of TRV2 and TRV2-CeTCP14 expression |
| pTRV2_R | ACACGGATCTACTTAAAGAA | | |
| TRV2_F | TGTTACTCAAGGAAGCACGATGAGCT | – | Used for vector construction sequencing and colony PCR detection |
| TRV2_R | GTACAGACGGGCGTAATAACGCTTA | | |

note: underlined for cleavage sites, bold for protective bases

sponding antibiotics for overnight culture. The bacteria were centrifuged at 3000x when OD reach to 1.2, and collected with MS containing MES (10 mM), acetyl syringone (200 μM) and $MgCl_2$ (10 mM) at pH = 5.6 and let stand at room temperature for 1–2 h. Afterwards, pTRV1 was mixed with pTRV2, TRV2-CePDS and PTRV2-CeTCP14 resuspension in equal volume respectively, the mixed solution was used for infection in subsequent research.

## Agrobacterium infection

Leaf injection[18] and bulb vacuum infiltration[8] were applied in agrobacterium infection. Infection solution containing these four combinations: pTRV1+pTRV2, pTRV1+pTRV2-PDS, pTRV1+pTRV2-CeTCP14, negetive control (suspensions). In the leaf injection method, 1mL syringe without needle was used, and plant material with 2–3 fully unfolded leaves were selected for infection material, about 1/3–1/2 of the leaf area were injected, and then cultured in seedling room with 16h of light and 8h of darkness at 22–24 °C (Fig. 1e). For bulb vacuum method, the cleaned little corms with holes punctured by needles that facilitate infection liquid entry, the pressure was 750 mm Hg for 30 min, then wash the surface of the taro after infection. The plants were planted in pot (10 cm × 10 cm) and moved to the seedling room (Fig. 1f). The temperature and light conditions were the same as those of leaf injection method. Leaf color change was continuously observed.

## The determination of gene expression, chlorophyll content and starch content

After 20 days of infection, photobleaching phenotype appeared, the leaves/corms sample were collected, the RNA was extracted using the polysaccharide polyphenol plant RNA extraction kit (0416-50, Huayueyang Biotechnology (Beijing) Co. LTD), and the expression levels of *CePDS* and *CeTCP14* were detected by RT-qPCR, actin with stable expression level was selected as the internal reference, and the relative expression level of genes was normalized by $2^{-\Delta\Delta CT}$ method with three biological repeats[19]. The primers were list in Table 1.

After photobleaching phenotype occurred in the VIGS-CePDS group, leaves without coarse leaf veins were collected. The samples were cut into small strips and about 0.2 g of fresh samples were put into 50 mL centrifuge tubes with three biological repeats, add 25 mL 95% ethanol, and let stand for 36h until leave become white under dark condition. The well mixed supernatant was taken for spectrophotometer determination at wavelengths of 649 nm and 665 nm respectively, OD value, $A_{649}$ and $A_{665}$ were recorded. chlorophyll a and chlorophyll b were calculate as follows: $C_a = 13.95 \cdot A_{665} - 6.88 \cdot A_{649}$, $C_b = 24.96 \cdot A_{649} - 7.32 \cdot A_{665}$, total chlorophyll content = $C_a + C_b$[19].

After 20 days of VIGS-CeTCP14 suspension infection, 0.5g fresh corms flesh sample at the same sites were collected to determine starch content with three biological repeats. The methods were referred to Gao[20].

# Results

## Appearance of photobleaching phenotype in the leaves of CePDS-silenced seedlings

To confirm TRV-based vector could induce gene silence in taro, we selected Ganyu No. 1 as material for preliminary test with *CePDS* as indicator by leaf injection at $OD_{600}$ = 0.6. After 20d, the leaves began to show photobleaching phenomenon (Fig. 2b), about 10d later, nearly entire leaf turn white (Fig. 2c), there was no colour change in the negative control group and the empty-vector group (Fig. 2a, d). Then total number of plants showing photobleaching phenomenon was counted, and the average value is 3.67 (30 plants each time with three biological replicates). Therefore, TRV-based vector could induce gene silence in taro, but the proportion with photobleaching phenotype is low (12.23%).

## Detection of *CePDS* silent seedlings in taro

To verify that the phenotypic changes were caused by endogenous *CePDS* silencing through RNA expression derived from pTRV1 and pTRV2-PDS, RT-PCR was applied to detection. Transcriptional analysis of pTRV1 coat protein (CP) and RNA-dependent RNA polymerase (RdRp) from pTRV2 confirmed the successful expression of pTRV1 and pTRV2 vectors in taro leaves (Fig 2e). The expression level of *CePDS* were about 59.34%–77.18% in VIGS-CePDS plants compared to the control through further detection (Fig. 2f), and the chlorophyll content

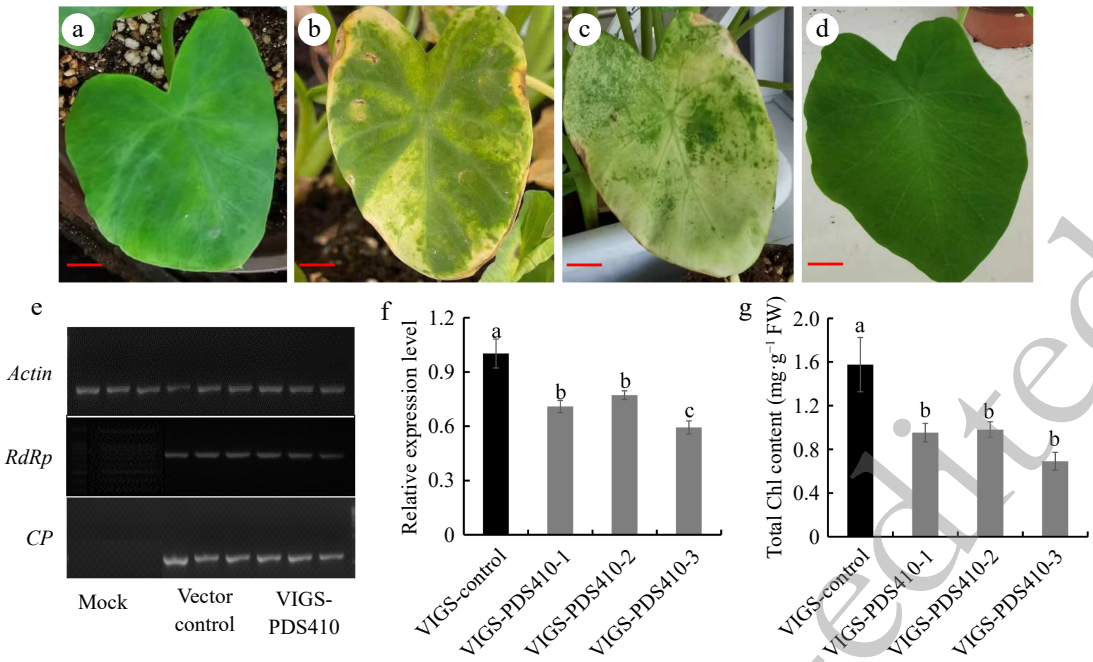


**Fig. 2** VIGS system constructing in taro. (a) Empty vector phenotype at 20d of leaf infection with OD600 = 0.6, (b) VIGS-PDS410 phenotype at 20d of leaf infection OD600 = 0.6, (c) VIGS-PDS410 phenotype at 30d of leaf infection with OD600 = 0.6, (d) Mock phenotype after 20d infection, (e) RdRp gene expression in TRV1 and CP gene expression in TRV2 were detected by RT-PCR with actin as the internal reference, (f) The detection of CePDS expression level for photobleaching phenotype plants, (g) Chlorophyll content detection for photobleaching phenotype plants. Scale bar = 3 cm.

decreased by 37.80%−56.11% in VIGS-CePDS plants (Fig. 2g). Phenotypic and molecular results of VIGS-CePDS seedlings clearly showed that *CePDS* expression in taro was targeted and silenced after VIGS, but the number of silenced lines was low and needed to be further improved.

## VIGS system optimization in taro

Studies have shown that factors such as infecting solution concentration, infecting method would influence on the silencing effect[8,18,21]. So we optimized taro VIGS system based on these two factors. Based on photobleaching phenotype, the results showed that when $OD_{600}$ = 1.0, the number of silent lines was the largest compared to $OD_{600}$ = 0.6 and $OD_{600}$ = 0.8, the proportion reached 27.77%, but there was no significant difference compared with $D_{600}$ = 1.2 at 30d after infection (Table 2). Subsequently, we compared the bulb vacuum infection and leaf injection infection, the number of photobleached lines showed no significant difference (Table 3). The above results indicated that the concentration of infective bacterial solution was a key factor affecting gene silencing in taro.

## Verification of the function of *CeTCP14* based on the established VIGS system

In our previous work, we found that *CeTCP14* presented obvious upregulated trend during the early stage of corm expansion, this gene was further selected to verify the robustness of constructed VIGS system. We silenced *CeTCP14* with $OD_{600}$ = 1.0 and bulb vacuum infiltration method to identify its function. RT-PCR results confirmed the successful expression of pTRV1 and pTRV2 vectors in taro corms (Fig. 3a), the expression level of *CeTCP14* in VIGS-CeTCP14 plants decreased by 36.66%~56.06% (Fig. 3b), this resulted in significant starch content decrease, only 70.88%~80.61% of the control (Fig. 3c).

**Table 2.** Bacterial concentrations optimization for VIGS system

| $OD_{600}$ | Total number of inoculation plants | The number of photobleaching phenotype | The percentage of photobleaching phenotype (%) |
|---|---|---|---|
| 0 | 10 | 0 c | 0 |
| 0.6 | 30 | 3.00 ± 1.00 bc | 10 |
| 0.8 | 30 | 5.33 ± 0.58 b | 17.77 |
| 1.0 | 30 | 8.33 ± 0.58 a | 27.77 |
| 1.2 | 30 | 7.67 ± 0.58 a | 25.57 |

**Table 3.** Comparsion of VIGS system based on infection type

| Infection type | Total number of inoculation plants | The number of photobleaching phenotype | The percentage of photobleaching phenotype (%) |
|---|---|---|---|
| Leaf injection | 30 | 8.33 ± 0.58 a | 27.77 |
| Bulb evacuation | 30 | 8.00 ± 0.58 a | 26.67 |

In maize, *ZmTCP7* regulated the starch content through bound to *ZmBt2* promoter[22], *CeTCP14* and *ZmTCP7* belongs to the same TCP class II, the factors interact with *CeTCP14* in taro to regulate starch content need further study.

## Discussion

At present, the genome of taro has been reported[23], which facilitates the gene mining that control of important agronomic traits. We cloned *CePDS* as a indicator gene from taro based on homologous alignment and constructed VIGS system, for color changes are the most intuitive way after gene silencing or gene edit[19,24–26].

Besides, virus silence-inducing vector is another important factor affecting the successful of VIGS system construction.

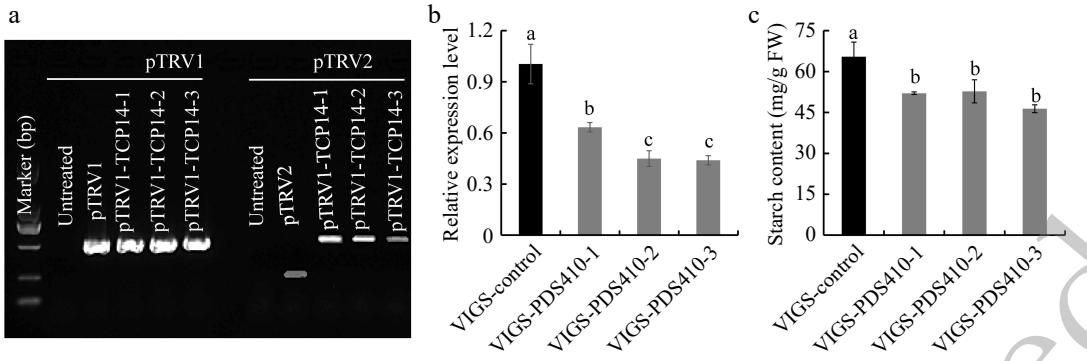


**Fig. 3** Detection of taro VIGS system for CeTCP14 in Ganyu No.2. (a) RdRp gene expression in pTRV1 and fragement expression in pTRV2 were detected by RT-PCR, (b) The detection of CeTCP14 expression at 20d after bulb vacuum infiltration. (c) Starch content detection for VIGS-CeTCP14 plants.

Although a variety of viral vectors have been used in VIGS, including RNA viruses, DNA viruses, and satellite viruses, the silencing efficiency induced by each viral vector is host-dependent. For example, the potato virus X (PVX) is a RNA viruses, studies in tomatoes have found that *in vitro* transcription is required before RNA infection[27]. DNA viral vectors are simple to construct, easy to operate and do not require *in vitro* transcription, such as beet curl top virus (BCTV) and tomato curl leaf virus (ToLCV) can be effectively used for gene silencing[28,29]. Satellite virus has a small genome, replicates quickly in the host and is easy to inherit. Chinese tomato yellow leaf curl virus (TYLCV) and tobacco stalk curl virus (TbCSV) of these virus vectors have been successfully used to verify gene function[30,31]. The virus silencing induction vector selected in this study is TRV, which belongs to RNA virus and is widely used because of its long silencing time, mild virus symptoms and high silencing efficiency[7,8]. In this study, TRV-based vector could produce silence effect in Ganyu No. 1 and Ganyu No. 2 which further verified TRV vector is suitable for gene silencing in taro, but the percentage of photobleaching phenotype is relatively low compared to banana. In banana VIGS research, another RNA virus, cucumber mosaic virus (CMV) performed better gene silencing effect with 95% infection rate and reduced target gene transcripts to 10%–18% of the control, the success of developing a banana VIGS system could be partially credited to the choice of utilizing a CMV isolate naturally infecting bananas during its construction[32], while in taro, TRV is not a serious taro-hosting virus, and greatly affected the infection rate, one way that can be used to improve the silencing effect is to increase the concentration of bacterial solution. Besides, it is reported that CMV could infect taro[16], both banana and taro are monocots and exhibit similar growth habits, it might be worth testing CMV in taro as well in the subsequent optimization experiments. The infecting bacterial solution concentration and infection method were also important factors affecting the silencing effect in VIGS. In the study, the best infection effect was achieved with $OD_{600}$ = 1.0, and this concentration was also applied in other plants of VIGS system construction[18,21]. It may be related to the bacterial vitality and infection ability at this concentration. But recent research of engineered TRV vector on tobacco turned out that $OD_{600}$ value of bacterial suspensions was $1x10^{-5}$ still can achieve good silence effect through one-strain/two-vector approach[33], this JoinTRV vectors that used pLX as back-bones have been tested in a range of agrobacterium strains, are notably more compact than any other TRV vector system currently documented, except for TRV is tobacco-hosting viruses, perhaps compact structure is another key factor to high efficiency at low concentration. The main VIGS inoculation methods are vacuum penetration[34,35], friction inoculation[36], root-filling method[37], leaf injection[38]. In corm crops, it usually turns out that vacuum penetration is superior to leaf injection[8,38]. However, the results in taro bulbs showed no significant difference between these two methods (Table 3). The possible reason is that taro bulbs are firm and full of starch with multiple buds. And the buds with less infection would display obvious growth advantage, this maybe an important factor resulted in no significant differences in the photobleaching phenomenon.

Currently available evidence has shown that most TCP class I genes are activators of plant development, whereas TCP class II members often function as a repressor of various growth and development pathways[12–14]. In maize, *ZmTCP7* affects the accumulation of starch by target to the promoter of *ZmBt2*[22], besides, *ZmTCP7* lacks transactivation activity in yeast system[10,12], this means that *ZmTCP7* may dimerize with other regulatory protein(s) or TF(s) to regulate *ZmBt2* to control the accumulation of starch. In this research, we found that after *CeTCP14* expression level downregulated by VIGS, the starch content decreased above 20% (Fig.3), this means *CeTCP14* contribute to the accumulation of starch as well. In VIGS-CeTCP14 plants, we also detected the expression level of *CeTCP13*, *CeTCP10* and starch biosynthesis gene (ADP-glucose pyrophosphrylase (*AGPL*), granule-bound starch synthase (*GBSS*), soluble starch synthase (*SSS*), starch branching enzyme (*SBE*), isoamylase (*ISA1*) in taro corms, they all present a significant down-regulated trend (data not show), these results means *CeTCP14* is a upstream regulatory gene in the starch biosynthesis pathway, but whether it interacts with *CeTCP13* and *CeTCP10* to regulate starch pathway remains to be investigated in future studies.

In all, VIGS is a powerful tool used in plant molecular biology to study gene function. VIGS allows for high-throughput functional analysis of plant genes. By silencing individual genes and observing the resulting phenotypic changes, researchers can elucidate the roles of specific genes in various biological processes such as development, stress response, and disease resistance.

## Conclusion

In this study, we constructed a VIGS system initially in taro with TRV as vector and *CePDS* as indicator gene, combined with phenotype change, gene expression and chlorophyll content determination. Through the optimization of bacterial solution concentration and infection mode, it was found that the silencing effect was the best at $OD_{600}$ = 1.0, and the silencing plants rate reached 27.77%. There was no significant difference in the silencing plants number between leaf injection method and bulb vacuum infiltration method. After silencing the *CeTCP14* based on the established system, the starch content in the bulb is reduced by more than 20%, preliminarily verified *CeTCP14* could promote starch accumulation in taro, which laid a foundation for rapid gene function verification in taro.

## Author contributions

Yanling GUI, Bicong LI, Yining HE, Yufeng ZHANG is the executor of this experiment, including complete data analysis and write the draft paper; Jiarui CUI, Rao PAN participate in experimental design and analysis of experimental results; Qianglong ZHU, Yingjin HUANG, Qinghong ZHOU, participated in the design of this experiment, Yao XIAO and Qinghong ZHOU aare the proposer and leader of the project, directing experimental design, data analysis, paper writing and revision. All authors have read and approved the manuscript.

## Data availability

The CePDS and CeTCP14 sequence used in this study is available in taro reference genome Taro_JAAS_v1.0 (BioProject: PRJNA587719), and gene number is EVM0008568.1 and EVM0000825.2, respectively.

## Acknowledgments

This research was supported by the National Natural Science Foundation of China (32060683), the Natural Science Youth Foundation of Jiangxi Province (20224BAB215025), and the Youth Foundation of Jiangxi Provincial Department of Education (GJJ210442).

## Conflict of interest

The authors declare that they have no conflict of interest.

## Dates

Received 25 March 2024; Accepted 7 June 2024; In press 17 June 2024

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