Plant Science 201–202 (2013) 25–41

Contents lists available at SciVerse ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Optimizing virus-induced gene silencing efficiency with *Cymbidium* mosaic virus in *Phalaenopsis* flower

Ming-Hsien Hsieh[a,b], Hsiang-Chia Lu[c], Zhao-Jun Pan[a], Hsin-Hung Yeh[c], Shyh-Shyan Wang[b], Wen-Huei Chen[d], Hong-Hwa Chen[a,d,*]

[a] *Department of Life Sciences, National Cheng Kung University, Tainan 701, Taiwan*
[b] *Tainan District Agricultural Research and Extension Station, Council of Agriculture, Tainan 712, Taiwan*
[c] *Department of Plant Pathology and Microbiology, National Taiwan University, Taipei 106, Taiwan*
[d] *Orchid Research Center, National Cheng Kung University, Tainan 701, Taiwan*

ARTICLE INFO

Article history:
Received 10 March 2012
Received in revised form 27 October 2012
Accepted 13 November 2012
Available online 26 November 2012

Keywords:
Cymbidium mosaic virus
PeMADS5
PeMADS6
PeUFGT3
Phalaenopsis
Virus-induced gene silencing

ABSTRACT

Virus-induced gene silencing (VIGS) is a good way to study floral gene functions of orchids, especially those with a long life cycle. To explore the applicability and improve viral silencing efficiency for application of *Cymbidium* mosaic virus (CymMV)-induced gene silencing, we examined several variables, including the optimal length of the DNA fragment, the effect of developmental maturation status of inflorescence, and suitable inoculation sites. A CymMV-based VIGS system can be used with orchids to silence genes including *PeUFGT3*, *PeMADS5* and *PeMADS6* and induce prominent phenotypes with silencing efficiency up to 95.8% reduction. The DNA fragment size used for silencing can be as small as 78–85 bp and still reach 61.5–95.8% reduction. The effect of cDNA location as a target in VIGS varies among genes because of non-target gene influence when using the 5′ terminus of the coding region of both *PeMADS5* and *PeMADS6*. Use of VIGS to knock down a B-class MADS-box gene (*PeMADS6*) in orchids with different maturation status of inflorescence allowed for observing discernable knockdown phenotypes in flowers. Furthermore, silencing effects with Agro-infiltration did not differ with both leaf and inflorescence injections, but injection in the leaf saved time and produced less damage to plants. We propose an optimized approach for VIGS using CymMV as a silencing vector for floral functional genomics in *Phalaenopsis* orchid with Agro-infiltration: (1) DNA fragment length about 80 bp, (2) a more mature status of inflorescence and (3) leaf injection.

1. Introduction

Orchidaceae is one of the largest families of angiosperms and includes approximately 25,000 species in 900 genera. Orchid flowers are morphologically diverse, as in the patterns of colors in sepals and petals and the modified dorsal petals (lips). The colorful lips serve as the landing platform for insect pollinators. In addition, the column (a fused structure of stamens and styles) helps facilitate pollination [1]. The genus *Phalaenopsis* is a popular pot plant because of its graceful appearance, long-lasting flowering and wide variety of species. *Phalaenopsis* orchids produce upright racemes that bloom in succession beginning with the lowest. Approximately eight to twelve flowers on a single inflorescence bloom consecutively about every other 3–4 days when plants are kept in a culture room inside a greenhouse with natural light and a controlled temperature of 27/22 °C (day/night) [2]. However, the molecular genetic information of orchids is still lagging behind that of Arabidopsis and rice. Further dissecting the regulation of floral morphological and physiological traits to improve our knowledge of floral development and enhance breeding for desirable traits is still challenging. Therefore, functional genomics tools are needed to accelerate the discovery of genes involved in floral traits of *Phalaenopsis*.

Forward and reverse genetics are commonly used to dissect how the genetic information contributes to a particular trait. Nonetheless, lack of high-resolution genetic maps leads to difficulty in studying orchid biology with forward genetics. Furthermore, because of low transformation rates and long regeneration time, reverse genetics with use of T-DNA insertion or transposon tagging is time-consuming in orchid research [3,4].

Virus-induced gene silencing (VIGS) is a viable alternative for high throughput functional analysis of plant genes because of its fast turn-around time and efficiency [5,6]. VIGS has been

* Corresponding author at: Department of Life Sciences and Orchid Research Center, National Cheng Kung University, Tainan 701, Taiwan.
Tel.: +886 6 275 7575x65521; fax: +886 6 235 6211.
E-mail address: hhchen@mail.ncku.edu.tw (H.-H. Chen).

0168-9452/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.plantsci.2012.11.003

successfully used for functional genomics of a range of dicot species, including Arabidopsis [7], *Pisum sativum* [8], soybean [9], tobacco [10], and tomato [11], and several monocot species, such as barley [12], wheat [13], rice [14], and maize [15]. VIGS has also been developed as a powerful alternative for functional characterization of orchid genes [16,17].

The *Cymbidium* mosaic virus-based VIGS vector (pCymMV-pro60) has been used to induce gene silencing in orchids [16]. Recently, Lu et al. (2012) successfully engineered a cloning vector (pCymMV-Gateway); the target gene fragment is easier to insert and more efficient to manipulate than with pCymMV-pro60 and can be inoculated into plants by Agro-inoculation. This new vector is suitable for high-throughput VIGS analysis in orchids. Lu et al. (2012) found no significant differences in VIGS phenotype or number of plants showing the phenotype with both the original vector (pCymMV-pro60) and the new vector (pCymMV-Gateway). Insert length of 150 bp did not induce a VIGS phenotype with the original vector, so a 500-bp conserved region was used, which is approximately the full length of the coding region [18]. Besides the effect of fragment size and location, several other variables such as inoculation method and developmental maturation status of inflorescence still need to be optimized to improve the silencing efficiencies of the new vector.

In this study, we investigated VIGS with *PeUFGT3* (UDP glucose: flavonoid 3-*O*-glucosyltransferase) and two B-class MADS-box transcription factors, *PeMADS5* (an *APETALA3* (*AP3*)-like ortholog) and *PeMADS6* (a *PISTILLATA* (*PI*)-like ortholog), as target genes because their functions have been previously studied. *PeUFGT3* encodes an enzyme involved in anthocyanin biosynthesis for red orchid flowers [17], and the two B-class MADS-box genes are involved in orchid floral morphogenesis [19,20]. Previously, four duplicated *AP3-like* genes (*PeMADS2* to *PeMADS5*) and a *PI-like* gene were found involved in orchid morphogenesis [19,20]. *PeMADS5* is predominantly expressed in petals and weakly in sepals and lips [19], whereas *PeMADS6* is more concentrated in the sepal, petal and lip primordium [20].

Another essential element of VIGS analysis is the confirmation of whether the orthologs and the paralogs of non-target genes are silenced simultaneously. In plants, siRNAs can silence target genes specifically by binding to corresponding mRNA. In addition, 22-nt siRNA molecules are sufficient to induce transcriptional gene silencing [21,22]. Minimizing or avoiding non-target silencing or identifying potential non-target genes is important for optimizing variables for VIGS efficiency in *Phalaenopsis* flowers with the CymMV-Gateway vector. These results may facilitate the use of pCymMV-Gateway in high-throughput analysis and will be important for functional genomics studies of orchid biology.

2. Materials and methods

2.1. Plant materials

The plants were purchased from Oxen Biotechnology Inc. (OBI; Tainan City, Taiwan). *Doritaenopsis* Taida Salu 'Sunset Fire' was used for analysis of *PeUFGT3*, *Dtps*. I-Hsin Sunrise Cinderella 'OX1357' for *PeMADS5* and *PeMADS6*, and *Phalaenopsis* Sogo Yukidian 'V3' and *Dtps*. OX Red Shoes 'OX1407' for *PeMADS6*. Because of the high rate of viral infection in *Dtps*. Taida Salu 'Sunset Fire' after the *PeUFGT3* experiment, we switched to the *Dtps*. I-Hsin Sunrise Cinderella 'OX1357' in the following experiments (Table S1). *Phalaenopsis* Sogo Yukidian 'V3' and *Dtps*. OX Red Shoes 'OX1407' were chosen to determine whether different cultivars affect VIGS efficiency. Plants were kept in a greenhouse with natural light and a controlled temperature at 27/22 °C (day/night). They were checked for the absence of two prevalent orchid viruses, *Cymbidium* mosaic virus (CymMV) and *Odontoglossum* ringspot virus (ORSV) by detecting the viral RNA encoding for viral coat protein. RT-PCR was performed with two primer pairs, ORSV-CP-F, ORSV-CP-R and CymMV-CP-F, CymMV-CP-R for amplifying the coat protein of the viruses [16,23] before VIGS experiments (Table 1).

Table 1
Primers for RT-PCR amplification of CymMV and ORSV.[a]

Primer name	Sequence (5′–3′)
CymMV-CP-F	GAAATAATCATGGGAGAGCC
CymMV-CP-R	AGTTTGGCGTTATTCAGTAGG
ORSV-CP-F	ACGCACAATCTGATCCGTA
ORSV-CP-R	ATCCGCAGTGAAAACCC

[a] CymMV, *Cymbidium* mosaic virus; ORSV, *Odontoglossum* ringspot virus.

2.2. Construction of pCymMV-Gateway plasmids

DNA fragments for *PeUFGT3* (EU427548), *PeMADS5* (AY378148.1) and *PeMADS6* (AY678299.1) for insertion into pCymMV-Gateway were obtained by PCR amplification with oligonucleotide primers (Table 2). Full-length cDNA clones for *PeUFGT3*, *PeMADS5* and *PeMADS6* [17,19,20] were used as PCR templates for amplification of VIGS inserts. Because we could not distinguish PCR products from the primers (approximately 50 nt of the primer contained the 29-nt attB recombination site by agarose gel electrophoresis when the length of the amplified PCR products was less than 140 nt. Thus, we used sequence determination with three randomly selected samples for genes with PCR products less than 140 nt (including *PeUFGT3-5*, *PeMADS5-2*, *PeMADS5-3*, *PeMADS5-4*, *PeMADS6-2*, *PeMADS6-3*, *PeMADS6-4*).

Ten specific fragments of *PeUFGT3* (*PeUFGT3-1* to *PeUFGT3-8*, *PeUFGT3-3a* and *PeUFGT3-7a*) were PCR amplified from *P. equestris* cDNA with ten pairs of primers: UFGT3-1F/UFGT3-4R, UFGT3-2F/UFGT3-4R, UFGT3-3F/UFGT3-4R, UFGT3-4F/UFGT4-4R, UFGT3-5F/UFGT3-4R, UFGT3-5F/UFGT3-5R, UFGT3-6F/UFGT3-6R, UFGT3-7F/UFGT3-7R, UFGT3-8F/UFGT3-8R, UFGT3-3aF/UFGT3-3aR and UFGT3-7aF/UFGT3-7Ar (Table 2).

Four specific fragments of *PeMADS5* (*PeMASD5-1* to *PeMADS5-4*) were PCR amplified from *P. equestris* cDNA with four pairs of primers, MADS5-1F/MADS5-1R, MADS5-2F/MADS5-2R, MADS5-3F/MADS5-3R and MADS5-4F/MADS5-4R (Table 2).

Four specific fragments of *PeMADS6* (*PeMASD6-1* to *PeMADS6-4*) were PCR amplified from *P. equestris* cDNA with four pairs of primers, MADS6-1F/MADS6-1R, MADS6-2F/MADS6-2R, MADS6-3F/MADS6-3R and MADS6-4F/MADS6-4R (Table 2).

The resulting products were cloned into pCymMV by *in vitro* recombination with BP Clonase II (Invitrogen) to form pCymMV–UFGT3-1 to pCymMV–UFGT3-8 and pCymMV–UFGT3-3a and pCymMV–UFGT3-7a. The PCR products were gel purified before being inserted into the VIGS vector by recombination. After recombination into the pCymMV-Gateway vector, each construct was confirmed by PCR with the gene-specific forward primer (Table 1) and the general reverse primer (CymMV 5351, 5′-CTTCTGTACCATACACATAG-3′) on the gateway vector sequence. In addition, each construct was sequenced from two to four randomly selected clones.

2.3. Agro-infiltration of plants

Agrobacterium tumefaciens containing pCymMV-Gateway-*PeUFGT3*, pCymMV-Gateway-*PeMADS5* or pCymMV-Gateway-*PeMADS6* was grown overnight at 28 °C to OD600 = 1. After centrifugation, bacterial cell pellets were re-suspended by adding 300 μl Murishige and Skoog medium containing 100 μM acetosyringone and allowed to stand at room