Scientific Research Publishing

American Journal of Plant Sciences, 2019, 10, 147-161
http://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

# RNA Silencing-Mediated Control of *Odontoglossum ringspot virus* (ORSV) Infection

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**How to cite this paper:** Zhang, X., Hu, Y.H., Chen, Z.J., Zhang, P.C., Li, H.M. and Shi, N.N. (2019) RNA Silencing-Mediated Control of *Odontoglossum ringspot virus* (ORSV) Infection. *American Journal of Plant Sciences*, **10**, 147-161.
https://doi.org/10.4236/ajps.2019.101013

**Received:** December 27, 2018
**Accepted:** January 18, 2019
**Published:** January 21, 2019

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## Abstract

*Odontoglossum ringspot virus* (ORSV) infects perennial orchids *(Phalaenopsis amabilis*) and causes a widespread viral disease. RNA-silencing of viral genes is a promising and effective way of controlling viral infection in plants. An inverted repeat (IR) fragment of the ORSV coat protein gene, *cp*, was inserted into the pXGY1 vector to generate the silencing construct, pXGY1-ORSV, which was introduced into *Nicotiana benthamiana* via Agrobacterium-mediated infiltration. A total of 15 homozygous pXGY1-ORSV transgenic *N. benthamiana* T1 plants were obtained from five transgenic lines, and ORSV *cp* gene multiplication was reduced by at least 75% - 95% in 12 T2 plants, demonstrating their increased resistance to ORSV. An infectious ORSV clone, pCAMBIA2300-ORSV, was generated to facilitate rigorous analyses of plant viral resistance. Semi-quantitative RT-PCR (sqRT-PCR) and northern-blot analyses revealed that levels of ORSV multiplication and ORSV coat protein were significantly reduced in pXGY1-ORSV transgenic *N. benthamiana*. Western-blot from pXGY1-ORSV inoculated leaves of ORSV infected *P. amabilis* also revealed the significant decrease and even degradation of ORSV-CP protein. Disease symptoms were not observed in transgenic plants. These results indicate a high level of ORSV-resistance in pXGY1-ORSV transgenic *N. benthamiana*.

## Keywords

ORSV, RNA Silencing, Agroinfiltration, Transient Expression, Transgenic Plant, Molecular Analysis

## 1. Introduction

Orchids (*Orchidaceae*) are a family of diverse and widespread flowering plants with high ornamental and economic value; however, during the cultivation

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process, they are easily infected by *Cymbidium mosaic virus* and *Odontoglossum ringspot virus* (ORSV). ORSV causes the most widespread viral disease in perennial orchids and is responsible for large economic losses in the orchid cultivation industry [1]. Infections with ORSV frequently cause leaf chlorosis and twisting and, when the condition becomes more serious, numerous symptoms appear, including necrotic lesions and ringspots, dwarfed plants, changes in the color and size of flowers, and even death of the entire plant [2]. ORSV can be transmitted by sap during cultivation operations. Numerous viral particles are replicated within infected host plant cells and translocated to surrounding cells *via* plasmodesmata [3]. Long-distance transport to the upper parts and root systems of plants, causing systemic infection, occurs via the sieve tubes of the vascular system [4]. Viruses generally alter the metabolic pathways of host cells and destroy their normal physiological functions. They utilize the host replication system, leading to the gradual emergence of obvious symptoms. The conventional method for elimination of orchid virus is the devastating and non-preventive process of isolating, burning, and discarding the remnants of symptomatic plants in landfill sites.

ORSV is a member of the *Tobamovirus* genus and has a positive-sense single-stranded RNA ($^+$ssRNA) genome of 6611 nucleotides. The genome contains three open reading frames (ORFs), ORF 1 encodes for a 126 kDa helicase polypeptide and a 183 kDa read-through product with helicase and polymerase motifs, ORF 2 and 3 are replicated as subgenomic RNAs and are translated into 31 kDa movement protein subgenomic RNA1 and 18 kDa capsid protein from subgenomic RNA2 [5]. Viral particles are frequently distributed in a lattice arrangement in host cells [3]. Orchids are perennial plants with low regeneration frequencies and need longer periods for development in tissue culture. *Nicotiana benthamiana* is a proven ORSV host and can be used as a model plant for studies of ORSV [6].

RNA silencing plays a crucial role in antiviral defense in plant cells [7] and can be induced by plant viruses (virus-induced gene silencing, VIGS), which enables the infected host plant cells to attack the viral genome [8]. If an siRNA molecule homologous to part of a viral genome can be permanently established in host cells, this usually leads to resistance of the host plant to the virus, providing there are no viral silencing-suppressor molecules present and active. Hence, RNA silencing, which is specific and highly efficient, has great potential for the generation of virus-resistant plants. For example, using the gene encoding the *Tobacco mosaic virus* (TMV) coat protein (CP) as a target, Zhao *et al.* (2006) constructed a vector capable of transcribing TMV siRNA and introduced it into tobacco via *Agrobacterium tumefaciens*-mediated inoculation and demonstrated that transient expression of the siRNA could indeed significantly inhibit TMV [9]. Similarly, Hu *et al.* (2011) generated transgenic *N. benthamiana* plants expressing a hairpin RNA-structure specific for part of the TMV movement protein gene and the *Cucumber mosaic virus* (CMV) replicase gene, to produce strong resistance against these two viruses [10]. Moreover, Himani *et al.*

(2008) constructed an RNA silencing vector and transformed it into rice to express dsRNA targeting the Rice tungro bacilliform virus (RTBV) and found that the level of virus within host rice cells was substantially reduced [11]. Regarding viruses infecting ornamental plants, only a few studies have used RNA silencing technology to generate resistant plants. Currently, the VIGS is applied to silence plant genes using recombinant viral genomes [12]. In addition to its broad application for the production of virus-resistant plants, this technology has also become one of the most attractive approaches for functional gene analyses in cereal, vegetable, and fruit crops [13] [14] [15] [16].

An Agrobacterium-mediated transient expression system for use in intact leaves was first established in Kapila's laboratory [17]. This system exhibits many advantageous characteristics, including safety, rapidity, effectiveness, and the ability to produce high levels of siRNA expression [17] [18] [19] [20] [21]. This transient expression technology has been applied to the study of the effects of gene silencing on *Grapevine A virus*, generating plants with strong resistance against this virus. The method could also provide timely information about the expression of disease-resistance genes in transgenic plants and prediction of their disease-resistance effects, particularly in perennial plants.

In this study, we constructed an infectious ORSV cDNA clone for inoculation and detection of virus resistance, and an ORSV-mediated RNA silencing vector by insertion of positive and inverse repeat fragments from the partial ORSV *cp* gene into the pXGY1 vector. We applied the Agroinfiltration-mediated transient expression system to the model host plant, *N. benthamiana*, with the aim of establishing RNA silencing-mediated control of ORSV infection.

## 2. Materials and Methods

### 2.1. Samples and Other Materials

Viral strains: *Phalaenopsis amabilis* infected with ORSV was obtained from Chuanhua Agricultural and Biotechnology Company in Hangzhou, Zhejiang, China. Wild-type *N. benthamiana*, supplied by our laboratory, was grown at 25˚C in a greenhouse with a light/dark cycle of 16/8 h. *Agrobacterium* strain LBA4404 and the pCAMBIA2300 vector were kept in our laboratory.

### 2.2. Primers

Primers (**Table 1**) were designed based on ORSV nucleotide sequences available in GenBank using Primer 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by Invitrogen (Shanghai, China) and/or Shanghai Shenggong Biology Engineering Technology Service Limited Company (Shanghai, China).

### 2.3. Virus Extraction, Mechanical Inoculation, and RT-PCR Detection

ORSV infected *P. amabilis* leaves were collected and ground with quartz sand

**Table 1.** Primers.

| Primer | Sequence (5′ → 3′) | Amplicon (bp) |
|---|---|---|
| ORSV-*cp*-F | AATGGTGTTAGTGATATTCG | 604 |
| ORSV-*cp*-R | CCACTATGCATTATCGTATG | |
| *EF1α*-F | TGCCTTGTGGAAGTTTGAGACC | 132 |
| *EF1α*-R | GGTGGAGTCAATAATCAGGACAGC | |
| 35S promoter-F | AAAGCAAGTGGATTGATGTGATGG | 400 |
| 35S promoter-R | TGCGAAGGATAGTGGGATTGT | |
| pXGY1-ORSV-F | GCATCTAATGTTTCCGTAGTTGTC | 329 |
| pXGY1-ORSV-R | ACTATTACAGACCCGTCTAAG | |
| 35S-ORUTR-Mer-KPN-F | AAGTTCATTTCATTTGGAGGTACC GGTATTGTTCGATTACTACAATCACAA | 3947 |
| OR Ndecom-R | CCTTAATCATATGTTTGTATTGATCA | |
| OR Nde-F | TGATCAATACAAACATATGATTAAGG | 2611 |
| OR-2300 3UTR1 | CGAACGAAAGCTCTGCAGGTCGACTG GGCCTCTACCCGAGGTAAGG | |
| OR ScaI-F | GCTAAGCAGGAGAAAAGTACTAT | 1551 |
| OR Apacom-R | GTAAGTTCGGTGGGCCCTCCTTCTGTT | |
| Intron-F | TGCTGGAACCAACTGTAATCAATC | 518 |
| Intron-R | CCCATAAATAGTAATTCTAGCTG | |

and TBS buffer (1:10 w/v), and then filtered. Leaf sap and TBS buffer (20 μL each) were separately dropped on to young leaves of healthy *N. benthamiana* at the 4 - 6 leaf growth stage. Inoculated plants were grown in a 16/8 h light/dark period at 25˚C. Total RNA was isolated using the Trizol method and quantified with a BioSpec-Nano spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific Inc.). First strand cDNA was synthesized using an M-MLV RT kit (PROMEGA), with addition of 0.5 μL of RNase inhibitor. PCR amplification was conducted using 2x Taq MasterMix (CoWin Biosciences) and qRT-PCR amplification was conducted with UltraSYBR Mixture (CoWin Biosciences). *EF1α* (elongation factor 1-alpha) was used as an internal reference gene.

## 2.4. Construction of a Full-Length ORSV Clone

Full-length ORSV clone was constructed following the procedure of Molecular Cloning from Cold Spring Harbor Laboratory (4th ed) [22]. An RNA sample extracted from *N. benthamiana* leaves systemically infected with ORSV was used as the template for synthesis of first strand cDNA. High fidelity KOD-plus-Neo DNA polymerase (TOYOBO) and the primer pairs, 35S-ORUTR-Mer-KPN-F/OR Ndecom-R and OR Nde-F/OR-2300 3UTR1, were used to amplify the ORSV fragments, OR-KpnI5-Nde (3947 bp) and OR-Nde-3'SalI (2611 bp), respectively. The ORSV 5' terminal fragment, OR-KpnI5-Nde, was digested with *Kpn* I and *Nde* I (New England Biolabs), while the 3' terminal fragment, OR-Nde-3'SalI,

was digested with *Sal* I and *Nde* I (New England Biolabs). The vector, pCAMBIA2300, was digested with *Kpn* I and *Sal* I, and the two digested fragments were ligated using T4 ligase (TAKARA). The recombinant vector, pCAMBIA2300-ORSV FL (**Figure 1(a)**), was transformed into *Agrobacterium* LBA4404. Colonies were screened by PCR with 2x Taq Mix (CoWin Biosciences), and the primer pairs OR ScaI-F/OR Apacom-R and ORSV-*cp*-F/ORSV-*cp*-R, and further verified by sequencing (Invitrogen, Shanghai, China) and phylogenic analysis (DNAMAN7.0).

## 2.5. Construction of the RNA Silencing Vector, pXGY1-ORSV

A partial ORSV *cp* fragment and its inverted repeat, the 35S promoter, a PDK intron, and NOS terminator sequences were ligated to generate a gene expression cassette, which was cloned into the expression vector, pXGY1, and transformed into *Agrobacterium*. *Agrobacterium* cell suspension was sprayed onto solid LB medium (lysogeny broth medium) containing kanamycin (50 g/mL), rifampicin (40 g/mL), and chloramphenicol (5 g/mL) (BBI Life science), and then incubated at 28˚C for 2 days. A single colony was picked, inoculated into LB liquid medium containing the same antibiotics, and cultured at 28˚C until reaching log growth phase. The resulting recombinant RNA silencing vector, pXGY1-ORSV (12383 bp) (**Figure 1(b)**), was extracted and verified by digestion, PCR, and sequencing.

## 2.6. *Agrobacterium* Infiltration

*Agrobacterium* cell suspension (1 mL) containing recombinant viral DNA was transferred into a 1.5 mL Eppendorf tube and centrifuged at 1150 ×g for 2 min. Pellets were suspended in acetosyringone buffer (1 mL), followed by centrifugation at 1150 ×g for 2 min. Then pellets were resuspended and centrifuged once more, resulting in suspensions with $OD_{600}$ values of 0.1 - 1.5. Suspensions (1 mL) were injected into leaf veins of *N. benthamiana* at the 6 - 8 leaf growth stage using a syringe. Two leaves of each plant were inoculated, and each treatment group included 8 - 10 plants. Empty vector suspension was also injected into leaves as a negative control. Two days after *Agrobacterium* infiltration, equal amounts of ORSV-containing leaf sap were inoculated into the infiltrated leaves; TBS buffer was inoculated as a negative control. After treatment for 1 - 2 weeks, symptomatic leaves were sampled for further analysis.

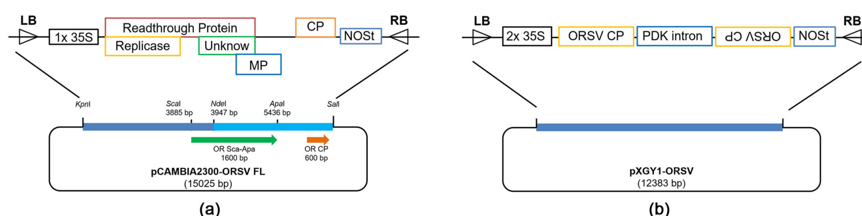


**Figure 1.** Structures of the ORSV full-length clone pCAMBIA2300-ORSV FL (a) and the ORSV-mediated RNA silencing vector pXGY1-ORSV (b).

### 2.7. Transformation and Detection

*N. benthamiana* leaf discs were pre-cultured at 25˚C for 24 h and then transferred to a suspension of *Agrobacterium* harboring the pXGY1-ORSV vector. Treated leaf discs were incubated in pre-culture medium for 48 h and then transferred to selective medium for the development of buds and roots at 25˚C with 24 h light/day. Seedlings were acclimatized, transferred to soil, and grown at a constant 25˚C with a 16/8 h light/dark period. Total DNA was extracted using DNeasy Plant Mini Kit (Qiagen) from seedlings for PCR analysis. T0 transgenic seeds were cultured in MS medium containing 500 μg/ml kanamycin at 25˚C with a 16/8 h light/dark cycle for single copy screening and further homozygous selection.

### 2.8. Molecular Hybridization Analyses

Southern and northern blots were conducted following the instructions provided with the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche). Probes were amplified with KOD-Plus-Neo DNA polymerase and labeled with DIG-High Prime. Leaf protein samples were extracted and their concentrations determined by measuring $OD_{595}$ of samples processed using the Bradford method with Coomassie brilliant blue G-250 solution. Western blots were performed by conducting SDS-PAGE (12.5%/3%) using BioRad (USA) Mini-PROTEAN® Tetra Vertical Electrophoresis Cell apparatus, following a protocol from "Molecular Cloning" (Cold Spring Harbor Laboratory Press 4th ed., 2012). Polyclonal antibodies against ORSV (1:51200) were supplied by our laboratory [23].

## 3. Results

### 3.1. Pathogenic Interference Using pXGY1-ORSV

*N. benthamiana* plants at the 6 - 8 leaf growth stage infiltrated with *Agrobacterium* harboring the pXGY1-ORSV silencing vector continued to grow normally without developing any symptoms. RT-PCR showed negative results for amplification of ORSV *cp*, indicating that ORSV was not present in plants infiltrated with the silencing vector. Nevertheless, *N. benthamiana* co-infiltrated with *Agrobacterium* harboring pXGY1-ORSV and ORSV inoculum exhibited different phenotypes: the majority of plants showed resistance or latency, with mild or no apparent symptoms, although the virus could be detected in *vivo*; some plants were immune, with no viral symptoms or virus detected (**Figure 2(a)**); and a few plants were susceptible, with both viral symptoms and virus detected. Plants inoculated with empty vector + ORSV inoculum as positive controls became infected and exhibited clear symptoms, whereas those treated with empty vector alone and/or TBS buffer as negative controls did not develop signs of infection (**Figure 2(a)**). Among a total of 19 *N. benthamiana* plants inoculated with pXGY1-ORSV+ORSV, 12 (63.2%) appeared to be resistant, latent, or immune to ORSV, while 7 (36.8%) were apparently susceptible. Thus, pXGY1-ORSV-mediated agroinfiltration could partially, but not completely reduce

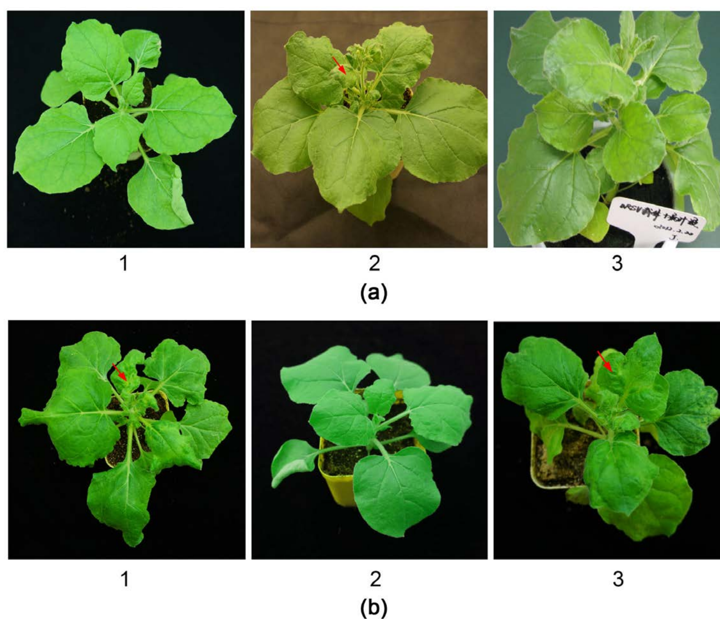


**Figure 2.** Growth status of *N. benthamiana* after co-inoculation with ORSV silencing vector pXGY1-ORSV-integrated *Agrobacterium* and virus sap at 14 dpi (a) 1. Healthy leaves; 2. ORSV-infected leaves; 3. Sap inoculated with pXGY1-ORSV + ORSV. Symptoms of *N. benthamiana* leaves inoculated with the pCAMBIA2300-ORSV-FL infectious clone (b) 1. pCAMBIA2300-ORSV-FL clone inoculated leaves; 2. Healthy leaves; and 3. Systemically infected leaves.

the ORSV infection rate. These data demonstrate that our ORSV *cp*-mediated RNA silencing vector can successfully degrade invading virus RNA.

### 3.2. Multiplication of the Virus *cp* Gene in *N. benthamiana* Plants Co-Infiltrated with *Agrobacterium* Harboring ORSV-Silencing Vector and Virus Sap

Total RNA was extracted from *N. benthamiana* leaves co-infiltrated with pXGY1-ORSV harbored *Agrobacterium* and ORSV. The multiplication level of the viral *cp* gene in co-infiltrated leaves was significantly lower than that of the positive control inoculated with ORSV alone (Figure 3(a)). Samples 3 and 8 in Figure 3(a) were negative for virus *cp* and developed no symptoms of ORSV infection, demonstrating that they were immune to the virus. Samples 1, 4, 5, 6, and 7 exhibited no apparent symptoms and low levels of ORSV *cp* multiplication, indicating resistance to ORSV. In sample 2, the *cp* multiplication level was relatively high and its leaves exhibited clear symptoms; however, its *cp* levels were lower than those of the positive control (Figure 3(a)).

Northern blotting showed the accumulated levels of ORSV genomic RNA, including sub-genomic RNA-1, encoding the movement proteins; and sub-genomic RNA-2, encoding the coat proteins (Figure 3(b)). The accumulated levels of ORSV genomes in samples 2, 5, and 7 were similar to those present in the positive control group, while those in samples 1, 4, and 6 were lower. No ORSV genomic RNAs were detected in samples 3 and 8.

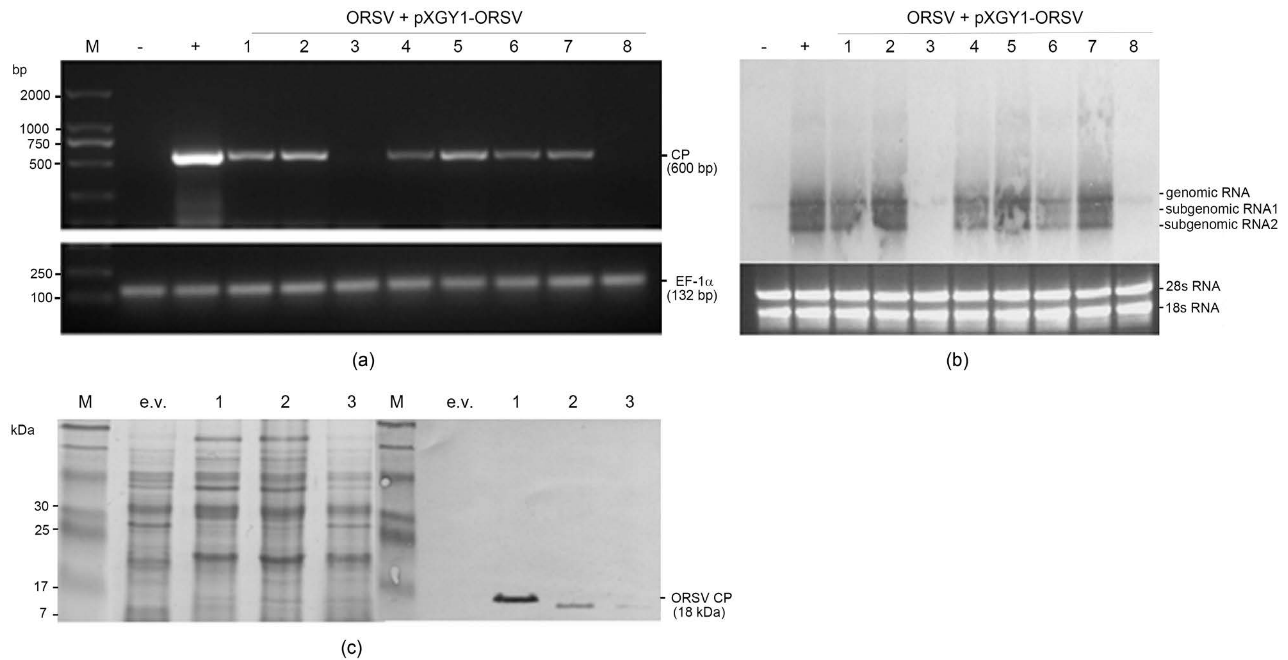


**Figure 3.** sqRT-PCR detection of expression of the ORSV coat protein gene in *N. benthamiana* co-inoculated with pXGY1-ORSV-integrated *Agrobacterium* and ORSV-containing sap, *EF1α*: internal-reference gene (a); +, co-inoculated with empty vector and ORSV; -, co-inoculated with empty vector and TBS buffer. Northern blot analysis of ORSV genome accumulation in *N. benthamiana* co-inoculated with pXGY1-ORSV-integrated *Agrobacterium* and virus sap (b); +, co-inoculated with empty vector and ORSV; -, co-inoculated with empty vector and TBS buffer. SDS-PAGE and western-blot analyses of viral coat protein time-course expression in ORSV-infected *Phalaenopsis* leaves after separate inoculation with pXGY1-ORSV (c); 1. Before inoculation; 2. 7 dpi; 3. 14 dpi; e.v., Inoculated with empty vector.

### 3.3. Expression of the *cp* Gene in ORSV-Infected *P. amabilis* Inoculated with *Agrobacterium* Harboring the ORSV-Silencing Vector

Leaves of ORSV-infected *P. amabilis* were inoculated with *Agrobacterium* harboring the pXGY1-ORSV silencing vector. Individual leaf samples were collected at 7 and 14 days post-infection (dpi), and total leaf proteins extracted and levels of viral coat protein assayed by SDS-PAGE and western blotting. Levels of ORSV CP were significantly lower at 7 dpi and reduced further at 14 dpi compared with levels prior to inoculation. The molecular weight of the expressed capsid protein was less than the expected 18 kDa, inferring that agro-inoculation harboring the ORSV-silencing vector likely led to reduced replication and expression of ORSV-CP, and degradation of the size of the viral coat protein, so that it became non-functional in the host (Figure 3(c)).

### 3.4. Regeneration of Putative pXGY1-ORSV-Transgenic *N. benthamiana* and Screening for Homozygous Transgenic Plants

Total DNA was extracted from putative pXGY1-ORSV transgenic *N. benthamiana* plants. Six T0 transgenic lines (OR-1, OR-4, OR-22, OR-26, OR-29, and OR-31) were screened by PCR amplification using primer-pairs specific for

pXGY1-ORSV, which amplified the 329 bp inserted ORSV *cp* fragment and the 400 bp 35S promoter (Figure 4). Five of them (OR-1, OR-4, OR-22, OR-26, and OR-31) were found to carry a single copy of the pXGY1-ORSV vector, and at least 15 homozygous transgenic seedlings were obtained after screening of these five lines (Table 2).

### 3.5. Infection with a Full-Length ORSV Clone

Sequencing analysis of the positive recombinant plasmid, pCAMBIA2300-ORSV-FL, and comparison of the sequence of this ORSV Hangzhou isolate with those of other isolates available in GenBank using BLAST revealed high homology, with levels up to approximately 99%. Hence, the 6613-nucleotide full-length ORSV genome was successfully inserted into the pCAMBIA2300 expression vector. Phylogenic analysis showed that Hangzhou ORSV isolate was most closely related to the Taiwan ORSV isolate 2, with the number of nucleotide substitutions per site smaller than 0.01, which therefore indicated their common origin (Figure 5(a)).

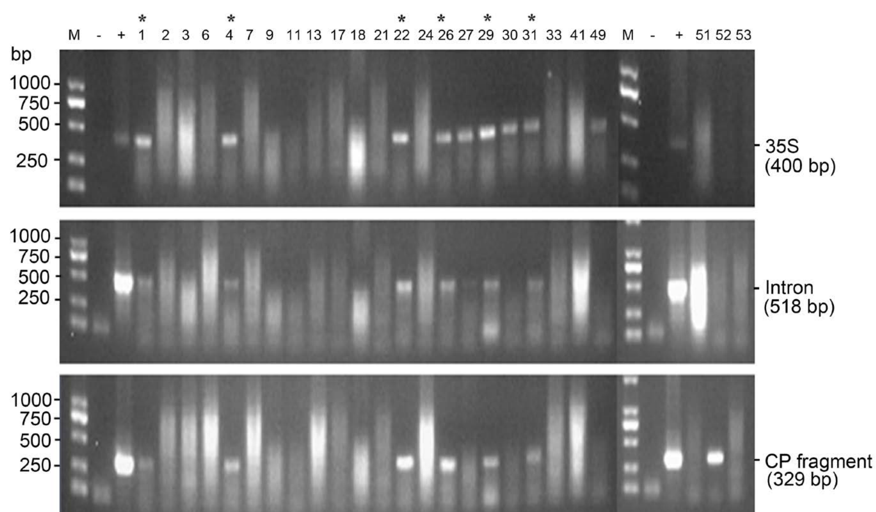


Figure 4. PCR detection of $T_0$ putative pXGY1-ORSV transgenic plants (stars: transgenic lines 1, 4, 22, 26, 29, 31) using primer-pairs specific for the ORSV *cp* fragment, the 35S promoter, and the PDK intron fragment. -, Wild-type *N. benthamiana* as a negative control; +, pXGY1-ORSV vector as a positive control.

Table 2. Identification of homozygotes from five $T_1$ single-copy pXGY1-ORSV transgenic lines.

| Transgenic line | No. of homozygous transgenic seedlings | Code for homozygote |
|---|---|---|
| OR-1 | 4 | B, C, E, J |
| OR-4 | 3 | C, D, H |
| OR-22 | 4 | F, G, H, J |
| OR-26 | 3 | C, G, H |
| OR-31 | 1 | B |

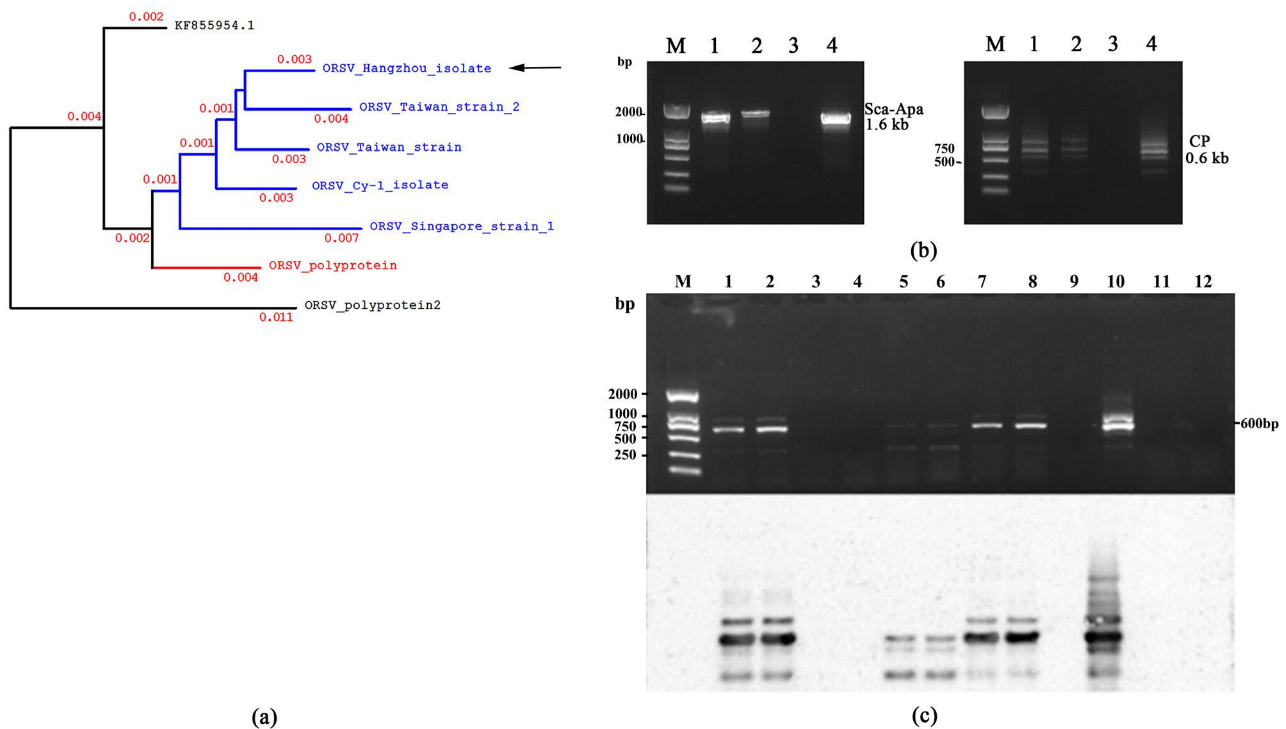


Figure 5. Phylogenic tree of ORSV isolates (a); arrow, ORSV Hangzhou isolate. PCR detection from leaves inoculated with the pCAMBIA2300-ORSV-FL infectious clone (b); M. DNA marker, 1. inoculated at 6 dpi, 2. inoculated at 10 dpi; 3. negative control/pCAMBIA2300 plasmid inoculated; 4. positive control /ORSV isolate. Southern blot detection of the RT-PCR amplified ORSV *cp* gene from new Nb leaves inoculated with the ORSV infectious clone (c); 1. Positive control/ORSV infected *Phalaenopsis*; 2. Positive control/ORSV sap inoculated Nb; 3-9. pCAMBIA2300-ORSV-FL infectious clone inoculated leaves; 10. Positive control/pCAMBIA2300-ORSV-FL plasmid; 11. Negative control/pCAMBIA2300 vector, and 12. Negative control/wide type Nb.

The positive clone was mechanically inoculated into *N. benthamiana* plants. ORSV-containing sap and pCAMBIA2300 empty vector were used as respective positive and negative controls. The results indicated that chlorotic spot, irregular twisting, and dwarfing symptoms developed in leaves inoculated with the pCAMBIA2300-ORSV-FL clone and systemically infected new leaves; these symptoms were comparable to those in *N. benthamiana* plants inoculated with ORSV sap, while no symptoms of viral infection were observed in empty vector-inoculated or untreated leaves (Figure 2(b)). RT-PCR from leaves at 6 dpi with pCAMBIA2300-ORSV-FL and systemically infected fresh leaves after 10 dpi resulted in positive amplification of the expected ORSV *Apa* and *cp* fragments (Figure 5(b)). Southern blot analysis confirmed the specificity of the amplified fragments (Figure 5(c)). Given the clear development of symptoms and robust molecular detection, we conclude that the full-length clone of the ORSV Hangzhou isolate, pCAMBIA2300-ORSV-FL, is highly infectious.

### 3.6. Resistance of pXGY1-ORSV Transgenic Plants

Approximately 30 T2 homozygous pXGY1-ORSV transgenic plants from five transgenic lines (OR-1, OR-4, OR-22, OR-26, and OR-31) were inoculated with the ORSV infectious clone pCAMBIA2300-ORSV-FL. At 10 dpi, the rate of in-

fection of transgenic plants was reduced by 40%, and the symptoms of those infected were significantly less pronounced than those of the positive control. sqRT-PCR analysis indicated that virus coat protein titers in these 30 transgenic plants were significantly and markedly reduced compared with the positive control. In particular, 12 transgenic plants (OR-1Eb2, OR-1Ja1, OR-1Jb1, OR-4Ca1, OR-22Fb1, OR-22Fb2, OR-22Ja2, OR-22Jb1, OR-22Jb2, OR-26Gb2, OR-31Ba1, and OR-31Ba2) exhibited reduction of virus coat protein multiplication by 75% - 95% relative to the positive control, indicating high levels of ORSV resistance (**Figure 6**).

## 4. Discussion

The ORSV *cp* RNA silencing vector is designed to be transcribed to generate homologous hairpin double-stranded RNA (dsRNA) in *N. benthamiana*, thereby initiating generation of viral small interfering RNA, triggering the plant's RNA silencing mechanism. When homologous ORSV virus invades *N. benthamiana*, VIGS will specifically block further virus replication, protecting the host from viral damage and effectively generating ORSV resistance.

Transient co-infiltration of *Agrobacterium* harboring pXGY1-ORSV with ORSV sap generated 63.2% of host plants with resistance and immunity to the virus. These results were further confirmed in ORSV-infected perennial *P. amabilis*, indicating that transient expression of pXGY1-ORSV can trigger the RNA silencing process within host cells and degrade the homologous viral genome. Further expression of ORSV coat protein is inhibited or defective, resulting in truncated, non-functional CP subunits.

A few pXGY1-ORSV vector and virus-sap co-inoculated plants remained susceptible to the virus. This may have been due to low levels of the pXGY1-ORSV vector, insufficient to diminish virus replication within host plant cells. To

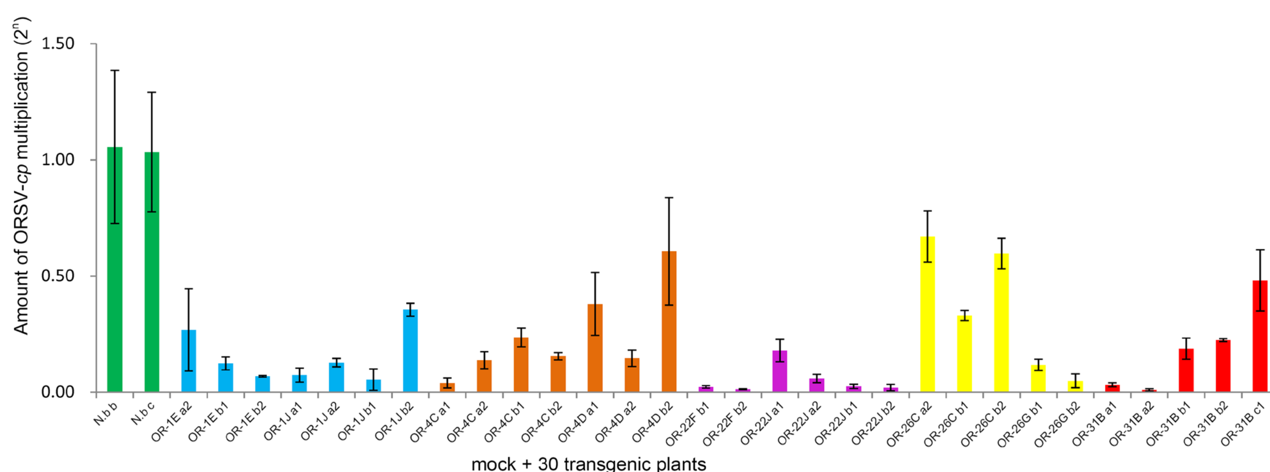


**Figure 6.** Evaluation of ORSV *cp* multiplication by qRT-PCR in 30 T2 homozygous pXGY1-ORSV transgenic plants from five lines at 10 dpi with the pCAMBIA2300-ORSV-FL infectious clone. Green bar, ORSV-infected *N. benthamiana* (negative control); Blue, brown, purple, yellow, and red bars represent the five T1 transgenic lines OR-1, OR-4, OR-22, OR-26, and OR-31 individually.

summarize, the transient expression of *Agrobacterium* harboring pXGY1-ORSV can effectively block or weaken ORSV infection of host plants, and has potential for use as an effective supplementary method for development of ORSV-resistant plants.

The ORSV infectious clone prepared in this study was originally derived from a Hangzhou isolate and phylogenic analysis revealed its close relationship with the ORSV strain Taiwan isolate 2. This is likely because a large proportion of the germplasm resources of *P. amabilis* growing in the eastern parts of China originate from Taiwan. During the processes of infection and transmission, ORSV interacts with its hosts and has developed various evolutionary mechanisms, resulting in differences in virulence and disease symptoms attributable to the same viral strain in different areas [24]. Therefore, it is necessary to construct isolate-specific ORSV infectious clones to improve their infection efficiency.

The application of RNA silencing-mediated antiviral genetic engineering is highly promising for effective prevention and control of plant viral diseases. Taking tobacco mosaic virus (TMV) coat protein as the target, Zhao's team synthesized specific siRNA and introduced it into tobacco via *Agrobacterium*, and transient expression of the designed interference sequences significantly inhibited TMV levels [9]. Moreover, Yan's team constructed an siRNA-silencing vector targeting TMV coat protein to transform tobacco and obtained TMV-resistant plants [25]. In this study, we produced five independent pXGY1-ORSV transgenic lines, OR-1, OR-4, OR-22, OR-26, and OR-31, using an RNA silencing approach. Fifteen T1 homozygous transgenic plants were obtained from these five lines, with at least 12 T2 homozygous transgenic plants (OR-1Eb2, OR-1Ja1, OR-1Jb1, OR-4Ca1, OR-22Fb1, OR-22Fb2, OR-22Ja2, OR-22Jb1, OR-22Jb2, OR-26Gb2, OR-31Ba1, and OR-31Ba2) exhibiting high levels of resistance to ORSV due to the anti-viral effect of *Agrobacterium*-mediated transformation with an pXGY1-ORSV silencing vector.

Transgenic plants carrying the ORSV-silencing vector, pXGY1-ORSV, contained partial positive and inverted repeat siRNA sequences targeting ORSV *cp*, and the ORSV genome sequence of the strain used for infection shared homology with the transcriptional hairpin structured products of the transgene. High levels of viral replication trigger endogenous RNA silencing defense mechanisms. These results further support the conclusion that introduction of dsRNA homologous to the viral gene into the host using a transgenic approach can lead plants to acquire an immune-like resistance against the virus [26] [27] [28]. The 12 highly ORSV-resistant transgenic plants obtained in this study represent valuable laboratory materials for further investigation of the mechanisms involved in ORSV resistance mediated by virus-induced RNA silencing vector, host-virus interactions, viral siRNA induced disease-resistant effects, and proteins involved in the plant host VIGS process.

## Acknowledgements

This research was supported the Free-Application Program on Agricultural

Sciences from Hangzhou Sci-Tech Bureau (20180432B01) and National Key R & D Program of China (2017YFE0110900).

## Compliance with Ethical Standards

1) Conflict of Interest: Nongnong Shi declares that she has no conflict of interest. Xian Zhang declares that he has no conflict of interest. Yihua Hu declares that she has no conflict of interest. Zhijuan Chen declares that she has no conflict of interest. Pengcheng Zhang declares that he has no conflict of interest. Hongmei Li declares that she has no conflict of interest.

2) Ethical Approval: This article does not contain any studies involving human participants or animals performed by any of the authors.

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