Genome-wide analysis of small RNAs from Odontoglossum ringspot virus and Cymbidium mosaic virus synergistically infecting Phalaenopsis

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SUMMARY

Cymbidium mosaic virus (CymMV) and Odontoglossum ringspot virus (ORSV) are the two most prevalent viruses infecting orchids and causing economic losses worldwide. Mixed infection of CymMV and ORSV could induce intensified symptoms as early as 10 days post-inoculation in inoculated Phalaenopsis amabilis, where CymMV pathogenesis was unilaterally enhanced by ORSV. To reveal the antiviral RNA silencing activity in orchids, we characterized the viral small-interfering RNAs (vsiRNAs) from CymMV and ORSV singly or synergistically infecting P. amabilis. We also temporally classified the inoculated leaf-tip tissues and noninoculated adjacent tissues as late and early stages of infection, respectively. Regardless of early or late stage with single or double infection, CymMV and ORSV vsiRNAs were predominant in 21- and 22-nt sizes, with excess positive polarity and under-represented 5’-guanine. While CymMV vsiRNAs mainly derived from RNA-dependent RNA polymerase-coding regions, ORSV vsiRNAs encompassed the coat protein gene and 3’-untranslated region, with a specific hotspot residing in the 3’-terminal pseudoknot. With double infection, CymMV vsiRNAs increased more than 5-fold in number with increasing virus titres. Most vsiRNA features remained unchanged with double inoculation, but additional ORSV vsiRNA hotspot peaks were prominent. The potential vsiRNA-mediated regulation of the novel targets in double-infected tissues thereby provides a different view of CymMV and ORSV synergism. Hence, temporally profiled vsiRNAs from taxonomically distinct CymMV and ORSV illustrate active antiviral RNA silencing in their natural host, Phalaenopsis, during both early and late stages of infection. Our findings provide insights into offence–defence interactions among CymMV, ORSV and orchids.

Keywords: anti-viral RNA silencing, Cymbidium mosaic virus, Odontoglossum ringspot virus, Phalaenopsis, small RNA sequencing, synergism, viral siRNA (vsiRNA).

INTRODUCTION

The Orchidaceae is an immense family of more than 800 genera, about 28 000 native species and more than 110 000 cultivars (Christenhusz and Byng, 2016; Sheehan, 2003). Because of their elegant flowers with a fascinating array of colours, long blooming duration and adaptability for growing indoors, orchids, especially Phalaenopsis, have been popular in the ornamental market. However, among numerous orchid pests and diseases, viruses are the most troublesome and we have no efficient measures to control virus diseases. Virus infections can result in retarded growth of orchids, even though the plants appear symptomless (Chia and He, 1999; Zettler et al., 1990); the hidden risk of latent symptom formation after shipment is a thorny problem (Inouye, 2008). Although large-scale cultivation by tissue culture propagation has become the trend in orchid production, mechanical transmission of orchid-infecting viruses via tissue propagation remains a major threat in the orchid industry (Ajikikutira and Wong, 2009; Chang et al., 2010).

Among more than 50 orchid-infecting viruses, Cymbidium mosaic virus (CymMV) and Odontoglossum ringspot virus (ORSV) are prevalent worldwide. The potexvirus CymMV has positive-sense single-stranded RNA ((+)-ssRNA) monopartite genome, consisting of five open reading frames (ORFs) encoding a 160-kDa RNA-dependent RNA polymerase (RdRp), three triple-gene-block proteins (TGBps) and a 24-kDa capsid protein (CP). The genomic RNA (gRNA) of about 6200-nt is 5’-capped and 3’-polyadenylated (Ajikikutira and Wong, 2009; Wong et al., 1997). ORSV, which belongs to the Tobamovirus genus, also harbours a (+)-ssRNA...
monopartite genome of about 6600 nt. The ORF1 encodes 126- and 183-kDa readthrough RdRp proteins. Other ORFs encode a 34-kDa movement protein (MP) and 18-kDa CP (Ajikuttira and Wong, 2009; Ryu and Park, 1995). The gRNA of ORSV has a 5ʹ structure but lacks the 3ʹ-poly(A) tail. Instead, its 3ʹ-untranslated region (3'-UTR) consists of a transfer RNA (tRNA)-like structure in the terminus and an upstream pseudoknot (PK) chain (Chng et al., 1996).

Both CymMV and ORSV are easily transmitted by mechanical wounding, with no recognized vectors in the field (Ajikuttira and Wong, 2009). Although taxonomically distinct, these two viruses commonly feature coinfection in orchid nurseries, and the synergistic effects of CymMV and ORSV coinfection often result in intensified symptoms (Ajikuttira et al., 2005; Hu et al., 1998; Ryu and Park, 1995). The gRNA of ORSV has a 5ʹ-capped structure but lacks the 3ʹ-poly(A) tail. Instead, its 3ʹ-untranslated region (3'-UTR) consists of a transfer RNA (tRNA)-like structure in the terminus and an upstream pseudoknot (PK) chain (Chng et al., 1996).

Both CymMV and ORSV have evolved viral suppressors of RNA silencing (VSRs) in plants. Plants respond to viral infection by producing double-stranded RNA (dsRNA) that can be sensed by RNA-dependent RNA polymerases (RdRps) to produce viral RNA replication intermediates or those with self-annealing hairpin structures, which can serve as templates for DCL processing. Virus-derived small interfering RNAs (siRNAs) usually accumulate at high levels in infected plants and are further recruited into an RNA-induced silencing complex (RISC), thereby guiding sequence-specific viral RNA cleavage. In addition, plant endogenous RdRps are involved in synthesizing dsRNAs from viral ssRNAs that serve as substrates for generation of secondary vsiRNAs, thus amplifying the antiviral response in plants. It is triggered by double-stranded RNAs (dsRNAs) or self-folding RNAs that are processed into functional small RNAs (sRNAs) that are 21- to 24-nt long by RNase III-type Dicer-like proteins (DCLs). On virus infection, viral dsRNAs, such as replication intermediates or those with self-annealing hairpin structures, can serve as templates for DCL processing. Virus-derived small interfering siRNAs (vsiRNAs) usually accumulate at high levels in infected plants and are further recruited into an RNA-induced silencing complex (RISC), thereby guiding sequence-specific viral RNA cleavage. In addition, plant endogenous RdRps are involved in synthesizing dsRNAs from viral ssRNAs that serve as substrates for generation of secondary vsiRNAs, thus amplifying the antiviral cascade (Angell and Baulcombe, 1997; Blevins et al., 2006; Dougherty et al., 1994; Kläve, 2010; Ruiz et al., 1998). Furthermore, siRNAs are mobile molecules and can spread throughout the plant via phloem (Dohnal et al., 2010), so vsiRNAs may act as signal molecules and confer systemic antiviral silencing. Counteractively, viruses have evolved viral suppressors of RNA silencing (VSRs) to interfere with the host endogenous sRNA pathways and inhibit the antiviral activity (reviewed in Bivalskar-Mehla et al., 2011; Csorba et al., 2015; Szittya and Burgyan, 2013). In addition to viral RNAs, vsiRNAs may potentially target plant transcripts, thereby dysregulating plant development (Qi et al., 2009; Wang et al., 2004, 2012b). Collectively, the RNA silencing machinery plays an important role in regulating plant growth and also constitutes a multifaceted layer of defence and counterdefence interactions between viruses and plants.

With advances in high-throughput sequencing technologies, the composition of vsiRNAs has been analysed in different plant–virus pathosystems, focusing on vsiRNA profiling of single pairs of a host and a virus or several viruses in single-inoculated plants (Donaire et al., 2009; Li et al., 2016; Naveed et al., 2014), the vsiRNA pattern with tissue or host specificity (Herranz et al., 2015; Lin et al., 2010; Mitter et al., 2013; Ogwok et al., 2016; Xu et al., 2012), and the effect of double infection on vsiRNA profiles (Cueiara et al., 2015; Li et al., 2013; Tatineni et al., 2014; Xia et al., 2016). Thus, by analysing the cleaved products and combined with other genetic assays, vsiRNA profiles could reflect the mode of action and components involved in antiviral RNA silencing (Szittya et al., 2010; Zhang et al., 2015).

This study documented CymMV and ORSV synergism in Phalaenopsis amabilis. We correlated viral synergism with temporal vsiRNA accumulation in infected orchids, with emphasis on comparing profiles of (1) early and late stages of infection, and (2) infection of Phalaenopsis with CymMV and ORSV alone or together.

RESULTS

CymMV and ORSV infection synergism in P. amabilis

ORSV and CymMV commonly infect orchids. Although viral infection could be detected in systemically infected orchid leaves over 2–8 weeks post-inoculation (Chan et al., 2005; Liao et al., 2004; Lu et al., 2007, 2009), the time course of infection progression for ORSV and CymMV in inoculated leaves of P. amabilis remains unclear. In our preliminary study, we first collected sample strips from ORSV-inoculated leaves at 2, 4, 7, 11 and 30 days post-inoculation (dpi) by tissue blotting. RNA blots revealed that mechanical infection with ORSV in leaf-tip tissues gradually spread to noninoculated leaf-base tissue by 15 days. However, in a similar assay, the time course of CymMV infection was obscure because the signal could not be differentiated from background (data not shown). Nevertheless, 10 dpi was considered the time when ORSV or CymMV titres were substantially raised and confined mostly in inoculated areas; therefore, in this study we collected inoculated leaves at 10 dpi for infection assays.

To determine the synergism of ORSV and CymMV coinfection in P. amabilis, leaf-tip tissues in virus-free Phalaenopsis were inoculated with CymMV or ORSV alone or combined (Fig. 1A). Virus infection was first confirmed by tissue blotting with digoxigenin (DIG)-labelled riboprobes (Fig. 1B). Tissue was collected separately from inoculated and noninoculated areas for RNA analysis. At 10 dpi, tissue blotting revealed ORSV single infection confined to the inoculated area in most inoculations (Fig. 1B, Oi). Similar to ORSV inoculation, in more than half of the inoculations (32/60 leaves), CymMV accumulation was confined to the inoculated area at 10 dpi (Fig. 1B, Ci). The results suggest that the system could be used to collect tissues of different infection stages by spatial separation. To avoid confusion with the scattered tracts of infected tissues over the separation line, we excluded leaves with virus infection detected in noninoculated tissues from further analysis. Of note, single-infected leaves were symptomless, but chlorotic

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lesions and ringspots appeared in double-infected leaves and were restrained to inoculated areas at 10 dpi (Fig. 1B, Di). In all 60 double-inoculated leaves, ORSV infection was detectable only in inoculated areas (Fig. 1B, Oi), without much difference from ORSV single infection (Fig. 1B, O). In contrast, double-infected leaves showed enhanced CymMV spreading to noninoculated tissues in 54/60 leaves (Fig. 1B, Dn).

To further confirm the viral accumulation, total RNA samples were separately extracted from inoculated (i) and noninoculated (n) tissues for northern blot analysis. Inoculated tissues of CymMV (C), ORSV (O) or CymMV mixed with ORSV (D, for ‘double’) were designated Ci, Oi and Di, respectively. Similarly, noninoculated adjacent tissues from corresponding inoculations were designated Cn, On and Dn. Mock (M) tissues with buffer inoculation and adjacent tissues were designated Mi and Mn, respectively (Fig. 1B). RNA blotting revealed much higher CymMV viral RNA accumulation in Di than Ci tissue (Fig. 2A), which suggests enhanced CymMV titres during coinfection with ORSV. ORSV accumulation was slightly reduced in double infection (Fig. 2A), which supports the tissue blotting results (Fig. 1B). Accordingly, sRNA blotting revealed a higher amount of CymMV vsiRNAs in Di than Dn or Ci tissues (Fig. 2B). Also, ORSV vsiRNA accumulation was higher in Di than in Dn tissues (Fig. 2B), so levels of vsiRNAs were associated with virus accumulation in infected tissues. Reverse transcription-PCR (RT-PCR) assay confirmed single or double infection in inoculated tissues and no virus in mock-inoculated tissues (Fig. 2C). Although CymMV and/or ORSV was undetectable in RNA blots (Figs 1B and 2A), RT-PCR reached higher sensitivity and

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**Fig. 1** Symptoms and tissue blotting detection of ORSV and/or CymMV-infection in Phalaenopsis. (A) Diagrams of leaf-tip inoculation and tissue-blotting detection in the infection assays. Phalaenopsis amabilis was inoculated with virions in the tip-half of the inoculated leaf. Red rectangles indicate areas that were used for collecting inoculated and noninoculated tissues. Right panel illustrates the sampling procedure of tissue blotting: the whole inoculated leaf was cut off and sequentially sliced from the base (noninoculated) to tip (inoculated). Each cut was pressed on nylon membranes. (B) Phenotype and tissue-blotting detection of ORSV and CymMV from mock- and inoculated leaves at 10 dpi. Mock (M) tissues with buffer inoculation and adjacent tissues were designated Mi and Mn, respectively. Dashed lines separate inoculated (right) and noninoculated (left) areas corresponding to (A). Labels indicate inoculated (i, orange characters) or noninoculated (n, green characters) tissues of mock (M), ORSV (O), CymMV (C) or CymMV and ORSV double (D)-inoculated leaves. The blots were hybridized with digoxigenin (DIG)-labelled CymMV- and ORSV-CP gene-specific probes.
revealed virus accumulation in noninoculated tissues (Fig. 2C, Cn, On and Dn), so the viruses had started invading these tissues at the initial stage of infection.

In summary, by leaf-tip inoculation, early (e.g. Cn and On tissues) and late (e.g. Ci, Oi and Di tissues) stages of infection could be distinguished at 10 dpi in noninoculated and inoculated tissues, respectively. Also, coinfection with CymMV and ORSV enhanced CymMV titres and spreading as well as symptom formation.

**Profiling sRNAs from virus-infected P. amabilis**

To analyse the vsiRNA profiles, we purified each sRNA pool from samples as described previously, then used Illumina high-throughput sequencing. We generated 50 342 804 raw reads from eight sRNA libraries. After quality trimming and size filtering, more than 2.9 million clean reads of 18–25 nt sRNAs were retrieved for each library (Table 1). Identical sequences were grouped into unique reads (tags) for further characterization (Table S1). The size distribution of total sRNA tags was similar among the libraries: the 24-nt length was most prevalent and represented more than 50% of total unique reads (Fig. 3B). For the redundant reads, 21-nt sequences were predominant in all libraries, followed by 24-nt sequences (Fig. 3A). Notably, the proportion of 24-nt reads was overwhelmingly lowered due to the much reduced number of 24-nt tags in the Di library (Fig. 3B).
## Table 1  
Summary of total reads in sRNA libraries constructed from mock- and virus-inoculated *Phalaenopsis* tissues

|                    | Mi* | Mn | Oi | On | Ci | Cn | Di | Dn |
|--------------------|-----|----|----|----|----|----|----|----|
| **Raw data input** |     |    |    |    |    |    |    |    |
| Total reads        | 5 384 213 | 5 230 602 | 11 447 603 | 6 911 927 | 5 049 276 | 5 680 202 | 5 326 905 | 5 312 076 |
| Adaptor and quality trimmed† | 5 056 536 | 4 933 398 | 11 094 651 | 6 737 444 | 4 739 283 | 5 375 120 | 5 082 378 | 5 066 946 |
| Low frequency filtered 18–25 nt reads‡ | 3 313 331 | 2 984 471 | 7 422 666 | 3 842 315 | 3 286 321 | 3 635 286 | 3 652 115 | 3 335 055 |
| **Viral siRNAs**    |     |    |    |    |    |    |    |    |
| ORSV$^*$            | 1 | 3 | 2 178 379 (29.34%) | 4577 (0.12%) | 50 | 87 | 439 373 (12.03%) | 237 (<0.01%) |
| CymMV               | 5 | 12 | 3 | 1 | 176 757 (5.37%) | 759 (0.02%) | 961 229 (26.32%) | 258 119 (7.74%) |
| **sRNAs from *Phalaenopsis*** |     |    |    |    |    |    |    |    |
| *P. aphrodite* ESTs | 2 044 888 | 1 871 853 | 2 762 900 | 2 597 837 | 1 771 682 | 2 197 024 | 1 042 558 | 1 924 590 |
| Structure RNA (Rfam) | 179 345 | 171 235 | 452 767 | 327 299 | 134 762 | 157 086 | 251 479 | 182 899 |
| miRNA               | 43 671 | 35 788 | 23 459 | 16 567 | 42 433 | 42 556 | 19 103 | 29 388 |
| Others              | 1 045 421 | 905 580 | 2 005 158 | 896 034 | 1 160 527 | 1 237 774 | 938 373 | 939 822 |

*Mi, Oi, Ci and Di: inoculated (i) tissues of mock (M)-, ORSV (O)-, CymMV (C)- and double (D)-inoculated plants. Mn, On, Cn and Dn: noninoculated (n) tissues of mock (M)-, ORSV (O)-, CymMV (C) - and double (D)-inoculated plants.

†Trimming steps, including sequencing quality, adapter removal and filtering poly-A/T/C/G or N (null)-calling bases containing reads.

‡Reads with only one count are defined as low-frequency reads and filtered before further analysis.$^*$ The numbers in parentheses indicate the percentage of ORSV and CymMV vsiRNAs in clean (low-frequency filtered 18–25 nt) reads.
CymMV and ORSV vsiRNAs were identified after searching against the viral genome for both viruses (Fig. S1). To tolerate possible sequence variations evolved during virus replication, genome sequences from 11 CymMV and five ORSV strains deposited in GenBank were used for annotation. Also, we confirmed that no read could be mutually mapped to both CymMV and ORSV genomes. About 29.3% (2,178,379 reads) and 0.1% (4,577 reads) of total annotated reads in the Oi and On libraries, respectively, were perfectly matched to the ORSV genomes (Table 1). A rather smaller proportion, about 5.4% and <0.1% total reads in the Ci and Cn libraries, respectively, were perfectly matched to the CymMV genomes (Table 1). As compared with Ci, CymMV vsiRNAs reached a much higher proportion, with 26.3% and 7.7% total reads in Di and Dn, respectively. In contrast, ORSV vsiRNAs contributed to 12.0% total reads (439,373 reads) for about 2-fold less accumulation than in the Oi library. Dn tissues showed an even larger gap between proportions of CymMV and ORSV vsiRNAs because less than 0.1% (237 reads) of total reads was identified as ORSV vsiRNAs. The more than 5-fold enrichment of CymMV vsiRNAs again demonstrated synergistic enhancement of CymMV accumulation.

**Size distribution, strand polarity and 5′-end nucleotide preference of CymMV and ORSV vsiRNAs**

We analysed the size distribution, strand polarity and 5′-end nucleotide preference in CymMV and ORSV vsiRNA populations. In the single-infected Ci and Oi libraries, 21-nt vsiRNAs were predominant (63.4% for CymMV and 61.3% for ORSV), followed by the 22-nt class (21.9% for CymMV and 17.2% for ORSV; Fig. 4). The size distribution of CymMV and ORSV vsiRNAs in double-infected tissues (Di and Dn) showed similar patterns, with 21 nt as
the major size class (59.8% in Di and 62.4% in Dn for CymMV vsiRNAs, 53.0% in Di and 54.4% in Dn for ORSV vsiRNAs) and 22 nt as the second most abundant (Fig. 4). The 21- and 22-nt reads represented 81.4% (CymMV) and 76.5% (ORSV) of total vsiRNAs, which suggests that antiviral RNA silencing in *P. amabilis* may be mediated mainly by DCL4 and DCL2 proteins.

We observed asymmetrical distribution of vsiRNAs in sense-strand polarity among all the libraries except the On library, in which vsiRNAs were derived almost equally from both polarities and possessed only a modest enrichment (54.6%) of (+)-strand reads (Fig. 4B, On). The prevalence of sense-strands remained in CymMV and ORSV double-infected tissues Di and Dn, accounting for 70.6% to 72.5% of total CymMV vsiRNAs (Fig. 4A, Di/Dn) and 58.2% to 62.4% of total ORSV vsiRNAs (Fig. 4B, Di/Dn).

When grouped by the 5′-end nucleotide composition, generally C- and U-terminated CymMV vsiRNAs were prevalent in the 21- and 22-nt classes in the CymMV single-infected Ci and Cn libraries (Figs 5A and S2A). This 5′-end C and U dominance of CymMV vsiRNAs was likewise observed among the Ci, Cn, Di and Dn libraries. In contrast, ORSV vsiRNAs showed an even distribution of 5′ A, C and U in the 21- and 22-nt classes in the Oi and On libraries (Figs S5B and S2B), whereas the preference was slightly shifted to 5′ U and C dominance in the Di and Dn libraries (Figs S5B and S2B). For vsiRNA species with other sizes, the pattern for 5′-end nucleotide preference seemed biased from low vsiRNA reads (Fig. S2). Generally, CymMV and ORSV vsiRNAs were infrequently 5′ G, regardless of size classes in all the libraries (Figs 5 and S2).

Collectively, we found a prevalence of 21- and 22-nt size classes and (+)-strand polarity for both CymMV and ORSV vsiRNAs in all the libraries, regardless of infection stages with single or double viruses. These results suggest that CymMV and ORSV trigger common gene silencing machinery to generate similar vsiRNA profiles as the defence response in *Phalaenopsis*.

**Genome mapping, coverage and hotspots of CymMV and ORSV vsiRNAs**

To study the vsiRNA origins across the entire viral genome, vsiRNAs were located according to their 5′-end sites along both positive and negative strands of the CymMV (AY571289) and ORSV (AY571290) genomes (Figs 6, S3 and S4). In the Di
library, the genome coverage was up to 91.0% and 98.8% for all sizes of CymMV and ORSV vsiRNAs, respectively. All the 21- to 24-nt size classes distributed continuously (Fig. S5) but with some genomic regions showing higher mapping frequencies. The CymMV vsiRNA distribution extensively encompassed the RdRp-coding region, and few were located in the TGBp- and CP-coding regions (Fig. 6A), and showed similar patterns in co-infection with ORSV or in early and late stages of infection (Figs 6A and S3). Northern blot hybridization confirmed the accumulation of RdRp-originated CymMV vsiRNAs in Ci, Di and Dn tissues (Fig. 2B). The origins of ORSV vsiRNAs had comparable origins and peak pattern were almost the same, especially for CymMV vsiRNAs between the Di and Di-2 libraries (Fig. S7C). When mapping the vsiRNAs to viral genomes, conserved origins and peak pattern were almost the same, especially for ORSV vsiRNAs between the two batches, some slight variation in peak pattern was observed (Fig. S7D). From the two replicates, we have identified 10 997 553 clean reads after quality control, with 20 777 593 raw reads from four libraries (Mi-2, Mn-2, Di-2 and Dn-2). In total, 10 997 553 clean reads were retained after quality and size filtering (Table S2). Although ORSV vsiRNAs accumulated to a higher level in the second batch of libraries with reduced (+)-strand predominance (Table S2 and Fig. S7), the other characteristics of vsiRNAs in the pool remained similar to the first profile. For instance, 21- and 22-nt size classes, (+)-strand polarity and 5′ U/C ends still prevailed in both CymMV and ORSV vsiRNA profiles (Fig. S7A,B). When mapping the vsiRNAs to viral genomes, conserved origins and peak pattern were almost the same, especially for CymMV vsiRNAs between the Di and Di-2 libraries (Fig. S7C). For ORSV vsiRNAs, although similar cleavage sites remained unchanged between the two batches, some slight variation in peak pattern was observed (Fig. S7D). The alteration may relate to the discrepancy in ORSV vsiRNA accumulation level and the shift in (+): (−)-strand ratio. Nevertheless, the enrichment of (−)-polarity ORSV vsiRNA hotspots in double infection was validated by northern blots (Fig. S7D). From the two replicates, we have identified and confirmed the general characteristics and major cleavage sites of CymMV/ORSV vsiRNAs in Phalaenopsis.

**Potential roles of CymMV/ORSV vsiRNAs in manipulating viral pathogenesis in infected orchids**

On the basis of sequence homology, previous studies have suggested vsiRNA-induced symptom formation via mediating host mRNA degradation (Navarro et al., 2012; Shimura et al., 2011). To search transcripts that could be targeted by CymMV or ORSV vsiRNAs, we examined possible pairings between vsiRNA tags and P. aphrodite EST sequences (Chao et al., 2014). A global search for all the sequenced tags revealed 22 021 and 24 772 orchid transcripts as potential targets by CymMV or ORSV vsiRNAs.
respectively. Assuming that vsiRNA tags with prevalent reads may have higher impact on the expression of target genes, we focused on targets of the 20 most abundant vsiRNAs in the Oi, Ci and Di libraries. The filter highlighted 52 targets for 24 CymMV vsiRNAs and 68 for 28 ORSV vsiRNAs (Tables S3 and S4 and Fig. S8). We observed high overlapping between sets from the Ci and Di libraries for targets of CymMV vsiRNAs versus ORSV vsiRNAs (Fig. S8), which reflects that the ORSV vsiRNA hotspot shift may have a specific impact on host gene regulation in synergistic infections.

Fig. 6 Distribution of vsiRNAs along CymMV (A) and ORSV (B) genome corresponding to reads from the CymMV (Ci), ORSV (Oi) and double inoculated (Di) inoculated tissue libraries. Upward dark-coloured and downward light-coloured lines represent (+) and (−) polarity reads, respectively. Genomic sites of RdRp, TGB (or MP) and CP gene coding regions are indicated below (numbers based on GenBank accession CymMV AY571289 or ORSV AY571290). The peak level is presented as reads per million (RPM).
To evaluate our hypothesis, we performed gene ontology (GO) enrichment analyses for functional interpretation of the vsiRNA targets. The annotations for CymMV vsiRNA targets were mainly enriched in autophagy, metabolic activities and transmembrane transport terms regardless of single (Ci) or double (Di) infections (Tables S5 and S6). However, the annotation of targets of ORSV vsiRNAs in single infection (Oi) encompassed a broader range of biological processes, including protein translocation, endoplasmic reticulum (ER) response, molecule modification, response to environmental stimulus and others (Table S7). Another 34 novel targets were identified for dominant ORSV vsiRNAs in double infection (Di), and GO terms linked to transcription and protein modification prevailed (Table S8). Of note, EKC/KEOPS complex subunit bud32 and tryptophan aminotransferase-related protein 2 (TAR2) were potential targets of ORSV 3′-UTR hotspot vsiRNAs. Also, two of the topmost (−)-strand hotspot ORSV vsiRNAs target chloroplastic nudix hydrolase 23 (NUDX23) and serine/threonine-protein kinase NAK (Table S4).

**Differential expression of P. amabilis RNAi components in response to CymMV and ORSV infection**

To identify the major components mediating vsiRNA generation, we determined transcript levels of *P. amabilis* DCL1/2/4, Argonaute proteins (AGOs) AGO1/4/5/10 and RNA-dependent RNA polymerases (RDRs) RDR1/2/6 with CymMV and ORSV single or double infection. All evaluated components except AGO4 and AGO10 were up-regulated with CymMV and ORSV double infection (Fig. 7). For single infections, AGO5, RDR1 and RDR6 expressions were readily induced by CymMV infection. Of note, several components including AGO4, AGO10, RDR2 and DCL4 were suppressed with ORSV single infection. These results show that the viruses have different effects on the RNAi genes, which may contribute to the varied profiles of CymMV and ORSV vsiRNAs.

**DISCUSSION**

In this study, we recorded the synergistic effects of CymMV and ORSV double infection in *P. amabilis*. Consistent with previous studies (Hadley et al., 1987; Lawson and Brannigan, 1986; Pearson and Cole, 1991), chlorotic lesions and ringspots appeared at about 7 dpi and continued enlarging afterwards in double-inoculated tissues (Fig. 1B and data not shown). Northern blot analysis further revealed enhanced CymMV titres (Fig. 2A) as well as about 5-fold accumulation of CymMV vsiRNAs in double- versus single-infected (Di vs Ci) tissues. In contrast, the viral titres of ORSV did not increase in Di tissues with CymMV coinfection and were scarcely detected in the noninoculated (Dn) tissues by northern blot or RT-PCR (Fig. 2A,C). The results clearly demonstrate an asymmetric synergism between CymMV and ORSV in double-infected *Phalaenopsis*. Previously, Hu et al (1998) reported that both CymMV and ORSV accumulation could be detected in *Dendrobium* protoplasts at earlier time points on mixed inoculation. In addition, ORSV genomic RNA accumulation was accelerated in double-infected *Nicotiana benthamiana* (Ajikuttira and Wong, 2009). Although we have observed intensified symptoms in mixed infected *Nicotiana* and orchid, both CymMV and ORSV titres reached similar levels between singly and doubly transfected protoplasts (Chen et al., 2019; Fig. S9). The discrepancy between our results and the other reports may be due to different virus strains in response to different host species. Their *Cattleya*-originated viral strains (Yu and Wong, 1998) differ from ours from *P. amabilis* with about 3% nucleotide variation (data not shown). This could be the result of coevolution between viruses and their natural hosts.

In reviewing asymmetric viral synergism, the classical example is potato virus Y (PVY) supporting potato virus X (PVX) accumulation (Vance, 1991). Similarly, PVX benefited more from mixed infection with tobacco mosaic virus (TMV) in tomato (Balogun et al., 2002). Of note, the accumulation of ‘helper’ viruses usually remained unchanged or slightly decreased in most combinations (Mascia and Gallitelli, 2016). This was also the case for ORSV in our study. TMV P126 protein has VSR activity that can increase the susceptibility of several viruses in *Nicotiana tabacum* (Harries et al., 2008). In addition, Ajikuttira et al. (2005) demonstrated that the MPs and CPs were functionally interchangeable between CymMV and ORSV, with the exception of long-distance movement of ORSV RNA by CymMV CP. In other tobamoviruses, the VSR and the ability of MP to increase the size exclusion limit of plasmodesmata have been documented (Wang et al., 2012a; Wolf et al., 1989). Together with the result of unchanged CymMV and ORSV viral titres in protoplasts (Chen et al., 2019; Fig. S9), we suggest that enhanced CymMV movement may be the major force for synergism. Whether ORSV exerts helper components to pave the way for vigorous CymMV infection requires further investigation.

The production of vsiRNAs is a hallmark of antiviral RNA silencing in host plants. In *P. amabilis*, although CymMV and ORSV outcompete the defence response and render the plant susceptible, vsiRNAs generated by ribonuclease cleavage against CymMV and ORSV viral RNAs were readily detectable in inoculated (Ci, Oi and Di) as well as neighbouring noninoculated (Cn, On and Dn) tissues (Fig. 2B), which indicates that antiviral RNA silencing was activated in the early stage of infection as well. The profile revealed that CymMV and ORSV vsiRNAs shared common features including (1) the predominance of 21- and 22-nt classes, which mirrors the conserved roles of DCL4 and DCL2 homologues in *P. amabilis* antiviral RNA silencing, as in many other plants; (2) asymmetry in strand polarity, which indicates that the biosynthesis of CymMV and ORSV vsiRNAs may involve self-annealing hairpin structures within viral ssRNA (Molnár et al., 2005) in addition to replicative intermediates and long dsRNA during viral replication (Ding, 2010); and (3) under-representation of guanine at the 5′-end nucleotide composition. The avoidance was clearly demonstrated because the base composition of ORSV genomic RNA is U (30.8%) > A (29.8%) > G (21.6%) > C (17.8%) (AY571290), and
CymMV genome is proportioned as C (29.2%) > A (26.5%) > U (24.7%) > G (19.6%) (AY571289). No preferential vsiRNA size class was associated with strand polarity and 5′ nucleotide preference, so the bias was not introduced by different DCL processing. Moreover, the 5′-end nucleotide identity is a key determinant for sorting sRNAs into distinct AGO proteins (Czech and Fig. 7 Expression of Phalaenopsis amabilis RNA silencing components with CymMV and/or ORSV infection. Quantitative reverse transcription PCR (RT-qPCR) analysis of mRNA accumulation of DCL1/2/4, RDR1/2/6 and AGO1/4/5/10. Mi, Ci, Oi and Di: inoculated tissues of mock (M)-, ORSV (O)-, CymMV (C)-, ORSV (O)- and double (D)-inoculated plants. Relative quantity (y-axis) was averaged from three biological replicates, presented as mean ± SD (n = 3). The average of mock (Mi) was arbitrarily set as one. Significant differences are marked with lower case letters at P ≤ 0.05 (Student–Newman–Keuls test).
Hannon, 2011; Kim, 2008), such as AGO1 preferring sRNAs with the 5’-terminal U, AGO2 and AGO4 the 5’ A, and AGO5 the 5’ C (Hu et al., 2011; Mi et al., 2008; Montgomery et al., 2008; Takeda et al., 2008). The absence of known 5’-G preferred AGO species may explain the avoidance of 5’-G vsiRNAs (Donaire et al., 2009). Furthermore, the 5’-A preference of ORSV vsiRNAs in single infection shifted to 5’-U and 5’-C dominance with double infection with CymMV, which coincided with up-regulation of AGO1 and AGO5 in double-infected tissues (Fig. 7). The results suggest that CymMV and ORSV vsiRNAs could be recruited into different AGO proteins with single or double infection.

For vsiRNA origins from viral genomes, the CymMV and ORSV vsiRNA profiles differed. In single-infected tissues, more hotspots resided in the RdRp of CymMV and the CP to 3’-UTR region of the ORSV genome (Fig. 6). We analysed the GC context of each viral segment but found no clear relevance (Table S9). This discrepancy may be due to higher accumulation of ORSV CP-expressed subgenomic RNA and more abundant CymMV gRNAs in the infected P. amabilis tissues, as shown on northern blots (Fig. 2A). ORSV single-infected tissues (Oi and On) showed a unique hotspot located in the 3’-UTR (Figs 6B and S4), which had one specific (+)-polarity tag. Modelling of the 3’-UTR secondary structure by Gultyaev et al. (1994) revealed that the hotspot vsiRNA resided in the first PK structure upstream of the tRNA-like structure (Fig. S6B), which was not a typical double-strand structure expected to be preferentially cleaved by DCL enzymes. Of note, we also identified similar hotspot vsiRNAs in TMV-infected tobacco that shared a conserved sequence and structural origin with the ORSV PK vsiRNA (Fig. S6C). These observations suggest the accessibility of DCL dicing at the PK structure. The 3’-UTR of tobamoviruses plays a central role in viral replication (reviewed in Chujo et al., 2015), especially as the two PKs upstream of the tRNA-like structure are indispensable for full translation and infectivity (Leathers et al., 1993; Takamatsu et al., 1990). However, how the RNA structure or the recruited factors are involved in generating the abundant hotspot vsiRNAs remains to be explored.

In addition, we identified three prominent hotspot peaks in the negative polarity of the ORSV genome specifically in double-infected tissues (Figs 6B and S4). Similarly, the sweet potato begomovirus isolate StV1 asymmetrically benefitted from coinfection with crinivirus sweet potato chlorotic stunt virus (SPCSV), and a specific change in vsiRNAs derived from SPCSV but not StV1 was observed (Cuellar et al., 2015). Moreover, vsiRNAs hotspot distribution was increased in asymmetrical synergism between maize chlorotic mottle virus and sugarcane mosaic virus (Xia et al., 2016) or between brassica yellows virus and pea enation mosaic virus 2 (Zhou et al., 2017). These studies suggest possible roles of potent VSR activity in altering the hosts’ RNA silencing machinery and leading to modified targeting to viral RNAs. In line with these hypotheses, vsiRNA profiles from wild-type or VSR-deficient CymRSV (Szittya et al., 2010) and tospoviruses (Margaria et al., 2015) revealed the influence of VSRs to DCL targeting of the viral genome. P25 of CymMV and P126 of ORSV are potential VSRs to suppress RNA silencing and affect vsiRNA generation. Along with DCL1/2/4 being highly induced in double-infected tissues (Fig. 7), the discrepancy in distribution of ORSV vsiRNA hotspots may be due to differences in the effects of VSRs and altered DCL accessibility to ORSV viral RNAs during interaction with CymMV.

In contrast to the viral RNA clearance activity of plant RNA silencing pathways, host transcripts could be targeted by vsiRNAs if the virus and host mRNAs share sequence complementarity (Navarro et al., 2012; Shimura et al., 2011; Smith et al., 2011). Our study predicted potential Phalaenopsis target genes of ORSV and CymMV vsiRNAs. For instance, targets of CymMV vsiRNAs include (1) serine/threonine kinases that harbour protein phosphorylation activity (Hardie, 1999) and several members reported to be involved in defence responses (Afzal et al., 2008; Rodriguez et al., 2010); (2) protochlorophyllide-dependent translocon component 52 involved in chlorophyll metabolism (Bartsch et al., 2008); and (3) autophagy-related proteins, which may contribute to antiviral responses or in turn facilitate viral replication (Clavel et al., 2017). On the other hand, EKC/KEOPS complex subunit bud32 and TAR2 were potential targets of the 3’-UTR ORSV hotspot vsiRNA. Novel targets such as NUDX23 and serine/threonine-protein kinase NAK were highlighted because unique ORSV vsiRNA hotspots were generated upon mixed infection with CymMV. EKC/KEOPS is a conserved complex for RNA modification (Srinivasan et al., 2011) and TAR2 has been linked to local auxin production (Stepanova et al., 2008). Because tobamoviruses are tRNA-tailed, and the auxin-response pathway confers an age-dependent defence against TMV (Collum et al., 2016; Padmanabhan et al., 2008), regulation of these two host genes may affect ORSV pathogenesis. NUDX23 is a chloroplastic flavin pyrophosphohydrolase in Arabidopsis involved in the antioxidant response (Maruta et al., 2012). Another pyrophosphohydrolase, NUDT7, was identified as a negative regulator of the defence response against bacteria (Ge and Xia, 2008). Because viral symptoms appeared only in Di tissues at 10 dpi, combined with the presence of unique ORSV vsiRNA hotspots and high abundance of CymMV vsiRNAs, dysregulation of the novel targets of ORSV vsiRNAs and/or enhanced targeting of CymMV vsiRNAs to host transcripts may lead to synergized viral pathogenesis under mixed infection. We expect future work will uncover the association between CymMV/ORSV viral synergism and vsiRNA-mediated silencing of Phalaenopsis genes.

### EXPERIMENTAL PROCEDURES

**Plant materials, virus inoculation, tissue blot and total RNA isolation**

Mericlone *P. amabilis* plants in 7.5-cm pots were purchased from I-hsin Biotechnology Inc. (Chia-yi, Taiwan). The plants were confirmed to be CymMV and ORSV-free by multiplex
RT-PCR as described (Lee and Chang, 2006), then grown in walk-in chambers with a day/night temperature of 28/25 °C. CymMV and ORSV isolates were kindly provided by Dr. Yau-Heiu Hsu (National Chung Hsing University, Taichung, Taiwan). After propagating in N. benthamiana, virions were purified as described (Lin and Chen, 1991; Lin et al., 1992). For inoculation assays, 1 μg of CymMV/ORSV or 0.5 μg of each CymMV and ORSV virions per leaf was inoculated on the first and second fully expanded leaves. Assuming the virus would spread along parallel veins, plants were mechanically inoculated with purified virions only at the tip halves. The mock-inoculated plants with 20 μL inoculation buffer (5 mM Tris-HCl, 1 mM EDTA, 5 mM phosphate buffer and 1 g/L bentonite, pH 8.0) were used as controls.

At 10 dpi, we first used tissue blotting to reveal virus infection and distribution in the inoculated leaves, as illustrated in Fig. 1A (Lin et al., 1990). Slices from inoculated and adjacent noninoculated tissues were separately collected. The blots were then hybridized with riboprobes specific to CymMV/ORSV full-length CP genes by using the DIG Nucleic Acid Detection Kit (Sigma-Aldrich, St Louis, MO, USA). The collected leaves were classified by infection stage as revealed by tissue blotting. Tissues of the same category from three leaves were pooled for total RNA extraction by using TriPure Isolation Reagent (Sigma-Aldrich) following the manufacturers’ instructions.

**Northern blot, sRNA detection and RT-PCR**

Accumulation of viral RNA was examined by northern blot analysis as described (Lin et al., 1996; Palani and Lin, 2007), following the hybridization methods used for tissue blotting. The northern blot method for total vsiRNAs was according to Lin et al. (2010). In brief, 25 μg total RNA was separated and electrophoresed onto to Hybond-N+ nylon membranes. The blots were hybridized with [γ − 32P] cytidine triphosphate (CTP)-labelled, carbonate buffer-fragmented RNA probes CymMV-RdRp or ORSV-CP3UTR (Table S11). To determine relative vsiRNA abundance of different genomic regions, a series of in vitro transcripts corresponding to CymMV and ORSV genomic segments (Table S11) were first blotted onto nylon membranes. Small RNAs were enriched by using miVana miRNA isolation kit (Invitrogen, Waltham, MA, USA) and the 5′-end labelled as described (Lin et al., 2010), then hybridized to the blots. Radioactive signals were recorded by using the phosphorimager Typhoon FLA7000 (GE Healthcare Life Sciences, Chicago, IL, USA).

For RT-PCR and RT-qPCR analyses, cDNA was reverse-transcribed from 1 μg total RNA by using the ToolsQuant II Fast RT Kit (BioTools, New Taipei City, Taiwan) with the primer pairs specific to CymMV/ORSV RdRp genes (CymRdRp-F, CymRdRp-R and OR-RdRp-F, OR-RdRp-R) to detect virus infection. To determine expression levels of P. amabilis RNA silencing components, putative DCLs/AGO/RDRs sequences were retrieved from OrchidBase 2.0 (Chao et al., 2014; Su et al., 2011) and OrchidBase 3.0 (Cai et al., 2015) by name search. Phylogenetic analysis by using Arabidopsis and Oryza sativa reference genomes clearly grouped DCL1/2/3/4, AGO1/4/5/6/7/10 and RDR1/2/3/6 homologues in Phalaenopsis. The sequences were cloned from P. amabilis for primer design (data not shown). Real-time PCR for major antiviral components involved the SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA) and QuantStudio 12K Flex System (Applied Biosystems). RT-qPCR reads were transformed into a relative expression index calculated by the ΔΔCt algorithm (Livak and Schmittgen, 2001), with ubiquitin (primer pairs Pa-Ubi-2F and Pa-Ubi-2R) as a reference gene. The mean of M1 tissue replicates was set as the secondary standard. The primer sequences are shown in Table S10.

**sRNA deep-sequencing and bioinformatic annotation**

The integrity of the RNA samples was checked by using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) before being submitted to sRNA library construction. For each library, 10 μg total RNA was size-fractionated on 15% Tris-borate-EDTA urea polyacrylamide gel. sRNAs recovered from the gel bands corresponded to 18–30 nt of the marker. Construction of cDNA libraries involved the TruSeq Small RNA Sample Preparation protocol (Illumina, San Diego, CA, USA). Bar-coded libraries were pooled in a single channel of the flow cell and sequenced by using the Illumina HiSeq 2000 at Yourgene Bioscience (New Taipei City, Taiwan).

The raw sequencing reads were first processed by using Trimomatic (Bolger et al., 2014). Customized Perl scripts were used to examine contamination from adapters, size markers, reads with N (null) or and poly-A/T/C/G callings. Clean reads were then grouped into unique reads (tags) by sequence identity. Low-frequency tags (i.e. tags with only one read in a single library that were not detected in any other libraries) and tags with sizes <18 nt or >25 nt were filtered out. CymMV- and ORSV-specific vsiRNAs were identified by alignment against viral genome sequences, which was conducted using Bowtie (Langmead, 2010). All the available CymMV/ORSV genome sequences were downloaded from GenBank (CymMV accession numbers AF016914, AY571289, JQ860108, HQ681906, EF125180, EF125179, EF125178, AM055720, AB197937, NC_001812, EU314803; ORSV accessions AY571290, DQ139262, NC_001728, KF855954, U34586). The nonredundant results from mapping to different isolates were summed as total mapped reads for CymMV/ORSV. To identify hotspots of vsiRNA origins, we selected the Taiwan isolates (CymMV AY571289 and ORSV AY571290) as representatives for genomic sites. The nonviral reads were further annotated with plant resources including databases for miRNAs (miRBase v. 21.0, Kozomara and Griffiths-Jones, 2013). The P. aphroditte transcriptome (OrchidBase 2.0), Rfam v. 10.1 (Navrocki et al., 2015) and other local databases, with the hierarchy and criteria indicated in Fig. S1. For TMV vsiRNA profile, sRNA sequencing data collected by
Pecman et al. (2017) were downloaded from Sequence Read Archive accession SRX2672447. Sequence quality and size trimming were performed as described for Phalaenopsis libraries. TMV vsiRNAs were identified by a perfect match to the TMV reference genome NC_001367.

Target gene prediction and GO enrichment analysis
Putative CymMV/ORSV vsiRNA targeting Phalaenopsis genes were predicted by using a scoring matrix (Weiberg et al., 2013). Because of the huge diversity, we selected only predicted targets of the top 20 abundant CymMV and ORSV vsiRNAs with single (Ci and Oi) or double infection (Di) for further analysis. GO annotations for the P. aphrodite transcriptome were downloaded from Orchidstra 2.0. A statistical test based on a hypergeometric distribution was performed using Matlab to determine whether a certain category was significantly enriched. The enriched terms with $P \leq 0.05$ were summarized into major categories using the web tool REVIGO (Supek et al., 2011).

Protoplast isolation, virus transfection and western blot analysis
Flower protoplasts were isolated from P. amabilis petals and sepals using methods previously described (Lin et al., 2018). In addition, the protoplasts were floated onto 20% sucrose gradient and centrifuged at 70 g for 5 min to remove oxalate crystals. Approximately $2 \times 10^7$ cells were transfected with 0.5 μg of CymMV/ORSV or 0.5 μg of each CymMV and ORSV virions by the PEG1450 method. The protoplasts were cultivated at 25 °C under light for 12 or 20 h before harvest. Virus accumulation was detected by western blots conducted with laboratory-generated rabbit anti-CymMV or anti-ORSV CP antiserum.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1 Annotation pipeline of deep sequencing data. The pre-processed reads were used as queries to search against virus genome, *Phalaenopsis* transcriptome, Rfam, and miRBase using Bowtie algorithm. The criteria and hierarchy are indicated. Reads that not annotated as known categories (classified as “others” at first) were mapped to *P. aphrodite* expression tags (ESTs) again and allowed 1 mismatch in the second round.

Fig. S2 Nucleotide composition of 5′-end of CymMV (A) and ORSV (B) vsiRNAs in inoculated *Phalaenopsis*. The proportion of adenine (A), cytosine (C), guanine (G) and uridine (U) within each size class. Libraries constructed from inoculated (i) or noninoculated (n) tissues of mock (M), ORSV (O), CymMV (C), or CymMV and ORSV double (D)-inoculated leaves are shown.

Fig. S3 Distribution of vsiRNAs along CymMV genome corresponding to reads from libraries of CymMV-infected tissue. Upward dark-coloured and downward light-coloured lines represent (+) and (−)-polarity reads, respectively. Genomic sites of RdRp, MP and CP gene coding regions are indicated below (numbers based on GenBank accession ORSV AY571290). Maps constructed from inoculated (i) or noninoculated (n) tissues with ORSV (O) single inoculation, or CymMV and ORSV double (D)-inoculated leaves are shown.

Fig. S5 Size-separated mapping of CymMV and ORSV vsiRNA hotspots. Peaks of (A) CymMV and (B) ORSV vsiRNA reads of 21-, 22-, or 24-nt classes are indicated in different-coloured lines.

Fig. S6 Analyses of sequence conservation and vsiRNA generation frequency of ORSV 3′-UTR. (A) Alignment of the 3′-UTR of some tobamovirus model species registered in ICTVdb. Red line indicates the ORSV 3′-UTR hotspot vsiRNA sequence. (B) Location and frequency of 3′-UTR-originating (+)-strand ORSV vsiRNAs. The hotspot vsiRNAs and its homologous sequences are indicated in red and blue, respectively. Grey numbers indicating the ORSV genome sites (based on AY571290). Reads per million (rpm) index of cumulative reads in Di library are presented in single-nucleotide resolution with different colour scales. (C) Pseudoknot (PK)-origin TMV vsiRNA identified at the 3′-terminus of the viral genome. Red numbers indicate the top 12 abundant TMV vsiRNA hotspots including a PK-origin vsiRNA that shared a conserved sequence with the ORSV 3′-UTR hotspot vsiRNA. The 12th peak is constituted by eight vsiRNA species as listed above the map. The ranking of each vsiRNA tag is shown, and the conserved nucleotides are indicated in red. In addition, seq1008031 shared an identical sequence with the ORSV 3′-UTR hotspot vsiRNA but did not contribute to a prominent peak on the map. Mapping of seq10083806 to the PK structure is highlighted in pink. The diagram of TMV 3′-UTR structure is modified from Osman et al. (2000).

Fig. S7 Abundance and size distribution of CymMV and ORSV vsiRNAs in the second batch of libraries. (A) Abundance and size distribution of vsiRNAs. Green and orange bars represent reads matched to a (+)- or (−)-strand viral genomic RNA, respectively. Percentages above the bar indicate the proportion of total CymMV or ORSV vsiRNAs within the library. The accumulation level is presented by reads per million (RPM). (B) Nucleotide composition of 5′-end of vsiRNAs. Shows the proportion of adenine (A), cytosine (C), guanine (G) and uridine (U) within each size class. Genomic sites of gene-coding regions are indicated below (numbers based on GenBank accession CymMV AY571289). Maps constructed from inoculated (i) or noninoculated (n) tissues with CymMV (C) single inoculation, or CymMV and ORSV double (D)-inoculated leaves are shown.

Fig. S8 Distribution of vsiRNAs along ORSV genome corresponding to reads from libraries of ORSV-infected tissue. Upward dark-coloured and downward light-coloured lines represent (+) and (−)-polarity reads, respectively. Genomic sites of RdRp, MP and CP gene coding regions are indicated below (numbers based on GenBank accession ORSV AY571290). Labels indicate libraries constructed from inoculated (Di-2) or noninoculated (Dn-2) tissues of CymMV and ORSV double-inoculated leaves. Maps from the first batch of sRNA sequencing (Di) are shown side by side to reveal consistency between the two batches. (D) The composition of CymMV and ORSV vsiRNAs originated from different genomic regions was validated by northern blot. The enriched sRNAs from CymMV/ORSV single- or double-infected total RNA samples were 5′-end labelled and hybridized to *in vitro* transcriptions.
corresponding to different genomic regions (indicated by 1-7, the genomic regions are listed in Table S11) on the blots.

Fig. S8 Venn diagrams showing the overlap of the top 20 vsiRNA species and predicted targets in single- or double-inoculation libraries. The top 20 abundant vsiRNA species were largely overlapped between Ci and Di libraries (A), whereas the list was totally exclusive between Oi and Di (B). VsirRNAs that did not predict with targets were discarded from the analysis, so the total number for each library was <20. The high overlap of CymMV vsiRNA species between Ci and Di led to highly overlapped target accessions as shown in (C). Correspondingly, there were no identical targets for top 20 ORSV vsiRNAs between Oi and Di (D).

Fig. S9 CymMV and ORSV accumulation in Phalaenopsis amabilis protoplasts. (A) Flower protoplasts were isolated from P. amabilis petals and sepals. (B) At 12 and 20 h, the transfected protoplasts were harvested for detecting CymMV and ORSV CPs by western blots using CymMV and ORSV CP antibodies. Mock (M), CymMV (C), ORSV (O), and CymMV plus ORSV double (D) transfection was indicated. Coomassie blue staining (CB) and actin detection are served as loading control.

Table S1 Summary of total unique reads in sRNA libraries constructed from mock- and virus-inoculated Phalaenopsis tissues.
Table S2 Summary of (A) total reads and (B) unique tags in sRNA libraries constructed from the second batch of mock- and virus-inoculated Phalaenopsis tissues.
Table S3 List of Phalaenopsis transcripts that were predicted as targets of the 20 topmost abundant CymMV vsiRNAs.
Table S4 List of Phalaenopsis transcripts that were predicted as targets of the 20 topmost abundant ORSV vsiRNAs.
Table S5 List of enriched GO terms (Biological process) for potential targets of top 20 CymMV vsiRNAs in Ci library.
Table S6 List of enriched GO terms (Biological process) for potential targets of top 20 CymMV vsiRNAs in Di library.
Table S7 List of enriched GO terms (Biological process) for potential targets of top 20 ORSV vsiRNAs in Oi library.
Table S8 List of enriched GO terms (Biological process) for potential targets of top 20 ORSV vsiRNAs in Di library.
Table S9 GC context of CymMV and ORSV viral segments.
Table S10 Primers used in this study.
Table S11 Information of viral probes used in this study.