Virus-based MicroRNA Silencing in Plants

Aihua Sha¹,² #, Jinping Zhao¹ #, Kangquan Yin¹ #, Yang Tang¹, Yan Wang¹, Xiang Wei¹, Yiguo Hong³, Yule Liu¹*

¹: MOE Key Laboratory of Bioinformatics, Center for Plant Biology, School of Life Sciences, Tsinghua University, Beijing 100084, China
²: Institute of Oil Crops Research, Chinese Academy of Agriculture Sciences, Wuhan 430062, China
³: Research Centre for Plant RNA Signaling, College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 310036, China

#: These authors contributed equally to this work

Corresponding author:
Name Yule Liu
Telephone +86-10-62794013
Fax +86-10-62794013
E-mail yuleliu@mail.tsinghua.edu.cn
Footnotes:

Financial source: This work was supported by the National Basic Research Program of China (Grant Nos. 2014CB138400, 2011CB910100), the National Natural Science Foundation of China (Grant Nos. 31071169, 31270182, 31000838, 31300134, 31370180); the National Transgenic Program of China (Grant Nos. 2013ZX08010-002, 2013ZX08009-003 and 2013ZX08005-001).

Present address: Yang Tang: Lilly China Research & Development Center, Eli Lilly and Company, Blog.8, No.338, Jia Li Lue Road, Zhangjiang Hi-Tech Park, Shanghai 201203, China

Corresponding author:
Yule Liu: yuleliu@mail.tsinghua.edu.cn
Abstract

MicroRNAs (miRNAs) play pivotal roles in various biological processes across kingdoms. Many plant miRNAs have been experimentally identified or predicted by bioinformatics mining of small RNA databases. However, functions of these miRNAs remain largely unknown due to the lack of effective genetic tools. Here, we report a virus-based miRNA silencing (VbMS) system that can be used for functional analysis of plant miRNAs. VbMS is performed through Tobacco rattle virus (TRV)-based expression of miRNA target mimics to silence endogenous miRNAs. VbMS of either miR172 or miR165/166 caused developmental defects in *Nicotiana benthamiana*. VbMS of miR319 reduced the complexity of tomato compound leaves. These results demonstrate that TRV-based VbMS is a powerful tool to silence endogenous miRNAs and to dissect their functions in different plant species.

Introduction

MiRNAs are genome-encoded 20-24-nucleotide small RNAs that act as posttranscriptional regulators in eukaryotes (Bartel, 2004; Vaucheret et al., 2004). In plant, mature miRNA is excised from the primary miRNA transcript by Dicer-like 1 (RNase III-like endoribonucleases) in a stepwise manner. The mature miRNAs are selectively loaded into RNA-induced silencing complex, which can cause either target mRNA degradation or translational repression directed by the miRNA with complete or partial complementarity to the target transcript (Sunkar et al., 2007; Ha et al., 2008).

Plant miRNAs play essential roles in various biological processes, such as development, signal transduction, protein degradation, response to abiotic and biotic stress, as well as the regulation of their own biogenesis (Zhang et al., 2006; Phillips et al., 2007; Sunkar et al., 2007; Jin, 2008; Lu et al., 2008; Shukla et al., 2008). To date, more than 6800 miRNAs in approximately 62 plant species have been identified (miRBase, Release 20.0, June 2013; Ambros et al., 2003; Griffiths-Jones et al., 2008). However, only a very limited number of miRNAs have been functionally characterized.

There were two reciprocal reverse genetic strategies to investigate the function of a
particular miRNA (Jones-Rhoades et al., 2006). One is to enhance miRNA activity through transgenic overexpression of the miRNA in plants. The other is to block miRNA function, which can be accomplished by either identifying an individual mutant of miRNA gene (Allen et al., 2007) or by expressing a miRNA-resistant target with silent mutations being introduced to avoid changing the encoded amino acids (Zhao et al., 2007). Recently, several alternative approaches have been developed for functional validation of miRNAs in plants, including miRNA target mimicry (Franco-Zorrilla et al., 2007), short tandem target mimic (STTM, Yan et al., 2012), transcriptional gene silencing (TGS) of miRNA gene promoters (Vaistij et al., 2010), and artificial miRNA (amiRNA) directed silencing of miRNA precursors (Eamens et al., 2011). However, all the aforementioned approaches rely on the time-consuming and costly process to generate the stable transgenic plants, which limits their utility for high throughput analysis.

Among various miRNA inhibition approaches, miRNA target mimicry and STTM have received more attention. The miRNA target mimicry was first reported to establish the mechanism of inhibition of miR399 activity by non-protein-coding RNA IPS1 (*INDUCED BY PHOSPHATE STARVATION1*) in response to low inorganic phosphate (Franco-Zorrilla et al., 2007). The *IPS1* mRNA contains a 23-nt sequence partially complementary to miR399 with a 3-nt mismatch at the expected miRNA cleavage site of miR399. Because of the mismatched bulge region, *IPS1* functions as a non-cleavable target mimic of miR399 that sequesters miR399 and arrests its cleavage activity to the target *PHOSPHATE 2* mRNA. By replacing the miR399 bulged target of the *IPS1* transcript with other miRNA target mimic sequences, the non-cleavable miRNA target mimics can be exploited to inhibit miRNAs other than miR399. Based on this strategy, two other miRNAs, miR156 and miR319, were sequestered by their target mimics respectively (Franco-Zorrilla et al., 2007). Furthermore, target mimics of 15 out of the 75 miRNA families (~20%) caused reproducible developmental defects in aerial tissues when expressed in transgenic Arabidopsis plants (Todesco et al., 2010). On the other hand, the newly developed STTM technology is an important approach to block miRNA function (Yan et al., 2012). STTM consists of two mimicking small RNA target sequences separated by a 48 to 88-nt artificially designed linker, whose expression leads
to the degradation of targeted small RNAs by small RNA degrading nucleases (Yan et al., 2012).

Plant viral vectors have been widely used for transient gene expression and for gene silencing in plants (Lu et al., 2003; Senthil-Kumar and Mysore, 2011; Hefferon, 2012). Viral vector-based techniques do not require the time-consuming procedure of generating stable transgenic plants whilst they allow characterization of phenotypes that might be lethal in stable transgenic lines. Therefore these technologies have the potential to become an attractive and quick approach to uncover miRNA function in plants, especially in those difficult for genetic transformation. Indeed, we have developed a Cabbage leaf curl virus-based vector for overexpression of miRNAs in plants (Tang et al., 2010). However, virus-based miRNA inactivation has not been reported.

Tobacco rattle virus (TRV) is a bipartite positive sense RNA virus and it can infect a broad range of plants (MacFarlane et al., 1999). TRV-based vectors (Liu et al., 2002b) have been widely applied as virus induced gene silencing (VIGS) to knock down gene expression in various plant species (Bachan and Dinesh-Kumar, 2012), and they have also been successfully modified for expression of foreign genes in plants (MacFarlane and Popovich, 2000). TRV, like all successful viruses, can escape host RNAi defense and infect host plants systemically because it encodes two weak gene silencing suppressors (Martin-Hernandez and Baulcombe, 2008; Deng et al., 2013). However, TRV induces only very mild symptoms in many host plants (Ratcliff et al., 2001), and it does not cause global deregulation of the miRNA-regulatory pathway (Martinez-Priego et al., 2008). In this study, we modified the TRV vector (Liu et al., 2002b) into a TRV-based T-DNA expression vector. Further, we developed a virus-based miRNA silencing (VbMS) system in which TRV-based expression of miRNA target mimics can effectively suppress endogenous miRNA activity in plants within a short period of time.

Results

Development of A TRV-based Expression Vector

TRV vectors have been used for the foreign gene expression by adding a fragment carrying the coat protein (CP) gene subgenomic promoter isolated from the Pea early
brown virus (PEBV) RNA genome (MacFarlane and Popovich, 2000). Here, we modified the TRV RNA2 derived vector pYL170 (Dong et al., 2007) by inserting the PEBV CP subgenomic promoter (Wang et al., 1997) and the ccdB gene with ligation independent cloning (LIC) adaptor sequences immediately downstream of the TRV CP gene to generate TRV expression vector pTRV2e (Fig. 1). This vector can be used to express RNA such as IPS1-based miRNA target mimics (MIM), STTM and foreign proteins-encoding genes as outlined in Figure 1. To assess whether pTRV2e can be indeed used for gene expression in plants, we cloned a GFP coding sequence into pTRV2e to generate pTRV-GFP. When plants agroinfiltrated with agrobacterium carrying pTRV-GFP and pTRV1 (Fig. 1), the GFP fluorescence was visible in the upper non-inoculated leaves at 4 days post inoculation (dpi), suggesting that the modified TRV vector can be used to express foreign genes in plants (Fig. 2A). RT-PCR analysis further confirmed GFP expression (Fig. 2B). In addition, TRV-GFP RNA was detected in the infected plants undergoing vegetative to reproductive growth (data not shown). Moreover, the infected plants were symptomless or only showed extremely mild symptom in N. benthamiana and tomato plants.

VbMS of miR172 Caused Flower Developmental Defects in N. benthamiana

In Arabidopsi and N. benthamiana, miR172 regulates the expression of floral homeotic gene APETALA2 (AP2). The AP2/miR172 regulatory circuit is conserved in higher plant species (Chen, 2004; Mlotshwa et al., 2006; Chuck et al., 2008; Zhu et al., 2009). Overexpression of miR172-resistant AP2 resulted in severe defects in floral patterning due to over-accumulated AP2 mRNA and protein (Chen, 2004; Mlotshwa et al., 2006). To test whether TRV-based VbMS via a miRNA target mimics can suppress miRNA activity, we used the modified TRV vector to express IPS1-based target mimic against miR172 (MIM172) in N. benthamiana (Fig. 3A). In each plant expressing MIM172, more than 1/3 of the flowers had dramatically reduced petal size compared with flowers in controls (Fig. 3B, right column). The shortened petals could not cover the androecium and anthers extending out of the fringe of petals. In contrast, all flowers in control TRV plants showed normal developmental patterns (Fig. 3B, left column). These phenotypes were exactly the same as those observed in transgenic N. benthamiana lines.
overexpressing miR172-resistant AP2 (Mlotshwa et al., 2006). Furthermore, RT-PCR assays indicated that MIM172 was expressed in TRV-MIM172 plants with flower developmental defects (Fig. S1A). Stem-loop RT-PCR assays indicated that miR172 level was reduced in TRV-MIM172 plants (Fig. 3C). It is known that NbAP2-like1 (NbAP2L1) is the target of miR172 in N. benthamiana (Mlotshwa et al., 2006). Thus we used real time RT-PCR to analyze the mRNA level of NbAP2L1. Indeed, the level of the NbAP2L1 mRNA was significantly higher in MIM172-expressing plants than in controls (Fig. 3D).

Further, we tested whether VbMS can inhibit miRNA activity by TRV-based STTM expression. For this purpose, we generated STTM against miR172 (STTM172) and cloned it into pTRV2e to generate TRV-STTM172 (Fig. 4A). Similar to TRV-MIM172 plants, about 1/3 of flowers of TRV-STTM172 inoculated plants had short petals with varied morphologic patterns (Fig. 4B). In certain extremes, flowers in plants infected with TRV-STTM172 developed extra petals or unclosed petals which could not enclose the interior stamens and carpels (Fig. 4C), suggesting that TRV-based VbMS of miR172 using the STTM approach was very effective to suppress normal miR172 function and caused many abnormal flower phenotypes. Accompanied with viral expression of STTM172 (Fig. S1B), miR172 level was lower (Fig. 4D) and the mRNA level of NbAP2L1 was higher in STTM172-expressing plants than in controls (Fig. 4E). Further, defective flowers were observed throughout the flowering periods.

Taken together, these results suggest that TRV-based VbMS using either MIM or STTM can effectively suppress miRNA function in N. benthamiana.

VbMS of miR319 Caused Smaller Leaves and Simpler Leaf Pattern in Tomato Plants

In tomato (Solanum lycopersicum), miR319/LANCEOLATE (LA) is a well-defined miRNA/target pair and misregulation of LA by miR319 led to a distinguishable phenotype in aerial organs (Ori et al., 2007). LA encodes a TEOSINTE BRANCHED/CYCLOIDEA/PCF family transcription factor (TF) whose mRNA sequence contains a miR319-binding site. The dominant La mutant with mutation in the
miR319-binding sequence conferred partial resistance against miR319-directed inhibition and led to elevated accumulation of LA protein, converting large compound leaves into small simple ones in tomato plants (Ori et al., 2007). To investigate whether VbMS works in tomato, we used the modified TRV vector to express IPS1-based target mimic against miR319 (MIM319, Fig. 5A). Approximately 20% of TRV-MIM319 plants showed similar phenotype to La mutant lines (Ori et al., 2007), with large compound leaves converted into small simple ones and reduced size of whole plants (Fig. 5B). RT-PCR confirmed that MIM319 was expressed in TRV-MIM319 plants (Fig. S1C). Further, the miR319 level was lower (Fig. 5C) and LA mRNA level was obviously higher in plants expressing MIM319 than that in controls (Fig. 5D). We monitored the VbMS for more than 3 months and the TRV-MIM319 plants continuously developed simplified leaves (Fig. S2), indicating VbMS of miR319 had an enduring impact on tomato leaf development.

We also tested whether VbMS can inhibit miR319 activity by TRV-based expression of STTM targeting miR319 (STTM319, Fig. 6A). More than 30% tomato plants infected with TRV-STTM319 showed a range of leaf simplification with reduced or no leaflets (Fig. 6B). RT-PCR confirmed that STTM319 was expressed in TRV-STTM319 plants (Fig. S1D). Further, the miR319 level was lower (Fig. 6C) and LA mRNA level is evidently higher in plants expressing STTM319 than that in controls (Fig. 6D).

Taken together, our results indicate that VbMS using either MIM or STTM can inhibit miRNA function in tomato.

VbMS of miR165/166 Reduced Apical Dominance in N. benthamiana

We have showed that VbMS can inhibit miRNA function quickly in N. benthamiana and tomato. To determine whether VbMS can be used to investigate the function of yet uncharacterized miRNAs, we performed VbMS of miR165/166 in N. benthamiana plants. The class III homeodomain-leucine zipper (HD-ZIP III) TFs have been clearly defined as miR165/166 target sets in Arabidopsis. MiR165/166s target and repress expression of the HD-ZIP III members, determining the behavior of shoot apical meristem and polarity of leaves (McConnell and Barton, 1998; Mallory et al., 2004; Kim et al., 2005; Jung and
Park, 2007; Sakaguchi and Watanabe, 2012). In *Nicotiana sylvestris*, the ortholog of HD-ZIP III TF PHAVOLUTA was shown to be regulated by miR165/166-directed cleavage of its mRNA (McHale and Koning, 2004). MiR165/166 has been also predicted and detected in *N. benthamiana* (Li et al., 2012). However, their exact function has not been characterized.

To determine the function of miR165/166, we performed TRV-based expression of STTM targeting miR165/166 (STTM165/166, Fig. 7A) in *N. benthamiana* plants. TRV control plants did not show any developmental defects. However, more than 20% of TRV-STTM165/166 plants showed reduced apical dominance and lacked the distinguishable main shoot (Fig. 7B). In certain extremes, ectopic leaf outgrowths on leaf middle vein were observed (Fig. 7C, arrows). These observations reflected the disruption of apical meristematic and leaf primordial functions caused by inhibition of miR165/166 (Zhong and Ye, 2004; Yan et al., 2012). However, we did not observe obvious change in leaf abaxial-adaxial polarity as observed in Arabidopsis over-expressing STTM165/166 (Yan et al., 2012). RT-PCR confirmed that STTM165/166 was expressed in TRV-STTM165/166 plants (Fig. S1E). Further, the miR165/166 level was reduced in TRV-STTM165/166 plants (Fig. 7D). One of the HD-ZIP III TFs was predicted to be the miR165/166 cleavable target in *N. benthamiana* ([TC21810](http://wmd3.weigelworld.org/cgi-bin/webapp.cgi), *N. benthamiana* EST NbGI 4.0). Indeed, in TRV-STTM165/166 plants, the mRNA level of TC21810 was much higher compared to the controls (Fig. 7E). These results demonstrated that the TRV-based VbMS can be applied to study the function of uncharacterized miRNAs in plants and there may be a conservation of miR165/166-HD-ZIP III partner in plant species.

**Discussion**

In this study, we demonstrate that VbMS by TRV-based expression of miRNA target mimics can be used to block the function of miRNAs in plants. Using this system, we have successfully silenced miR172 in *N. benthamiana* and miR319 in tomato. VbMS of miR172 in *N. benthamiana* led to typical defects in flower organs, confirming that miR172 is a functionally conserved miRNA between tobacco and Arabidopsis. VbMS of miR319 in tomato reduced the complexity of tomato compound leaves. We also found
that miR165/166 could regulate the development of the shoot apical meristem in *N. benthamiana*, which is consistent with their function in Arabidopsis (Eckardt, 2012; Yan et al., 2012). These results indicated the TRV-based VbMS by overexpressing miRNA target mimics was efficient to silence plant endogenous miRNAs. To the best of our knowledge, this is the first report of using a viral vector to investigate miRNA function by blocking miRNA activity in plants.

We found that only about 20-30% of plants expressing target mimics against miR319 and miR165/166 showed expected phenotype. In addition, only about 1/3 of the flowers were defective in each plant expressing target mimics against miR172 although all plants had defective flowers. This could be caused by nonuniform and incomplete miRNA silencing because TRV cannot infect 100% meristematic cells that finally divide and differentiate to form the tissues and organs of the plant. This is not surprising, because VIGS does not result in 100% uniform silencing (Liu et al., 2002a). There may be other unknown factors that contribute to this variability, because even different progenies from the same stable transgenic lines expressing target mimics gave various phenotype (Todesco et al., 2010), and only about 60% of flowers exhibited defects in the transgenic plant lines expressing mi172-resistant *AP2* (Mlotshwa et al., 2006).

Although transgenic plants expressing miR172-resistant *AP2* show severe flower defects in both *N. benthamiana* and Arabidopsis (Chen, 2004; Mlotshwa et al., 2006), the flowers of transgenic plants expressing MIM172 are normal (Todesco et al., 2010). However, we found that TRV-based expression of either MIM172 or STTM172 caused abnormal flowers in *N. benthamiana*, suggesting that VbMS could be more effective to block miR172 function than stable transgene expressing *IPS1*-based miRNA target mimics.

We found that VbMS of miR165/166 using STTM technology caused the loss of apical dominance. However, VbMS of miR165/166 using *IPS1*-based miRNA target mimics did not cause any visible phenotype in *N. benthamiana* (data not shown). These observations suggest that STTM is superior to *IPS1*-based miRNA target mimics to suppress miRNA function through viral approach. Indeed, disruption of miR165/166 function using STTM approach also results in a more severe phenotype than that using *IPS1*-based miRNA target mimic in transgenic Arabidopsis (Eckardt, 2012; Yan et al.,
It should be noted that transgenic Arabidopsis plants expressing STTM165/166 exhibited severe loss of apical dominance and loss of leaf symmetry phenotypes (Yan et al., 2012). However, VbMS of miR165/166 using STTM technology caused the loss of apical dominance but no obvious changes in leaf symmetry in *N. benthamiana*. The phenotypic discrepancy caused by miR165/166 silencing between Arabidopsis and *N. benthamiana* is probably due to different miRNA silencing efficiency between different approaches. It is possible that STTM transgenic approach give stronger phenotype than virus-mediated STTM. Transgenic STTM block miRNAs in each cell of the transgenic plants from the very beginning without any difference between fast dividing cells and developed cells, while the virus mediated STTM has less effect in fast dividing cells such as shoot apical meristems due to much diluted virus concentration in fast dividing cells. Thus, there may be the limitation of VbMS as a method to inhibit miRNA function due to its relatively weak phenotype. Nevertheless, VbMS is a great complementary method to the transgenic target mimics or STTM for a fast screening for functions of miRNAs.

Besides the aerial parts such as leaf and flower, TRV-based vectors are able to trigger VIGS in fruits (Hanania et al., 2007; Jia et al., 2011) and underground tissues (Valentine et al., 2004; Kaloshian, 2007) in various plant species. On the other hand, TRV-based vectors have been used to express foreign genes in systemically infected leaves (MacFarlane and Popovich, 2000) and in hairy roots (Larsen and Curtis, 2012). Further, TRV-based expression has been successfully used in the functional characterization of scent-related genes, protein compartmentalization studies and nontransgenic genome modification (Spitzer-Rimon et al., 2010; Spitzer-Rimon et al., 2012; Spitzer-Rimon et al., 2013). Therefore, TRV-based VbMS should also be able to inhibit miRNA in organs other than leaves and flowers.

Moreover, the currently described TRV-based VbMS possesses several advantages over other functional assays for plant miRNAs. First, VbMS is efficient and quick and it usually can result in miRNA silencing-mediated phenotypes within 2-4 weeks. Second, VbMS does not require the tedious and time-consuming plant transformation procedure and only needs the simple agroinfiltration technique for miRNA silencing. This is particularly useful for functional characterization of miRNAs whose knockout or
knockdown might cause embryonic lethality in transgenic lines, and for plant species which are not amenable to stable genetic transformation. Third, TRV has a wide host range and TRV vectors have been applied in a wide range of plant species (Bachan and Dinesh-Kumar, 2012). Thus, TRV-based VbMS should be applicable for miRNA functional analysis in these plants.

Our finding also implies that many other available viral vectors, for example, *Apple latent spherical virus* (Igarashi et al., 2009; Yamagishi et al., 2011), *Brome mosaic virus* (Ding et al., 2006) and *Barley stripe mosaic virus* (Holzberg et al., 2002) could be used in a similar strategy as the TRV VbMS vector for functional analysis of miRNAs in a diverse range of eudicot and monocot crops. In addition, besides *IPS1*-based miRNA target mimicry and STTM methods, several other techniques, such as transcriptional gene silencing (TGS) of miRNA gene promoters (Vaistij et al., 2010), artificial miRNA (amiRNA) directed silencing of miRNA precursors (Eamens et al., 2011), and miRNA decoy (Ivashuta et al., 2011), have successfully been used to investigate miRNA/target interactions in transgenic plants. Given the performance of VbMS by TRV-based expression of miRNA target mimics, these miRNA silencing approaches can be widely adapted to VbMS to elucidate small RNA functions in plants.

**Methods**

**Plasmid Construction**

The pTRV1 (pYL192) was described previously (Liu et al., 2002b). The pTRV2-derived expression vector pTRV2e was constructed as follow: The PEBV CP subgenomic promoter was amplified from a PEBV RNA2 vector (kindly provided by Professor Daowen Wang). The *ccdB* gene with ligation-independent cloning (LIC) adaptor sequences was amplified using pTRV2-LIC (Dong et al., 2007) as template. pTRV2e was obtained by cloning the digested DNA fragments of PEBV CP sub-genomic promoter and *ccdB* gene containing LIC adaptor sequences into pYL170, a TRV RNA2 VIGS vector (Dong et al., 2007). The pTRV1 can be obtained from Arabidopsis Biological Resource Center (ABRC, stock name is YL192), and pTRV2e will be available in ABRC (stock name is pTRV2e).
The *GFP* coding sequence was amplified from TMV-GFP (Liu et al., 2002b). Artificial miRNA target mimicry sequences were engineered into *IPS1* backbone by overlapping PCR as described (Franco-Zorrilla et al., 2007). A 48-nucleotide oligo was synthesized as template for PCR amplification of STTM (Yan et al., 2012). Primers with LIC adaptor, corresponding target mimic of miRNA and STTM optimal spacer were used to PCR amplify STTM molecules. The *GFP*, *MIM* and *STTM* fragments were cloned into pTRV2e using the LIC protocol as described (Dong et al., 2007). All constructs were confirmed by DNA sequencing. Primers used in this study were listed in Supplemental Table S1.

**Plant Growth, Agroinfiltration, and GFP Imaging**

*N. benthamiana* and tomato plants (Moneymaker) were grown in pots at 25°C in growth chambers under 16 h light/8 h dark cycle with 60% humidity. For TRV-based expression or VbMS, pTRV1 and pTRV2e or its derivatives were introduced into Agrobacterium strain GV3101 (An et al., 1988). A 5-ml culture was grown overnight at 28°C in the appropriate antibiotic selection medium, then amplified in a 50-ml LB medium containing antibiotics and grown overnight in a 28°C shaker. Agrobacterium cells were harvested and re-suspended in infiltration media (10 mM MgCl₂, 10 mM MES, 200 μM acetosyringone), adjusted to an OD₂₆₀ of 1.0 and left at room temperature for 3 h before infiltration for *N. benthamiana* or adjusted to an OD₂₆₀ of 2.0 and incubated at room temperature for 6 h before infiltration for tomato. Agrobacterium was infiltrated using a 1-ml syringe without needle into leaves of *N. benthamiana* before flowering or into cotyledons of tomato before true leaf emerged. The infiltrated plants were grown and observed until phenotypes appeared. For each VbMS construct, at least five plants were used for agro-infiltration and replicated for at least six times. GFP imaging was illuminated under long-wavelength UV light, and photographs were taken using a digital camera.

**RNA Isolation and RT-PCR Analysis**

Total RNA was extracted from developing flowers (for VbMS of miR172), shoot apical tissues (for VbMS of miR165/166) and developing leaves (for VbMS of miR319)
using the Trizol reagent (Invitrogen) and treated with RNase-free DNase I (Sigma-Aldrich). First strand cDNA was synthesized using 2-5 μg of total RNA with oligo-d(T) primer or TRV specific primer and M-MLV reverse transcriptase (Promega). Primers were designed with Primer Premier 9.0 and listed in Supplemental Table S1.

**Real-time RT-PCR Analysis**

Real time RT-PCR was performed using Power SYBR Green PCR master mix (ABgene), eIF4a and Tubulin was used as internal control for *N. benthamiana* and tomato respectively for normalization. Stem-loop RT-PCR were performed as described with the SYBR Green assay (Varkonyi-Gasic and Hellens, 2011) and the miRNA complementary regions in reverse transcript primers were elongated to cross the cleavage site to exclude STTM contamination. Primers were designed with Primer Expression 3.0 (ABI) and listed in Supplemental Table S1. The values were calculated using the comparative normalized Ct method and all the experiments were repeated at least three times. Data were analyzed and plotted with Origin 8.1. Data shown were from at least 3 repeated experiments.

Sequence data from this article can be found in the GenBank data libraries under accession numbers: PEBV subgenomic promoter (NC_001368); TRV CP (AF406991); GFP (SCU87973); AtIPS1 (NM_180219); NbAP2L1 (CK287095); LA (EF091571); tomato Tubulin (XM_004244485); and in DFCI *N.benthamiana* Gene Index under TC Annotator: eIF4a (TC19454). miRNA sequence can be found in miRBase [http://www.mirbase.org](http://www.mirbase.org).

**Acknowledgements**

We thank Professor Daowen Wang at Institute of Genetics and Developmental Biology, Chinese Academy of Science, Beijjing, China for providing PEBV vectors.

**References**


Hanania U, Velcheva M, Or E, Flashman M, Sahar N, Perl A (2007) Silencing of chaperonin 21, that was differentially expressed in inflorescence of seedless and seeded grapes, promoted seed abortion in tobacco and tomato fruits. Transgenic research 16: 515-525


Liu Y, Schiff M, Dinesh-Kumar SP (2002a) Virus-induced gene silencing in tomato. Plant J 31: 777-786

Liu Y, Schiff M, Marathe R, Dinesh-Kumar SP (2002b) Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. Plant J 30: 415-429


Zhao L, Kim Y, Dinh TT, Chen X (2007) miR172 regulates stem cell fate and defines the inner boundary of APETALA3 and PISTILLATA expression domain in Arabidopsis floral meristems. Plant J 51: 840-849


Figure Legends

Figure 1. Schema of TRV-based VbMS Vector.

pTRV1 is TRV RNA1 T-DNA vector; pTRV2e is a PEBV CP subgenomic promoter (sgP)-containing TRV RNA2 T-DNA vector, which cDNA of TRV RNA2 is cloned between CaMV 35S promoter with the duplicated enhancers (2×35S) and NOS terminator (NOS). pTRV2e contains the ligation independent cloning (LIC) cassette for the insertion of the target gene sequences. LB: left border of T-DNA; RdRP: RNA-dependent RNA polymerase; MP: movement protein; 16K: 16-kD cysteine rich protein; Rz: self-cleaving ribozyme; RB: right border of T-DNA; CP: TRV coat protein. IPS1-based miRNA target mimic (MIM) and STTM sequences can be cloned into
pTRV2e by LIC reaction. MIM contains an \textit{AtIPS1} backbone but the target mimic motif of miR399 is changed to that of corresponding miRNAs. STTM contains two tandem target mimics separated by a 48nt imperfect stem-loop linker (48nt).

**Figure 2. Visualisation of GFP Expressed by the Modified TRV Vector in \textit{N. benthamiana}.**

(A) The TRV-GFP infiltrated plants were photographed at 4 days post-inoculation under white light (left) or UV illumination (right). Arrows indicate the upper un-infiltrated leaves. “T” indicate the infiltrated leaves. Green color under UV light indicates the GFP signal. (B) RT-PCR detection of TRV RNA in upper un-infiltrated leaves. RNA samples were extracted from TRV-GFP and TRV control plants, and RT-PCR was performed with \textit{GFP} and \textit{TRV CP} specific primers.

**Figure 3. VbMS of miR172 using \textit{ISP1}-based miRNA Target Mimicry Caused Flower Defects in \textit{N. benthamiana}.**

(A) Diagrammatic representation of MIM172. (B) The flowers of plants infected with TRV control (left) and with TRV-MIM172 (right) were photographed at 12 days post flowering. Shown are typical flowers with sepal (top row) and sepal removed (bottom row). Scale bars correspond to 1 cm. (C) Stem-loop RT-PCR detection of miR172 level in plants infected with TRV control and with TRV-MIM172. (D) Real-time RT-PCR analysis of mRNA levels of miR172 target \textit{NbAP2LI} in TRV control and plants expressing \textit{MIM172}. Bars show ±SD.

**Figure 4. VbMS of miR172 using STTM Approach Caused Varied Floral Defects in \textit{N. benthamiana}**

(A) Diagram of STTM172. (B) The varied morphology of flowers caused by TRV-based expression of STTM172. Scale bars represent 1 cm. Each flower was photographed with sepal (top row) and sepal removed (bottom row). (C) In some TRV-STTM172 infected
plants, there are abnormal petals which could not enclose interior flower organs (top row, Arrow indicate) and ectopic genesis of petal like tissues (bottom row, Arrow head indicate). Scale bars represent 1 cm. 

(D) Stem-loop RT-PCR detection of miR172 level in plants infected with TRV control and with TRV-STTM172. (E) Real time RT-PCR analysis of miR172 target NbAP2LI in VbMS plants. Bars show ±SD.

Figure 5. VbMS of miR319 using ISP1-based Target Mimicry Approach Converted Large Compound Leaves into Small Simple Ones in Tomato

(A) Diagram of MIM319. (B) The plants (left column) and the 3rd leaf excised from the left-sided plants (right column) of TRV control (bottom row) and plants expressing MIM319 (top row) were photographed at 20 dpi. Leaf orders are signed with number. Scale bars represent 1 cm. (C) Stem-loop RT-PCR detection of miR319 level in plants infected with TRV control and with TRV-MIM319. (D) Real-time RT-PCR analysis of miRNA levels of the miR319 target LA in TRV and plants expressing MIM319. Bars show ±SD.

Figure 6. VbMS of miR319 using STTM Approach Caused Smaller and Simpler Leaves in Tomato Plants.

(A) Diagram of STTM319. (B) The whole stature and the 3rd to 6th leaves of plants infected with TRV (left row) and with TRV-STTM319 (right row) were photographed at 10 dpi. L1 to L7 indicates leaf. Scale bars represent 1 cm. (C) Stem-loop RT-PCR detection of miR319 level in plants infected with TRV control and with TRV-STTM319. (D) Real-time RT-PCR analysis of miR319 target LA. Bars show ±SD.

Figure 7. VbMS of miR165/166 using STTM Approach Caused the Loss of Apical Dominance in N. benthamiana

(A) Diagram of STTM165/166. (B) The whole plants infected with TRV-STTM165/166 (right column) or TRV (left column) were photographed at 28 dpi. Photos were captured
from top view (top row) and side view (bottom row). Arrow indicates the branched shoot apex. (C) Outgrowth of ectopic leaf. Ectopic leaf was photographed in front view and side view. Arrows indicate ectopic leaf tissues, “mv” and arrow head indicate middle vein. Scale bars correspond to 1cm. (D) Stem-loop RT-PCR detection of miR165/166 level in plants infected with TRV control and with TRV-STTM165/166. (E) Real-time RT-PCR analysis of relative mRNA levels of the putative miR165/166 target genes TC21810 in TRV control plants and TRV-STTM165/166 plants. Bars show ±SD.

Supporting Information

Supplemental Figure S1. RT-PCR Confirmation of TRV Infection and Expression of Target Mimics in VbMS Plants.

RT-PCR confirmed successful TRV infection as indicated by presence of CP transcripts in all plants (CP) and TRV-based expression of MIM172 (A), STTM172 (B), MIM319 (C), STTM319 (D) and STTM165/166 (E) in plants expressing the corresponding miRNA target mimic.

Supplemental Figure S2. VbMS of miR319 Caused Developmental Defects of Tomato Plants in Later Growth Stages.

(A) VbMS of miR319 at 3 months post inoculation. Plants infected with TRV-MIM319 developed simplified leaves. Shoot apical regions are magnified in the top row. Arrows indicate the simpler-shaped leaves. Scale bars represent 5 cm. (B) RT-PCR confirmed the existence of RNAs of TRV and MIM319. Real time RT-PCR revealed the reduction of miR319 level (C), and the increase of mRNA level of the miR319 target LA (D) in TRV-MIM319 plants compared to that in TRV control plants. Bars show ±SD.
Supplemental Table S1. Primers Used in Vector Construction and PCR Analysis.

a, Restriction site used for cloning is underlined; b, Target mimic sequence is underlined; c, STTM mimic sequence is underlined; d, MiRNA sequence is underlined; e, Reverse-complement sequence of miRNA is underlined.
Figure 1. Schema of TRV-based VbMS Vector.

pTRV1 is TRV RNA1 T-DNA vector; pTRV2e is a PEBV CP subgenomic promoter (sgP)-containing TRV RNA2 T-DNA vector, which cDNA of TRV RNA2 is cloned between CaMV 35S promoter with the duplicated enhancers (2×35S) and NOS terminator (NOS). pTRV2e contains the ligation independent cloning (LIC) cassette for the insertion of the target gene sequences. LB: left border of T-DNA; RdRP: RNA-dependent RNA polymerase; MP: movement protein; 16K: 16-kD cysteine rich protein; Rz: self-cleaving ribozyme; RB: right border of T-DNA; CP: TRV coat protein. *IPS1*-based miRNA target mimic (MIM) and STTM sequences can be cloned into pTRV2e by LIC reaction. MIM contains an *AtIPS1* backbone but the target mimic motif of miR399 is changed to that of corresponding miRNAs. STTM contains two tandem target mimics separated by a 48nt imperfect stem-loop linker (48nt).
Figure 2. Visualisation of GFP Expressed by the Modified TRV Vector in *N. benthamiana*.

(A) The TRV-GFP infiltrated plants were photographed at 4 days post-inoculation under white light (left) or UV illumination (right). Arrows indicate the upper un-infiltrated leaves. “I” indicate the infiltrated leaves. Green color under UV light indicates the GFP signal. (B) RT-PCR detection of TRV RNA in upper un-infiltrated leaves. RNA samples were extracted from TRV-GFP and TRV control plants, and RT-PCR was performed with *GFP* and *TRV CP* specific primers.
Figure 3. VbMS of miR172 using *ISP1*-based miRNA Target Mimicry Caused Flower Defects in *N.benthamiana*.

(A) Diagrammatic representation of MIM172. (B) The flowers of plants infected with TRV control (left) and with TRV-MIM172 (right) were photographed at 12 days post flowering. Shown
are typical flowers with sepal (top row) and sepal removed (bottom row). Scale bars correspond to 1 cm. (C) Stem-loop RT-PCR detection of miR172 level in plants infected with TRV control and with TRV-MIM172. (D) Real-time RT-PCR analysis of mRNA levels of miR172 target NbAP2L1 in TRV control and plants expressing MIM172. Bars show ±SD.
Figure 4. VbMS of miR172 using STTM Approach Caused Varied Floral Defects in *N. benthamiana*.

(A) Diagram of STTM172. (B) The varied morphology of flowers caused by TRV-based expression
of STTM172. Scale bars represent 1 cm. Each flower was photographed with sepal (top row) and sepal removed (bottom row). (C) In some TRV-STTM172 infected plants, there are abnormal petals which could not enclose interior flower organs (top row, Arrow indicate) and ectopic genesis of petal like tissues (bottom row, Arrow head indicate). Scale bars represent 1 cm. (D) Stem-loop RT-PCR detection of miR172 level in plants infected with TRV control and with TRV-STTM172. (E) Real time RT-PCR analysis of miR172 target NbAP2L1 in VbMS plants. Bars show ±SD.
Figure 5. VbMS of miR319 using *ISP1*-based Target Mimicry Approach Converted Large Compound Leaves into Small Simple Ones in Tomato

(A) Diagram of MIM319. (B) The plants (left column) and the 3rd leaf excised from the left-sided plants (right column) of TRV control (bottom row) and plants expressing MIM319 (top row) were photographed at 20 dpi. Leaf orders are signed with number. Scale bars represent 1 cm. (C) Stem-loop RT-PCR detection of miR319 level in plants infected with TRV control and with TRV-MIM319. (D) Real-time RT-PCR analysis of miRNA levels of the miR319 target *LA* in TRV and plants expressing MIM319. Bars show ±SD.
Figure 6. VbMS of miR319 using STTM Approach Caused Smaller and Simpler Leaves in Tomato Plants.

(A) Diagram of STTM319. (B) The whole stature and the 3rd to 6th leaves of plants infected with TRV (left row) and with TRV-STTM319 (right row) were photographed at 10 dpi. L1 to L7 indicates leaf. Scale bars represent 1 cm. (C) Stem-loop RT-PCR detection of miR319 level in plants infected with TRV control and with TRV-STTM319. (D) Real-time RT-PCR analysis of miR319 target LA. Bars show ±SD.
Figure 7. VbMS of miR165/166 using STTM Approach Caused the Loss of Apical Dominance in *N. benthamiana*

(A) Diagram of STTM165/166. (B) The whole plants infected with TRV-STTM165/166 (right column) or TRV (left column) were photographed at 28 dpi. Photos were captured from top view (top row) and side view (bottom row). Arrow indicates the branched shoot apex. (C) Outgrowth of ectopic leaf. Ectopic leaf was photographed in front view and side view. Arrows indicate ectopic leaf tissues, “mv” and arrow head indicate middle vein. Scale bars correspond to 1cm. (D) Stem-loop RT-PCR detection of miR165/166 level in plants infected with TRV control and with TRV-STTM165/166. (E) Real-time RT-PCR analysis of relative mRNA levels of the putative miR165/166 target genes *TC21810* in TRV control plants and TRV-STTM165/166 plants. Bars show ±SD.