MicroRNAs (miRNAs) are genome-encoded 20- to 24-nucleotide small RNAs that act as posttranscriptional regulators in eukaryotes (Bartel, 2004; Vaucheret et al., 2004). In plants, mature miRNA is excised from the primary miRNA transcript by DICER-LIKE1 (RNase III-like endoribonucleases) in a stepwise manner. The mature miRNAs are selectively loaded into an RNA-induced silencing complex, which can cause either target miRNA 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 6,800 miRNAs have been identified 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 are 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 a miRNA gene (Allen et al., 2007) or 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 of miRNA gene promoters (Vaisj et al., 2010), and artificial miRNA-directed silencing of miRNA precursors (Eamens et al., 2011). However, all the aforementioned approaches rely on time-consuming and costly processes 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 mimic was first reported to establish the mechanism of inhibition of miR399 activity by the non-protein-coding RNA INDUCED BY PHOSPHATE STARVATION1 (IPS1) in response to low inorganic phosphate (Franco-Zorrilla et al., 2007). The IPS1 mRNA contains a 23-nucleotide sequence partially complementary to miR399 with a three-nucleotide mismatch at the expected miRNA cleavage site of miR399. Because of the mismatched bulge region, IPS1 functions as a noncleavable target mimic of miR399 that sequesters miR399 and arrests its cleavage activity to the target PHOSPHATE2 mRNA. By replacing the miR399 bulged target of the IPS1 transcript with other miRNA
target-mimic sequences, the noncleavable 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 (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 (Arabidopsis thaliana) 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-nucleotide 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, while they allow the 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 functions in plants, especially in those difficult for genetic transformation. Indeed, we have developed a cabbage leaf curl virus-based vector for the 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 that 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 the expression of foreign genes in plants (MacFarlane and Popovich, 2000). TRV, like all successful viruses, can escape host RNA interference defense and infect host plants systemically because it encodes two weak gene-silencing suppressors (Martín-Hernández 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 (Martínez-Priego et al., 2008). In this study, we modified the TRV vector (Liu et al., 2002b) into a TRV-based T-DNA expression vector. Furthermore, we developed a virus-based microRNA 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 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 pYL70 (Dong et al., 2007) by inserting the PEBV CP subgenomic promoter (Wang et al., 1997) and the ccdB (for control of cell death B) gene with ligation-independent cloning (LIC) adaptor sequences immediately downstream of the TRV CP gene to generate the TRV expression vector pTRV2e (Fig. 1). This vector can be used to express RNAs such as IPS1-based miRNA target mimics (MIM), STTM, and foreign protein-encoding genes as outlined in Figure 1. To assess whether pTRV2e can be used for gene expression in plants, we cloned a GFP coding sequence into pTRV2e to generate pTRV-GFP. When plants were infiltrated with Agrobacterium tumefaciens carrying pTRV-GFP and pTRV1 (Fig. 1), GFP fluorescence was visible in the upper noninoculated leaves at 4 dpi, suggesting that the modified TRV vector can be used to express foreign genes in plants (Fig. 2A). Reverse transcription (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 symptoms in Nicotiana benthamiana and tomato (Solanum lycopersicum) plants.

**VbMS of miR172 Caused Flower Developmental Defects in N. benthamiana**

In Arabidopsis and N. benthamiana, miR172 regulates the expression of the 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 floral defects in floral patterning due to overaccumulated AP2 mRNA and protein (Chen, 2004; Mlotshwa et al., 2006). To test whether TRY-based VbMS via a miRNA target mimic can suppress miRNA activity, we used the modified TRV vector to express an IPS1-based target mimic against miR172 (MIM172) in N. benthamiana (Fig. 3A). In each plant expressing MIM172, more than one-third 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 (Supplemental Fig. S1A). Stem-loop RT-PCR assays indicated that the 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).

Furthermore, 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 one-third 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 that 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 (Supplemental Fig. S1B), miR172 level was lower (Fig. 4D) and the mRNA level of NbAP2L1...
was higher (Fig. 4E) in STTM172-expressing plants than in controls. Furthermore, 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 a Simpler Leaf Pattern in Tomato Plants

In tomato, miR319/LANCEOLATE (LA) is a well-defined miRNA/target pair, and misregulation of LA by miR319 led to a distinguishable phenotype in aerial
LA encodes a TEOSINTE BRANCHED/CYCLOIDEA/PROLIFERATING CELL FACTORS family transcription factor 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

Figure 4. VbMS of miR172 using the STTM approach caused varied floral defects in N. benthamiana. A, Diagram of STTM172. 48nt, 48-nucleotid imperfect stem-loop linker. B, The varied morphology of flowers caused by TRV-based expression of STTM172. Bars = 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 that could not enclose interior flower organs (top row, arrow) and ectopic genesis of petal-like tissues (bottom row, arrowheads). Bars = 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. Error bars show SE. [See online article for color version of this figure.]
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 an IPS1-based target mimic against miR319 (MIM319; Fig. 5A). Approximately 20% of TRV-MIM319 plants showed a 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 (Supplemental Fig. S1C). Furthermore, the miR319 level was lower (Fig. 5C) and the LA mRNA level was obviously higher (Fig. 5D) in plants expressing MIM319 than in controls. We monitored the VbMS for more than 3 months, and the TRV-MIM319 plants continuously developed simplified leaves (Supplemental Fig. S2), indicating that 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% of 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 (Supplemental Fig. S1D). Furthermore, the miR319 level was lower (Fig. 6C) and the LA mRNA level was evidently higher (Fig. 6D) in plants expressing STTM319 than in controls.

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 shown that VbMS can inhibit miRNA function quickly in N. benthamiana and tomato. To determine

Figure 5. VbMS of miR319 using an IPS1-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 third 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 indicated with numbers. Bars = 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. Error bars show so. [See online article for color version of this figure.]
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) transcription factors have been clearly defined as miR165/166 target sets in Arabidopsis. miR165/166 target and repress the expression of the HD-ZIP III members, determining the behavior of the shoot apical meristem and the 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 the HD-ZIP III transcription factor PHAVOLUTA was shown to be regulated by miR165/166-directed cleavage of its mRNA (McHale and Koning, 2004). miR165/166 also have been 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*

![Diagram of STTM319](image)

**Figure 6.** VbMS of miR319 using the STTM approach caused smaller and simpler leaves in tomato plants. A, Diagram of STTM319. 48nt, 48-nucleotid imperfect stem-loop linker. B, The whole stature and the third to sixth leaves of plants infected with TRV (left column) and with TRV-STTM319 (right column) were photographed at 10 dpi. L1 to L7 indicate leaves 1 to 7. Bars = 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. Error bars show so. [See online article for color version of this figure.]
Figure 7. VbMS of miR165/166 using the STTM approach caused the loss of apical dominance in N. benthamiana. A, Diagram of STTM165/166. 48nt, 48-nucleotid imperfect stem-loop linker. B, Whole plants infected with TRV-STTM165/166 (right
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 the leaf middle vein were observed (Fig. 7C, arrows). These observations reflected the disruption of apical meristematic and leaf primordial functions caused by the inhibition of miR165/166 (Zhong and Ye, 2004; Yan et al., 2012). However, we did not observe obvious changes in leaf abaxial-adaxial polarity, as observed in Arabidopsis overexpressing STTM165/166 (Yan et al., 2012). RT-PCR confirmed that STTM165/166 was expressed in TRV-STTM165/166 plants (Supplemental Fig. S1E). Furthermore, the miR165/166 level was reduced in TRV-STTM165/166 plants (Fig. 7D). One of the HD-ZIP III transcription factors was predicted to be the miR165/166 cleavable target in N. benthamiana (TC21810; http://wmd3.weigelworld.org/cgi-bin/webapp.cgi; N. benthamiana EST NbG1 4.0). Indeed, in TRV-STTM165/166 plants, the mRNA level of TC21810 was much higher compared with 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 the 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* 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% to 30% of plants expressing target mimics against miR319 and miR165/166 showed the expected phenotype. In addition, only about one-third 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 progeny from the same stable transgenic lines expressing target mimics gave various phenotypes (Todesco et al., 2010), and only about 60% of flowers exhibited defects in the transgenic plant lines expressing miR172-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 at suppressing miRNA function through the viral approach. Indeed, disruption of miR165/166 function using the STTM approach also results in a more severe phenotype than that using an IPS1-based miRNA target mimic in transgenic Arabidopsis (Eckardt, 2012; Yan et al., 2012).

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 efficiencies between different approaches. It is possible that the STTM transgenic approach gives a stronger phenotype than virus-mediated STTM. Transgenic STTM blocks miRNAs in each cell of the transgenic plants from the

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Figure 7. (Continued.)

column) or TRV (left column) were photographed at 28 dpi. Photographs were captured from top view (bottom row) and side view (top row). Arrows indicate the branched shoot apex. C, Outgrowth of an ectopic leaf. The ectopic leaf was photographed in front and side views. Arrows indicate ectopic leaf tissues, and “mv” and arrowhead indicate the middle vein. Bars = 1 cm. 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 gene TC21810 in TRV control plants and TRV-STTM165/166 plants. Error bars show so. [See online article for color version of this figure.]
very beginning without any difference between fast-dividing cells and developed cells, while the virus-mediated STTM has less of an effect in fast-dividing cells such as shoot apical meristems due to much diluted virus concentrations in fast-dividing cells. Thus, there may be a 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 species. On the other hand, TRV-based vectors have been used to express foreign genes in systemically infected species. 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 to 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 that 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, such as 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 to 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 of miRNA gene promoters (Vaistij et al., 2010), artificial miRNA-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.


Virus-Based MicroRNA Silencing in Plants


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
