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TITLE:

Potato Virus X-based microRNA Silencing (VbMS) in Potato

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SUMMARY:

We present a detailed protocol for potato virus X (PVX)-based microRNA silencing (VbMS) system to functionally characterize endogenous microRNAs (miRNAs) in potato. Target mimic (TM) molecules of miRNA of interest are integrated into the PVX vector and transiently expressed in potato to silence the target miRNA or miRNA family.

ABSTRACT:

Virus-based microRNA silencing (VbMS) is a rapid and efficient tool for functional characterization of microRNAs (miRNAs) in plants. The VbMS system has been developed and applied for various plant species including *Nicotiana benthamiana*, tomato, Arabidopsis, cotton, and monocot plants such as wheat and maize. Here, we describe a detailed protocol using PVX-based VbMS vectors to silence endogenous miRNAs in potato. To knock down the expression of a specific miRNA, target mimic (TM) molecules of miRNA of interest are designed, integrated into plant virus vectors, and expressed in potato by *Agrobacterium* infiltration to bind directly to the endogenous miRNA of interest and block its function.

INTRODUCTION:

Plant microRNAs (miRNAs) are characterized as 20–24 nucleotide-long, nuclear-encoded regulatory RNAs[1] and play fundamental roles in almost every aspect of plant biological processes, including growth and development[2,3], photosynthesis and metabolism[4-7], hormone synthesis and signaling[8,9], biotic and abiotic responses[10-13], and nutrient and energy regulation[14,15]. The regulatory roles of plant miRNAs are well-programmed and fulfilled typically at post-transcriptional levels by either cleaving or translationally repressing the target mRNAs.

Tremendous progress has been made towards the identification, transcriptional profiling, and target prediction of miRNAs in potato[16-21]. However, the functional characterization of miRNAs in plants, including potato, has lagged behind other organisms due to the lack of efficient and high-throughput genetic approaches. It is challenging to perform functional analysis of individual miRNA by standard loss-of function analysis, because most miRNAs belong to families with considerable genetic redundancy[22]. In addition, a single miRNA can control multiple target genes[23] and several different miRNAs can modulate the same molecular pathway collaboratively[24,25]. These properties make it difficult to characterize the function of a specific miRNA or a miRNA family.

Much of the functional analysis of miRNAs has relied heavily on gain-of-function approaches that have obvious limitations. The artificial miRNA (amiRNA) method exploits the endogenous primary transcripts (pri-miRNAs) to produce miRNAs at a high level, leading to inhibition of target gene expression[26-29]. However, activation tagging and miRNA overexpression using a strong constitutive 35S promoter often lead to heightened expression of miRNAs that are not representative of in vivo conditions and therefore may not reflect the endogenous function of miRNAs[30]. An alternative approach has been developed involving expression of miRNA-resistant forms of target genes that contain uncleavable mutations in the binding and/or cleavage sites[31-33]. But this approach can also potentially cause misinterpretation of the phenotype derived from the miRNA-resistant target transgene due to transgenic artifacts. Therefore, conclusions from these gain-of-function studies should be drawn with caution[34]. Another major limitation of the above-described approaches is that they require transformation, which is labor-intensive and time-consuming. Furthermore, the transgene-dependent approaches are hardly applicable for transform-recalcitrant plant species. Therefore, it is essential to develop a fast and efficient loss-of-function approach to unravel the functions of miRNAs.

To bypass the prerequisite of the transformation procedure, virus-based microRNA silencing (VbMS) has been established by combining the target mimic (TM) strategies with virus-derived vectors. In the VbMS system, artificially designed TM molecules are transiently expressed from a virus backbone, offering a powerful, high-throughput, and time-saving tool to dissect the function of plant endogenous miRNAs[35,36]. VbMS was initially developed in *N. benthamiana* and tomato with the tobacco rattle virus (TRV)[35-37] and has been extended to Arabidopsis, cotton, wheat, and maize using various other virus expression systems, including potato virus X (PVX)[38], cotton leaf crumple virus (ClCrV)[39], cucumber mosaic virus (CMV)[40-42], Chinese wheat mosaic virus (CWMV)[43] and barley stripe mosaic virus (BSMV)[44,45].

Potato (*Solanum tuberosum*) is the fourth most important food crop and the most widely grown noncereal crop in the world primarily because of its high nutritional value, high energy production, and relatively low input requirements[46]. Several features of potato make it an attractive dicotyledonous model plant. It is a vegetatively propagated polyploid crop with high outcrossing rate, heterozygosity, and genetic diversity. However, to date, there is no report characterizing the function of miRNAs in potato using VbMS. Here, we present a ligation independent cloning (LIC)-adapted potato PVX-based VbMS approach to evaluate the function of miRNAs in potato plants[38]. We selected the miR165/166 family to illustrate the VbMS assay

because the miR165/166 family and their target mRNAs and Class III homeodomain/Leu zipper (HD-ZIP III) transcription factors have been extensively characterized[22,47,48]. The *HD-ZIP III* genes are key regulators of meristem development and organ polarity, and suppression of miR165/166 function leads to increased expression of the *HD-ZIP III* genes, resulting in pleotropic developmental defects such as reduced apical dominance and aberrant patterns of leaf polarity[22,35,38,41]. The readily scorable developmental phenotypes correlated with silencing of miRNA165/166 enable accurate evaluation of the effectiveness of the PVX-based VbMS assay.

In this study, we demonstrate that the PVX-based VbMS system can effectively block the function of miRNAs in potato. Because the PVX-based virus-induced gene silencing (VIGS) system has been established in a number of potato varieties[49-52], this PVX-based VbMS approach can be likely applied to a broad range of diploid and tetraploid potato species.

PROTOCOL:

1. Grow Potato Plants.

1.1. Propagate in vitro potato plants in culture tubes (25 x 150 mm) with Murashige and Skoog (MS) media with Gamborg's vitamin (MS basal salt mixture, Gamborg's vitamin, 30 g/L sucrose, 3.5 g/L agar, pH = 5.7). Place the tubes in the growth room under 20–22 °C, 16 h light/8 h dark photoperiod, and light intensity 120 µmol/m^2·s^1.

NOTE: New shoots and roots normally develop in 1–2 weeks from in vitro plants. Propagate in vitro plants with fresh MS/Gamborg's vitamin media every month.

1.2. Four weeks later, transplant in vitro plants into soil and grow the plants in a greenhouse under 20–22 °C, 16 h light/8 h dark photoperiod, and light intensity 120 µmol/m^2·s^1.

NOTE: The in vitro plants with newly developed roots and leaves are suitable for transplanting. Maintain moisture for the freshly transplanted plants for the first 3–4 days.

2. Construct the VbMS vectors.

2.1. Design and clone the short tandem TM molecules (STTM, **Figure 1**)[22,53] for the miRNA of interest.

NOTE: Acquire miRNA sequences based on experimental data or from the miRbase database[54-59]. The miR166 sequence used in this study has been described previously[60].

2.1.1. Design the TM module by inserting a mismatch sequence, normally 5'-CTA-3', into the reverse complement sequence of miRNA at the site corresponding to the 10^{th}–11^{th} nucleotides of the miRNA.

NOTE: For example, the *Stu-miR160* sequence is 5'-UGCCUGGCU**CC**CUGUAUGCC-3'[61], where the 10th–11th nucleotides is in bold. The reverse complement sequence (in deoxynucleotides) is 5'-GGCATACAG**GG**AGCCAGGCA-3' and the mismatch bulge insertion site is shown in bold. The TM molecule sequence (deoxynucleotide) should be 5'-GGCATACAG**G-**CTA**-G**AGCCAGGCA-3'.

2.1.2. Design primers for cloning the STTM fragment (**Figure 1**). Use DNA oligonucleotides with the 48 nt spacer sequence as template for PCR cloning. The forward primer consists of a LIC1 linker (5'-CgACgACAAgACCgT-3'), the forward sequence of the above designed TM molecule, and the partial 5' sequence of the 48-nt spacer (5'-GTTGTTGTTGTTATGGT-3'). The reverse primer consists of a LIC2 linker (5'-gAggAgAagAgCCgT-3'), the reverse complement sequence of the TM molecule, and a partial reverse complement to the 3' sequence of the 48-nt spacer (5'-ATTCTTCTTCTTTAGACCAT-3').

NOTE: The 48-nt spacer sequence is 5'-GTTGTTGTTGTTATGGTCTAATTTAAATATGGTCTAAAGAAGAAGAAT-3'. For example, for STTM-miR160, the forward primer should be 5'-CgACgACAAgACCgT-GGCATACAG**G-**CTA**-G**AGCCAGGCA-GTTGTTGTTGTTATGGT-3'; the reverse primer should be 5'-gAggAgAagAgCCgT-TGCCTGGCT**C**-TAG-**C**CTGTATGCC-ATTCTTCTTCTTTAGACCAT-3' (**Figure 1**).

2.1.3. Amplify the STTM fragment in a volume of 50 µL by PCR using the synthesized universal 48-nt spacer as the template and a high fidelity DNA polymerase.

2.1.3.1. Set up the PCR reaction by mixing 0.5 µL of each primer (40 µM), 0.5 µL of 48-nt spacer oligo (40 µM), 5 µL of 10x PCR buffer, 1 µL of dNTP mixture (10 mM each), 0.1 µL of high fidelity PCR DNA polymerase (10 U/µL), and 43 µL of ddH_2O to a total volume of 50 µL. Perform standard PCR amplification as follows: 94 °C for 3 min, 32 cycles of 94 °C for 45 s, 60 °C for 45 s and 72 °C for 60 s.

2.1.4. Purify the STTM fragment by ethanol precipitation. Add 2.5 volumes of ethanol and 1/10 volume of 3 M sodium acetate (pH = 4.0) to the PCR products. Mix vigorously and centrifuge at 14,000 x *g* for 10 min. Remove the supernatant and rinse the pellet with 1 mL of 70% ethanol. Dry the pellet and dissolve it in 20 µL of ddH_2O.

NOTE: Use DNA electrophoresis to check the amplification of the STTM PCR products.

2.1.5. Set up the T4 DNA polymerase reaction on ice by mixing 2.5 µL of purified STTM PCR product, 0.5 µL of 10x T4 DNA polymerase buffer, 0.05 µL of 1 M dithiothreitol (DTT), 0.25 µL of 100 mM dATP, 0.1 µL of T4 DNA polymerase (3 U/µL), and 1.6 µL of ddH_2O to a total volume of 5 µL. Incubate the mixture at 37 °C for 15 min, and treat the products at 75 °C for 20 min to inactivate the T4 DNA polymerase.

2.2. Prepare the PVX-based VbMS construct.

2.2.1. Digest 5 µg of PVX-LIC plasmid[38] with 2.5 µL of *Sma* I(20 U/µL) in a volume of 100 µL.

2.2.2. Add an equal volume of phenol:chloroform:isopropanol (25:24:1, pH = 6.7/8.0) to the digested PVX-LIC products and mix vigorously. Centrifuge at 14,000 x *g* for 10 min and transfer the supernatant to a new centrifuge tube. Add an equal volume of chloroform:isopropanol (24:1) and vortex vigorously. Centrifuge at 14,000 x *g* for 10 min.

NOTE: The PVX-LIC vector harbors the LIC cassette for cloning. The LIC cassette of PVX-LIC vector contains a *ccdB* gene and a chloramphenicol-resistant gene and needs to be maintained/propagated in the *E. coli* strain DB3.1 using LB medium containing kanamycin (50 mg/L) and chloramphenicol (15 mg/L).

2.2.3. Transfer the supernatant to a new centrifuge tube. Add 2.5 volumes of ethanol and 1/10 volume of 3 M sodium acetate (pH = 4.0) and mix vigorously. Centrifuge at 14,000 x *g* for 10 min and remove the supernatant.

2.2.4. Rinse the pellet with 1 mL of 70% ethanol and vortex vigorously. Centrifuge at 14,000 x *g* for 10 min and remove the supernatant. Dry the pellet and dissolve the digested PVX-LIC plasmid with 100 µL of ddH_2O.

2.2.5. Set up the T4 DNA polymerase reaction on ice by mixing 2.5 µL of digested PVX-LIC vector DNA, 0.5 µL of 10x T4 DNA polymerase buffer, 0.05 µL of 1 M DTT, 0.25 µL of 100 mM dTTP, and 0.1 µL of T4 DNA polymerase (3 U/µL) in a total volume of 5 µL. Incubate the mixture at 37 °C for 15 min and treat the products at 75 °C for 20 min to inactivate the T4 DNA polymerase.

2.3. Clone the STTM sequence into the PVX-LIC vector using a LIC reaction. Mix the T4 DNA polymerase-treated STTM PCR products (5 µL) and the T4 DNA polymerase-treated PVX-LIC plasmids (5 µL). Incubate at 70 °C for 5 min, cool down to 22 °C at a ramp of 0.1 °C/s, and keep at 22 °C for 30 min using a PCR machine.

NOTE: Extend the incubation time to overnight at 4 °C to achieve higher efficiency of LIC cloning.

2.4. Transform 5 µL of the LIC reaction products into the *E. coli* DH5α and grow on an LB plate containing 50 µg/mL kanamycin[62,63]. Pick and verify positive colonies by PCR with the cloning primers and universal primer for the PVX-LIC vector and sequencing.

2.4.1. Perform colony PCR with the forward primer for PVX-LIC (5'-GTGTTGGCTTGCAAACTAGAT-3') in combination with the reverse primer for STTM cloning to identify positive clones. The size of the PCR band should be ~300 bp.

NOTE: Verify the sequences of STTM fragments by terminator cycle sequencing[64,65].

2.5. Isolate the PVX-STTM plasmids from the validated clones and transform them into *Agrobacterium* strains GV3101, GV2260, or EHA105[62,63]. Verify *Agrobacterium* colonies by PCR.

NOTE: Confirm *Agrobacterium* colonies by PCR using the forward primer for PVX-LIC and the reverse primer for STTM cloning.

3. Perform PVX-based VbMS assay in potato plants.

3.1. Transplant 4-week-old in vitro potato plants into soil. The transplanted plants will be subjected to VbMS assay 3–4 days later.

3.2. Inoculate potato plants with *Agrobacterium* containing the PVX-STTM plasmids.

NOTE: For the VbMS assay in potato, *Agrobacterium*-mediated infiltration and toothpick-scratching inoculation are performed simultaneously.

3.2.1. Pick and inoculate positive transformants containing PVX-STTM vectors into 50 mL of liquid LB containing 50 µg/mL kanamycin and 50 µg/mL rifampicin. Grow in a 28 °C incubator at 220 rpm for 16 h until OD_{600} = 1.0.

3.2.2. At the same time, streak the positive *Agrobacterium* colonies onto at least two new LB plates containing 50 µg/mL kanamycin and 50 µg/mL rifampicin and grow at 28 °C for 1 day. Include the PVX-LIC[38,66] vector as a control and PVX-GFP[67,68] to monitor virus spread.

3.2.3. Collect the *Agrobacterium* liquid culture by centrifugation at 3,400 x *g* for 10 min at room temperature. Resuspend *Agrobacterium* cells with an equal volume of infiltration buffer (10 mM $MgCl_2$, 10 mM MES, and 200 µM acetosyringone, pH = 5.6) and adjust to OD_{600} = 1.0. Incubate at room temperature for 6 h.

3.2.4. Infiltrate the *Agrobacterium* culture into the abaxial side of fully expanded leaves with a 1 mL needleless syringe.

3.2.4.1. Flip and hold a leaf with one hand, then use one finger to support the leaf lamina from the adaxial side at the infiltration site. Keep the syringe vertical to the leaf surface with the other hand and infiltrate the *Agrobacterium* culture into the abaxial side of lamina.

3.2.5. Scrape the *Agrobacterium* culture from the LB plates and scratch the stem surface of the first one or two internodes of the infiltrated potato plants with a toothpick. Gently scratch the epidermis of the stem. Avoid piercing through the stem, which may cause severe damage to the plants.

3.3. Grow the infiltrated plants at 22 °C with a 16 h light/8 h dark photoperiod and light intensity of 120 µmol/$m^2 \cdot s^1$ in a greenhouse.

NOTE: Phenotypes caused by miRNA silencing usually appear in 2–4 weeks postinoculation (**Figure 2**, **Figure 3**). It typically takes 10–20 days for the VbMS phenotype to appear after

infiltration. The vbMS phenotype depends on the properties of the specific miRNA and target genes, growth conditions, and potato varieties.

4. Perform expression analysis.

4.1. When phenotypes appear at 2–4 weeks postinoculation, collect tissues such as shoots, leaves, flowers, or roots with phenotypes from the VbMS plants and tissues from the control plants with scissors. Isolate total RNAs from the collected tissues.

4.2. Check the RNA quality by electrophoresis[62,63] and quantify the RNA concentration by measuring the OD_{260} absorbance with a spectrophotometer.

4.3. Use stem-loop real-time reverse transcription PCR (RT-PCR) to analyze the miRNA expression.

4.3.1. For specific miRNAs, design a stem-loop reverse transcription primer. Stem-loop RT primers contain a universal 5' backbone and a 3' 6 nt extension of a specific miRNA. Design a 5' universal backbone (5'- GTCTCCTCTG***GTGCagggtccgaggt***attcGCACCAGAGGAGAC-3') that forms a stem-loop structure. (The upper case corresponds to an inverse-repeated sequence and the lower case to the loop region).

4.3.2. Part of the 5' backbone sequence that forms a loop serves as the reverse primer for subsequent PCR amplification (bold-italic sequence in the backbone sequence). Add a 6 nt extension sequence that is reverse-complementary to the 3' end of the miRNA of interest to the stem-loop primer to provide specificity for reverse transcription (**Supplemental Figure S1 A**).

NOTE: Design the stem-loop reverse transcription primer as described by Chen et al.[69] and Erika Varkonyi-Gasic et al.[70-72]. For example, the stem-loop reverse transcription primer for *Stu-miR160* is designed as 5'-GTCTCCTCTG***GTGCagggtccgaggt***attcGCACCAGAGGAGAC**GGCATA**-3'. The stem-loop reverse transcription primer for the potato miR165/166 family is designed as 5'-GTCTCCTCTG***GTGCagggtccgaggt***attcGCACCAGAGGAGAC**GGGG(A/G)A**-3'. (The bold uppercase sequences provide the specificity of reverse transcription for specific miRNAs).

4.3.3. Set up the reverse transcription reaction by mixing 50–200 ng of total RNA, 1 µL of the stem-loop reverse transcription primer (100 µM), 2 µL of 10x buffer, 0.2 µL of RNase inhibitor (40 U/µL), 0.25 µL of dNTPs (10 mM each), and 1 µL of reverse transcriptase (200 U/µL) on ice. Add nuclease-free ddH_2O to a total volume of 20 µL.

4.3.4. Perform reverse transcription using the pulsed reverse transcription procedure. Incubate the reverse transcription reaction mixture at 16 °C for 30 min, perform a temperature cycle of 30 °C for 30 s, 42 °C for 30 s and 50 °C for 1 s, for a total of 60 cycles, and inactivate the reverse transcriptase by incubation at 85 °C for 5 min.

4.3.5. For real-time PCR analysis of miRNA expression, design the forward primer based on the miRNA sequence but do not include sequences that overlap with the above designed stem-loop reverse transcription primer. Add a 3–7 nt extension to the 5' of the forward primer to optimize the length, melting temperature, and GC content (**Supplemental Figure 1A**,**B**).

NOTE: The universal reverse primer is 5'-GTGCAGGGTCCGAGGT-3'. For example, the stem-loop reverse transcription primer for *Stu-miR160* is designed as 5'-**CGGC**TGCCTGGCTCC-3'. The stem-loop reverse transcription primer for miR165/166 family is 5'-**CGGC**TCGGACCAGGCTT-3'. The bold sequences serve as 5' extensions for primer optimization. The stem-loop reverse transcription primers and the real-time PCR primers for miRNA can be designed using the miRNA Primer Design Tool[73].

4.4. Synthesize cDNAs of the target mRNAs by standard reverse transcription PCR (RT-PCR). Incubate the reverse transcription reaction mixture at 37 °C for 60 min and inactivate the reverse transcriptase by heating to 85 °C for 5 min.

NOTE: (1) Predict target mRNAs using the psRNATarget program[74] if the target mRNAs are not known. (2) The universal reverse transcription primer for mRNA is an anchored primer 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3'.

4.5. Set up the real-time PCR reaction for both the miRNA of interest and the target mRNAs. Mix 0.5 µL of template cDNA, 5 µL of 2x real-time PCR buffer with SYBR green, 0.05 µL of each forward and reverse primer (40 µM), and 4.4 µL of ddH_2O in a total volume of 5 µL on ice.

4.6. Incubate at 95 °C for 3 min, 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Perform a subsequent melting curve analysis as follows: Incubate at 95 °C for 15 s, cool down to 60 °C at a ramp of 20 °C/s, keep at 60 °C for 60 s, heat to 95 °C at a ramp of 0.2 °C/s, and keep at 95 °C for 15 s. Analyze the C_t-values using the $\Delta\Delta C_t$ methods[76,77] and plot the means with standard errors.

NOTE: (1) The real-time PCR primers for target mRNAs can be designed as described[75]. (2) Potato *polyubiquitin 10* gene can serve as an internal control for normalization in potato. The forward primer for the potato *polyubiquitin 10* gene is 5'-ATGTTGCCTTTCTTATGTGTGGTTG-3' and the reverse primer 5'-TTATTTATTCACATAAACGACAGTTCAACC-3'. (3) For real-time PCR analysis, contamination and primer-dimer formation may generate false positive results. To monitor nonspecific amplification and increase the liability of real-time PCR analysis, it is recommended to include controls with no template and controls with no reverse transcription for real-time PCR assays.

REPRESENTATIVE RESULTS:

Figure 2 shows the PVX-STTM165/166 potato plants (Katahdin) with ectopic growth of leaf tissues from the abaxial side of leaf lamina along the veins. More severe phenotypes such as trumpet-shaped leaf formation have also been observed. In contrast, no phenotypic abnormality was observed in the PVX control plants. These results show that the VbMS system was effective in suppressing endogenous miRNA function in tetraploid potato plants and the PVX-VbMS system

was a robust genetic tool to determine the function of specific miRNAs or miRNA families.

Figure 3 shows the PVX-STTM165/166 potato plants (Russet Burbank) with ectopic leaf tissue growth from the abaxial side of the leaf lamina along the veins. These results show that the PVX-VbMS system could be applied to other potato species, including a major potato cultivar.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic diagram of PVX-based VbMS vectors and the PVX-STTM165/166 structure. LB = T-DNA left border; RB = T-DNA right border; 35S = cauliflower mosaic virus 35S promoter; NOST = nopaline synthase terminator; RdRP = RNA-dependent RNA polymerase; TGB1 = triple gene block protein 1; TGB2 = triple gene block protein 2; TGB3 = triple gene block protein 3; sgP = PVX subgenomic RNA promoter; CP = coat protein; LIC Cassette = ligation independent cloning cassette; 48 nt = 48 nucleotide imperfect stem-loop linker. STTM165/166 consists of tandem TM sequences of miR165/166 separated by a 48 nt imperfect stem-loop linker sequence. The green arrowhead in the PVX-LIC vector indicates the start site of the PVX subgenomic RNA harboring the STTM sequence. The triple minus hyphens in miRNA sequences indicate the cleavage sites.

Figure 2: VbMS of miR165/166 in diploid potato Katahdin. Phenotypes of the potato plants (Katahdin) expressing the PVX vector control or PVX-STTM165/166. Magenta arrows denote ectopically generated leaf structures in the leaf veins. The orange arrowhead denotes trumpet-like leaf structures. Bars = 1 cm.

Figure 3: VbMS of miR165/166 in diploid potato Russet Burbank. Phenotypes of the potato plants (Russet Burbank) expressing PVX vector as control or PVX-STTM165/166. Magenta arrows denote ectopically generated leaf structures in the leaf veins. Bars = 1 cm.

Supplemental Figure 1: Schematic diagrams of stem-loop RT-PCR analysis of miRNAs and real-time PCR primer design. (**A**) Stem-loop RT-PCR analysis of miRNAs. During reverse transcription, the binding of the stem-loop primer to the 3' miRNA initiated the reverse transcription and cDNA was synthesized. PCR products were amplified with a specific forward primer of the miRNA of interest and the universal reverse primer. (**B**) Real-time PCR primer design. The forward and reverse primers for Stu-miR160 and Stu-miR165/166 are shown. The forward primer was designed based on the miRNA sequence but did not include sequences that overlapped with the designed stem-loop reverse transcription primer. A 3–7 nt extension was added to the 5' of the forward primer to adjust the length, melting temperature, and GC content.

DISCUSSION:

We present a PVX-based miRNA silencing system to characterize the function of endogenous miRNAs in potato by integrating the STTM design into the PVX vector. The VbMS system proved to be effective in silencing miRNA165/166 in potato, a highly conserved miRNA family across plant species.

The TM approach has been developed to interfere with the expression of miRNAs based on an

artificial miRNA target mimic that is designed to create a mismatch loop at the expected cleavage site within the miRNA complementation sequence that results in sequestration of targeted miRNA and arrest of its activity[22,35,78,79]. The pairing between TM molecules and the target miRNAs blocks the function of the miRNAs by knocking down the levels of a specific miRNA or a miRNA family, which leads to upregulation of the target mRNAs. Several TM technologies have been developed for silencing of miRNAs, including endogenous miRNA target mimicry (eTM)[79,80], eTM-based miRNA mimics (MIMs)[35,78], short tandem target mimics (STTMs)[22,53], a miRNA decoy approach with TMs integrated into the 3' UTR of protein-coding transcripts[81], and miRNA SPONGEs containing miRNA binding sites with two central mismatches to target miRNAs (cmSPs)[82]. STTM consists of two miRNA binding sites with a 3 nt mismatch bulge, linked by a 48 nt spacer that was empirically optimized. STTM triggers efficient inhibition of target miRNAs[22,53]. The STTM technology has recently been successfully applied to a large-scale functional analysis of miRNAs from the model plant Arabidopsis and major crops such as rice and maize. This led to the discovery of unprecedented roles of several endogenous miRNAs involved in yield and hormone control, which hold great promise in improving crop breeding[47]. Based on these advantages of STTM design, we chose STTM and integrated it into the PVX vector for functional characterization of the miRNAs in potato. It is worth noting that the various designs of TM molecules, such as cmSPs, MIMs, and STTMs, have variable efficacies in blocking the function of different miRNAs[82]. Therefore, using various TM design strategies may help to achieve more effective miRNA suppression. The length and sequence context of the unmatched bulge as well as the nucleotide alterations adjacent to the miRNA binding sites may also need to be optimized for a specific miRNA silencing outcome[22,36,38,78,83]. Furthermore, design of TM molecules under guidance of computational predictions together with experimental analysis will probably lead to more reliable inhibition of miRNAs[84].

It was shown that the PVX-based VIGS system is effective in triggering RNA silencing in both diploid and cultivated tetraploid Solanum species. The PVX-based systemic silencing is induced and maintained throughout the foliar tissues on in vitro propagated potato plants for several cycles and on in vitro generated microtubers[85]. We have recently reported that the PVX-based VIGS system can silence genes of interest in several tetraploid potato cultivars, such as Ancilla, Arran Pilot, Marius Bard, and Serrana[86]. It remains to be determined whether the PVX-based VbMS effect can be transmitted and sustained for several generations through vegetative propagation in potato. Transgenic approaches to introduce TM molecules stably into potato plants are still recommended when the silencing effects of miRNA(s) of interest need to be maintained in the subsequent generations.

Numerous miRNAs involved in potato growth and development have also identified. RNA-seq, genome sequencing, and bioinformatic prediction have greatly facilitated identification of miRNAs and their targets[17,19-21]. So far, three potato genomes have been sequenced, including a doubled monoploid *S. tuberosum* Group Phureja clone DM1-3, a wild diploid species *S. commersonii,* and a diploid inbred clone M6 of *S. chacoense*[87-89]. Up to date, only a limited number of potato miRNAs have been functionally characterized, in most cases using TM technology. For example, the *FLOWERING LOCUS T (FT)* homolog *SP6A* acts as a mobile signal to control tuberization in potato and is targeted by a miRNA, suppressing expression of *SP6A* (*SES*),

which mediates heat-induced cleavage of the *SP6A* transcript[90,91]. STTM-mediated overexpression of *SES* blocks the activity of *SES* miRNA and facilitates tuberization even under continuous heat conditions[91]. Knockdown of miR160, a miRNA involved in immune response, by the eTM approach showed that miR160 is required in both local and systemic acquired resistance against *Phytophthora infestans* in potato[92].

Using a bioinformatic approach, eight unique families of miRNAs that target nucleotide binding site leucine-rich repeat (NLR) immune receptors in potato and tomato were identified[93]. One of the miRNA families, miR482/2118, targets several NLRs that confer resistance to various pathogens and suppression of the miR482/2118 family miRNAs mediated by transgenic expression of STTM constructs leads to enhanced resistance in tomato against *P. infestans* and *Pseudomonas syringae*[13]. Increasing evidence suggests that small RNAs produced in pathogens and hosts can travel between the two organisms and suppress each other's gene expression mediated by cross-kingdom RNA interference[94-97]. For example, target mimics of an oomycete pathogen-derived sRNAs can scavenge these invading sRNAs and reduce pathogen infection[61]. It would be interesting to examine whether the present VbMS system can be employed to target pathogen-derived sRNAs to improve resistance in plants.

In summary, virus-based miRNA silencing system is rapid and cost-effective and can be performed in a high-throughput format. The PVX-based VbMS system provides an efficient and robust genetic tool to determine the function of specific miRNAs or miRNA families and the target genes.

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DISCLOSURES:

None.

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Figure 1

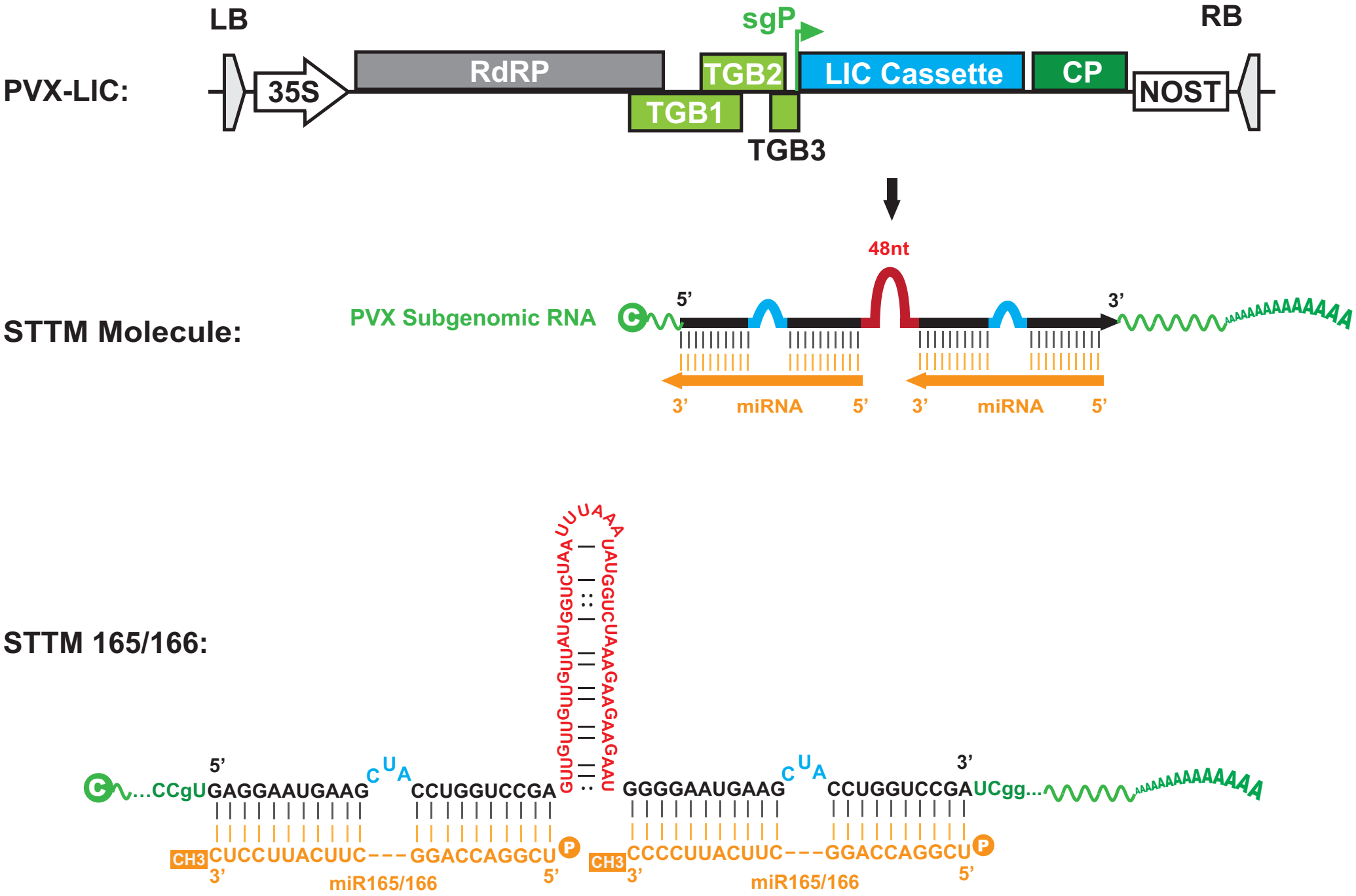


Figure 2

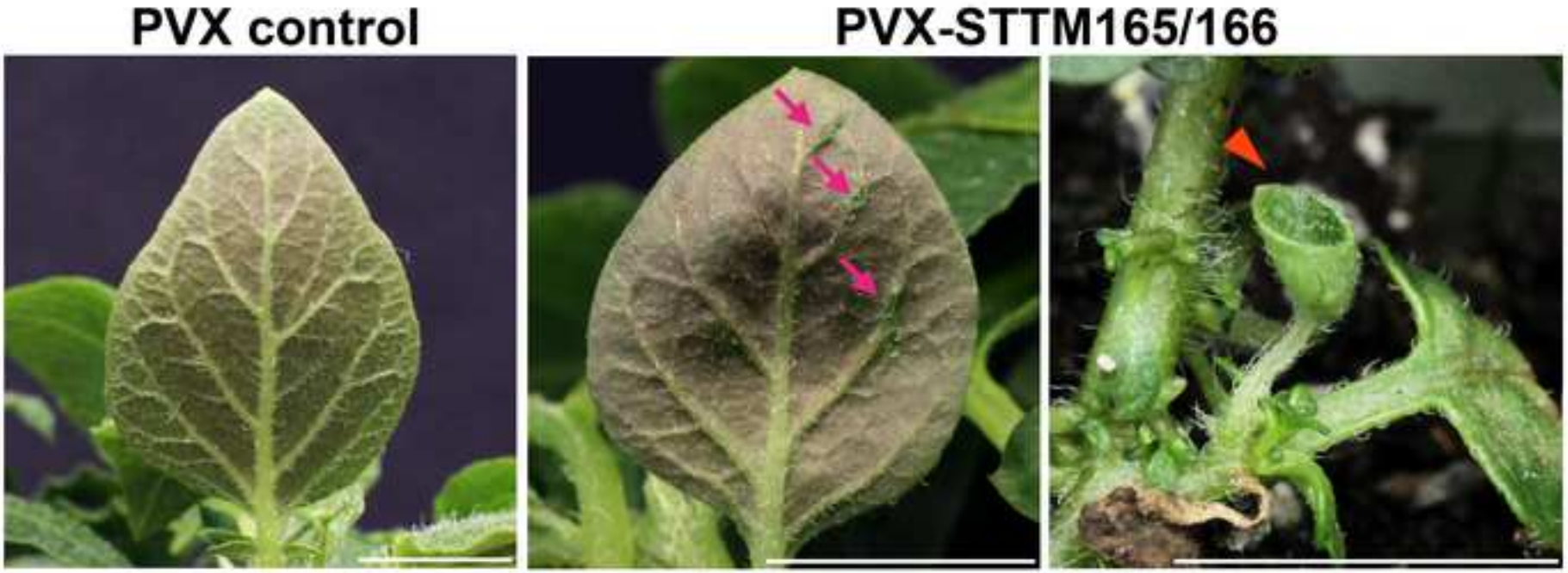
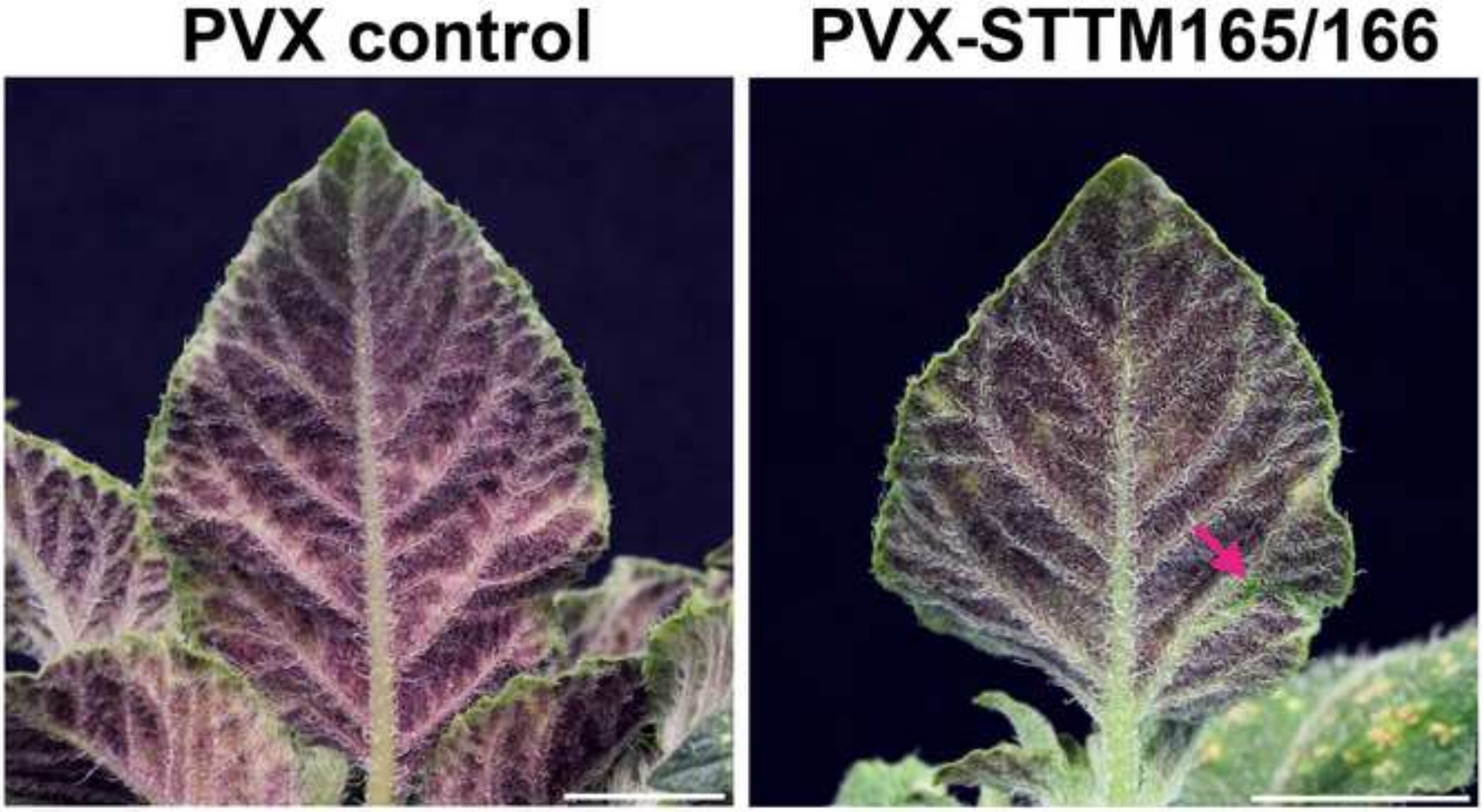


Figure 3



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
100 µM dATP and 100 µM dTTP	Omega Bio-tek, Inc., Norcross, Norcross, GA 30071 , USA	TQAC136	
3 M Sodium acetate, pH 4.0.	Teknova, Hollister, CA 95023, USA	#S0297	
Acetosyringone	TCI America, Portland, OR 97203, USA	D2666-25G	
Agrobacterium tumefaciens strains: GV3101, GV2260 or EHA105.			
Chloroform	VWR Corporate, Radnor, PA 19087-8660, USA	VWRV0757-950ML	
Dimethyl sulfoxide, DMSO	TCI America, Portland, OR 97203, USA	D0798-25G	
DTT	VWR Corporate, Radnor, PA 19087-8660, USA	VWRV0281-25G	
E. coli DB3.1			for maintenance of PVX-LIC and pTRV2e containing the *ccdB* gene
E. coli DH5α			for the destination constructs generated by LIC cloning
Fertilizer: Peters Peat Lite Special 15-0-15 Dark Weather Feed	ICL Specialty Fertilizers, Summerville, SC 29483, USA	G99260	
High fidelity PCR reagents: KAPA HiFi DNA Polymerase with dNTPs	Roche Sequencing and Life Science, Kapa Biosystems, Wilmington, MA, USA	7958960001	
Isoamyl alcohol	VWR Corporate, Radnor, PA 19087-8660, USA	VWRV0944-1L	

Koptec Pure Ethanol – 200 Proof	Decon Labs, King of Prussia, PA 19406 , USA	V1005M	
MES	TCI America, Portland, OR 97203, USA	M0606-250G	
$MgCl_2$	ThermoFisher, Waltham, MA 02451, USA	MFCD00149781	
M-MuLV Reverse Transcriptase	New England BioLabs, Ipswich, MA 01938-2723 USA	M0253L	
Nano-drop spectrometer: NanoDrop One^C Microvolume UV-Vis Spectrophotometer with Wi-Fi	ThermoFisher, Waltham, MA 02451, USA	ND-ONEC-W	
PCR machine: Bio-Rad MyCycler PCR System	Bio-Rad, Hercules, California 94547, USA	170-9703	
PCR machine: Eppendorf Mastercycler pro	Eppendorf, Hauppauge, NY 11788, USA	950030010	
pH meter	Sper Scientific, Scottsdale, AZ 85260, USA	Benchtop pH / mV Meter - 860031	
Phenol:chloroform:isoamyl alcohol (25:24:1), pH 6.7/8.0.	VWR Corporate, Radnor, PA 19087-8660, USA	VWRV0883-400ML	
Phytagel: Gellan Gum	Alfa Aesar, Tewksbury, MA 01876, USA	J63423-A1	
PVX VIGS vector: PVX-LIC			Zhao et al., 2016
Real-time PCR machine: QuantStudio 6 Flex Real-Time PCR System	ThermoFisher, Waltham, MA 02451, USA	4485697	
Real-time PCR reagent: KAPA SYBR® FAST qPCR Master Mix (2x) Kit	Roche Sequencing and Life Science, Kapa Biosystems, Wilmington, MA 01887, USA	7959389001	

Restriction enzyme: *Sma* I	New England BioLabs, Ipswich, MA 01938-2723 USA	R0141S
Reverse transcription reagents: qScript cDNA SuperMix	Quanta BioSciences, Gaithersburg, MD 20877 , USA	95107-100
RNA extraction Kit: E.Z.N.A. Plant RNA Kit	Omega Bio-tek, Inc., Norcross, Norcross, GA 30071 , USA	SKU: D3485-01
RNase Inhibitor Murine	New England BioLabs, Ipswich, MA 01938-2723 USA	M0314L
RNAzol RT	Sigma-Aldrich, St. Louis, MO 63103, USA	R4533
Soil: Metro-Mix 360	Sun Gro Horticulture, Agawam, MA 01001-2907, USA	Metro-Mix 360
T4 DNA polymerase and buffer	New England BioLabs, Ipswich, MA 01938-2723 USA	M0203S

Dear Dr. Alisha DSouza,

Thank you very much for your constructive comments on our manuscript. We appreciate the opportunity to revise and improve our manuscript. In the revised version, we have addressed all the editorial comments in the manuscript.

We have added one supplemental Figure and made changes to the highlighted text for visualization as you suggested. We hope that this revised version meets the standards of JoVE.

Thank you for your consideration.

Sincerely,

Junqi Song