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Xiaoyan Cao
Editor
JoVE

October 28, 2019

Dear Dr. Xiaoyan Cao

Thank you very much for giving us the opportunity to revise our manuscript entitled “Screening and identification of RNA silencing suppressors in plant pathogens secreted effectors” (JoVE60697). We greatly appreciate the careful reviews and constructive comments of the reviewers and yourself. We have comprehensively modified the manuscript to address the editorial comments and the reviewers’ concerns.

We also thoroughly revised the main text and figure elements by two native English speakers and a Bioediting service (https://www.bioedit.com/). Please see as attached.

We have added one coauthor (Dr. Yushuang Guo) in our revised manuscript because of his contribution of revised version

Thank you for your consideration of our revised manuscript. We believe we have sufficiently addressed the previous concerns and hope that the revised manuscript is suitable for publication. Please do not hesitate to contact me if you require any further information.

Sincerely yours,

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TITLE:

Screening and Identification of RNA Silencing Suppressors from Secreted Effectors of Plant Pathogens

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KEYWORDS:

agroinfiltration, effector, local RNA silencing, *Nicotiana benthamiana*, *Phytophthora sojae*, plant pathogens, RNA silencing suppressor, systemic RNA silencing

SUMMARY:

Here, we present a modified screening method that can be extensively used to quickly screen RNA silencing suppressors in plant pathogens.

ABSTRACT:

RNA silencing is an evolutionarily conserved, sequence-specific gene regulation mechanism in eukaryotes. Several plant pathogens have evolved proteins with the ability to inhibit the host plant RNA silencing pathway. Unlike virus effector proteins, only several secreted effector proteins have showed the ability to suppress RNA silencing in bacterial, oomycete, and fungal pathogens, and the molecular functions of most effectors remain largely unknown. Here, we describe in detail a slightly modified version of the co-infiltration assay that could serve as a general method for observing RNA silencing and for characterizing effector proteins secreted by plant pathogens. The key steps of the approach are choosing the healthy and fully developed leaves, adjusting the bacteria culture to the appropriate optical density (OD) at 600 nm, and observing green fluorescent protein (GFP) fluorescence at the optimum time on the infiltrated leaves in order to avoid omitting effectors with weak suppression activity. This improved protocol

will contribute to rapid, accurate, and extensive screening of RNA silencing suppressors and serve as an excellent starting point for investigating the molecular functions of these proteins.

INTRODUCTION:

Over the past two decades, acceleration in genome sequencing of microorganisms that cause plant diseases has led to an ever increasing knowledge gap between sequence information and the biological functions of encoded proteins[1]. Among the molecules revealed by sequencing projects are effector molecules that suppress innate immunity and facilitate host colonization; these factors are secreted by destructive plant pathogens, including bacteria, nematodes, and filamentous microbes. To respond to these threats, host plants have evolved novel receptors that recognize these effectors, enabling restoration of the immune response. Hence, effectors are subject to various selective pressures, leading to diversification of effector repertoires among pathogen lineages[2]. In recent years, putative effectors from plant pathogens have been shown to disrupt plant innate immunity by impeding host cellular processes to benefit the microbes in a variety of ways, including dysregulation of signaling pathways, transcription, intracellular transport, cytoskeleton stability, vesicle trafficking, and RNA silencing[3-5]. However, the vast majority of pathogen effectors, particularly those from filamentous pathogens, have remained enigmatic.

RNA silencing is a homology-mediated gene inactivation machinery that is conserved among eukaryotes. The process is triggered by long double-stranded RNA (dsRNA) and targets the homologous single-stranded RNA (ssRNA) in a sequence-specific manner, and it manipulates a wide range of biological processes, including antiviral defense[6]. To surmount innate immune responses of the host, some viruses have evolved to offset RNA silencing, including the ability to replicate inside intracellular compartments or escape from the silencing reorganized signal. Nevertheless, the most general strategy by which viruses protect their genomes against RNA silencing-dependent loss of gene function is to encode specific proteins that suppress RNA silencing[7,8]. Several mechanistically different approaches have been established to screen and characterize viral suppressors of RNA silencing (VSRs), including the co-infiltration of *Agrobacterium tumefaciens* cultures, transgenic plants expressing putative suppressors, grafting and cell culture[9-13].

Each of these assays has advantages and disadvantages, and identifies VSRs in its own distinct manner. One of the most common approaches is based on the co-infiltration of individual *A. tmuefaciens* cultures harboring the potential viral protein and a reporter gene (typically green fluorescent protein [GFP]) on the *Nicotiana benthamiana* 16c plants constitutively expressing *GFP* under the control of the cauliflower mosaic virus 35S promoter. In the absence of an active viral silencing suppressor, GFP is identified as exogenous by the host cells and is silenced within 3 days post-infiltration (dpi). By contrast, if the viral protein possesses suppression activity, the expression level of GFP remains steady beyond 3−9 dpi[9]. This co-infiltration assay is simple and fast; however, it is neither highly stable nor sensitive. Nonetheless, the assay has identified numerous VSRs with diverse protein sequences and structures in many RNA viruses[7,8].

Recently, several effector proteins that can inhibit the cellular RNA silencing activity have been

characterized from bacterial, oomycete, and fungal plant pathogens[14-16]. These findings imply that RNA silencing suppression is a common strategy for facilitating infection that is used by pathogens in most kingdoms. In theory, many, if not all, of the effectors might encode RNA silencing suppressors (RSSs); to date, however, only a few have been identified, mainly due to the shortage of the reliable and general screening strategy. Moreover, suppressors of RNA silencing have not been investigated in the vast majority of plant pathogens[17].

In this report, we present an optimized and general protocol for identifying plant pathogen effectors that can suppress local and systemic RNA silencing using the agro-infiltration assay. The foremost objective of this study was to emphasize the key aspects of the protocol and describe the steps in detail, thereby providing a screening assay that is suitable for almost all effectors of plant pathogens.

PROTOCOL:

NOTE: All steps of the procedure should be conducted at room temperature (RT).

CAUTION: Deposit all media containing pathogenic microbes, as well as the plants and plant tissue used in the assay, into the appropriate waste containers and autoclave before discarding.

1. Preparation of plasmid constructs containing putative effectors

1.1. Select putative secreted effectors that are highly expressed during infection, as determined by RNA sequencing (RNA-Seq), and further by quantitative real time PCR (qRT-PCR) technique.

1.2. Determine the signal peptide cleavage site of each effector using a software tool such as SignalP[18].

1.3. Design primers and perform PCR to amplify the gene encoding the effector of interest using high fidelity DNA polymerase. Perform agarose gel electrophoresis to check the PCR product, then clone the amplicon into Gateway entry vector pQBV3 using the ligation-free cloning kit (**Table of Materials**), and subsequently into the destination expression vector pEG100 using the LR recombination reaction[19].

1.4. Transform 3−5 µL of LR reaction mixture into 100 µL of competent *E. coli* cells (e.g., TOP10, DH5α), spread the transformed bacterial cells on Luria-Bertani (LB) agar medium supplemented with 50 µg/mL kanamycin, and incubate at 37 °C for 16−20 h.

NOTE: Positive transformants can be identified by colony PCR[20] and further analyzed by plasmid miniprep and sequencing.

1.5. Introduce 50−100 ng of recombinant plasmids carrying effector protein into 100 µL of chemically competent *A. tumefaciens* cells (e.g., GV3101 or C58C1), mix gently, and then freeze in liquid nitrogen for 1 min. Thaw the cells in a 37 °C water bath for 5 min.

1.6. Add 500 µL of LB broth and incubate at 30 °C for 4−6 h with gentle shaking, and then transfer and spread all the agrobacteria cells on LB agar medium supplemented with 50 µg/mL kanamycin and 50 µg/mL rifampicin at 30 °C for 2 days.

NOTE: Positive clones can be verified by colony PCR.

1.7. Use a single transformed colony for agro-infiltration experiments unless otherwise directed.

NOTE: In the present research, none of the tested effector genes contained predicted introns; each of the genes was directly amplified from genomic DNA of a *Phytophthora sojae* isolate. A Gateway plant expression vector without any tag is recommended, but not necessary.

2. Preparation of *N. benthamiana* 16c plants

2.1. Prepare potting soil mixes consisting of (by volume) 50% peat moss, 30% perlite, and 20% vermiculite, and autoclave at 120 °C for 20 min.

2.2. Soak autoclaved soil mixes with plant fertilizer solution (1 g/L) and sub-package them into smaller pots (80 mm x 80 mm x 75 mm) stored in a larger tray (540 mm x 285 mm x 60 mm).

2.3. Sow one or two seeds of *N. benthamiana* 16c onto the soil surface of each pot. Cover the tray with a plastic dome and allow seeds to germinate.

2.4. Place the tray under light and temperature-controlled growth chambers with a temperature of 23−25 °C, 50−60% relative humidity, and a long-day photoperiod (14 h light/10 h dark, with illumination at 130−150 $\mu E \cdot m^{-2}s^{-1}$).

2.5. Take the plastic dome off after the seeds germinate (3−4 days) and allow the seedlings to grow under the same conditions used for the germination step.

2.6. Add an appropriate amount of water, keeping soil moist but not soaking, every 2−3 days; add fertilizer every 10 days to promote further growth. Maintain *N. benthamiana* 16c plants under normal conditions until they are ready for use; at this stage the plant should have at least five fully developed true leaves and no visible axillary or flower buds, and the leaves should have a healthy green appearance).

2.7. Use 3−4 week-old leaves of *N. benthamiana* 16c for local RNA silencing assays, and young *N. benthamiana* 16c plants (10−14 days old) for systemic RNA silencing assays.

NOTE: Plant growth conditions and facilities vary across laboratories; choose healthy, well-developed and fully expanded leaves for infiltration.

3. Preparation of *Agrobacterium* culture for infiltration

3.1. Pick a positive colony from the LB plate and inoculate the cells into glass tube containing 5 mL of LB medium supplemented with 50 µg/mL kanamycin and 50 µg/mL rifampicin. Grow the cells at 30 °C with shaking at 200 rpm for 24−48 h.

3.2. Transfer 100 µL of culture into 5 mL of LB medium supplemented with same antibiotics, 10 mM 2-(N-morpholino) ethanesulfonic acid (MES; pH 5.6) and 20 µM acetosyringone (AS). Grow bacteria at 30 °C with shaking at 200 rpm for 16−20 h.

3.3. Centrifuge cells at 4,000 x *g* for 10 min. Pour off the supernatant and resuspend the pellet in 2 mL of 10 mM $MgCl_2$ buffer.

3.4. Repeat step 3.3 to ensure the complete removal of antibiotics.

3.5. Determine the density of the *Agrobacterium* culture by measuring the optical density at 600 nm (OD_{600}). Adjust to an OD_{600} of 1.5−2.0 with 10 mM $MgCl_2$ buffer.

3.6. Add 10 mM MES (pH 5.6) and 150 µM AS to the final suspension cultures and incubate the cells at RT for at least 3 h without shaking.

NOTE: Do not leave the final suspension cultures overnight.

4. Co-infiltration *N. benthamiana* leaves

4.1. Mix equal volumes of an *Agrobacterium* culture containing 35S-GFP with an *Agrobacterium* culture containing 35S- cucumber mosaic virus suppressor 2b (CMV2b), putative effector, or empty vector (EV).

4.2. Carefully and slowly infiltrate the mixed agrobacterium suspensions on the abaxial sides of *N. benthamiana* 16c leaves using a 1 mL needleless syringe.

4.3. Remove the remaining bacterial suspension from the leaves with soft tissue wipers and circle the margins of the infiltrated patches with a maker pen.

4.4. Leave the infiltrated plants in the growth chamber under the same growth conditions.

NOTE: For safety and health reasons, protective eye goggles and a mask should be worn during infiltration. To prevent cross-contamination, gloves should be changed or sterilized with ethanol between infiltrations. Normally at least 1 mL of agrobacterium suspensions per leaf is needed for infiltration. For systemic silencing, at least two leaves will be required for infiltration.

5. GFP imaging analysis

5.1. Visually detect GFP fluorescence in newly grown leaves of whole plants 2 weeks post-

infiltration (for systemic RNA silencing) or infiltrated patches of leaves 3–4 dpi using a long-wave ultraviolet (UV) lamp without leaves collection.

5.2. Photograph collected plants and/or detached leaves with a digital camera fitted with both UV and yellow filters.

NOTE: For assays of local silencing suppression, investigate infiltrated patches of *N. benthamiana* 16c leaves, whereas for systemic silencing suppression activity, investigate newly grown leaves. RNA silencing suppression activity may vary across individual effectors. Therefore, observe the patches or leaves over a few days starting at 3 dpi. Use CMV2b and EV as positive and negative controls, respectively.

6. Northern blot analysis of GFP mRNA levels in infiltrated leaves

6.1. Isolation of total RNA from leaf tissue using the RNA isolation reagent (**Table of Materials**)

6.1.1. Collect leaf tissues from infiltrated *N. benthamiana* 16c patches at 4–7 dpi, pulverize in liquid nitrogen to a fine powder, and transfer the powder to a sterile 2 mL tube.

6.1.2. Add the RNA isolation reagent (1 mL/100 mg tissue) immediately to the sampled tube in a hood, shake vigorously to homogenate, and incubate at RT for 5 min.

6.1.3. Add chloroform (200 µL/1 mL RNA isolation reagent) to each tube in a hood, shake vigorously for 15 s, and incubate at RT for 5 min. Centrifuge the homogenate at 12,000 x *g* for 15 min at 4 °C.

6.1.4. Transfer the supernatant into a new RNase-free tube and discard the pellet. Add 0.7 volume of isopropanol to the supernatant, gently invert several times, and incubate the mixture at RT for 10 min.

6.1.5. Precipitate the RNA pellet by centrifugation at 12,000 x *g* for 15 min at 4 °C.

6.1.6. Discard the supernatant, wash the pellet with 70% ethanol, and air-dry the pellet in a hood. Dissolve the RNA in diethyl pyrocarbonate (DEPC)-treated water by incubating in a 65 °C water bath for 10–20 min.

6.2. Northern blot analysis of GFP mRNA level in the infiltrated leaves

6.2.1. Prepare a 1.2% formaldehyde denaturing agarose gel in 1x MOPS running buffer.

6.2.2. Mix RNA 1:1 with RNA loading dye and denature by incubation at 65 °C for 10 min, immediately chill the denatured samples on ice for 1 min.

6.2.3. Load the samples on the gel and electrophorese at 100 V for 50 min until the RNA is well

separated.

6.2.4. Rinse the gel in 20x saline-sodium citrate (SSC) buffer to remove formaldehyde and transfer the gel to nylon membrane by capillary transfer in 20x SSC buffer overnight. Soak the membrane in 2x SSC and fix the RNA to the membrane by exposing the wet membrane to UV cross linking.

6.2.5. Add the appropriate amount of hybridization buffer (10 mL per 100 cm^2 membrane; **Table of Materials**) in a hybridization tube and agitate at 60 °C in a hybridization oven.

6.2.6. Put the membrane into the hybridization tube and incubate for 60 min at 60 °C. Dilute a 5'DIG-labeled DNA probe (final concentration, 50 ng/mL) into the hybridization solution containing prewarmed hybridization buffer.

6.2.7. Discard the prehybridization buffer and immediately replace with prewarmed hybridization solution containing the DIG-labeled probe. Incubate the blot with probe at 60 °C overnight, with gentle agitation.

6.2.8. After hybridization, wash the membrane twice in buffers of increasing stringency at 60 °C for 20 min each. Gently wipe the membrane to remove extra washing buffer and add reagents as suggested in the chemiluminescent hybridization and detection kit (**Table of Materials**).

6.2.9. Detect hybridized signals by chemiluminescent reaction combined with the imaging system.

REPRESENTATIVE RESULTS:

Above, we describe the step-by-step procedure for an improved screening assay for assessing the RSS activity of *P. sojae* RxLR effectors. Altogether, the experiment takes 5−6 weeks. Subsequently, the RSSs identified by the assay can be further characterized in terms of function and molecular mechanism. As an example of our approach, we used the *P. sojae* RxLR effecto Phytophthora suppressor of RNA silencing 1 (PSR1), which is secreted and delivered into host cells through haustoria during infection.

To confirm that PSR1 has RSS activity and is thus suitable for this method, each transformed agrobacterium strain was mixed with a strain harboring 35S-GFP and was infiltrated into leaves of *N. benthamiana* 16c. EV and CMV2b were used as negative and positive controls, respectively (**Figure 1**). GFP expression reached the highest level in leaves infiltrated with all mixtures at 2−3 dpi. Green fluorescence intensity remained strong in patches co-infiltrated with 35S-GFP plus CMV2b during a 6−9-day period of observation[21]. Co-infiltration of GFP with PSR1 also resulted in stronger GFP fluorescence during a 3.5−4.5 day period of observations, as demonstrated by elevated fluorescence in the infiltrated patches. However, the fluorescence intensity in patches infiltrated with 35S-GFP and EV weakened at 3 dpi. At 4−5 dpi, GFP fluorescence was hardly detectable (**Figure 1**).

Northern blot revealed that GFP mRNA accumulated higher in the leaves expressing 35S-GFP plus 35S-CMV2b or 35S-GFP plus 35S-PSR1 than in the leaves expressing 35S-GFP plus EV (**Figure 2**).

Thus, transcriptional expression of PSR1 as well as CMV2b contributed to the stabilization of GFP mRNA, which resulted in elevated GFP fluorescence.

We also used the agro-infiltration assay to evaluate the spread of the silencing signal in the leaves of 2-week-old *N. benthamiana* 16c seedlings. For this purpose, we usually selected 3−4 larger leaves for whole leaves injection. At 14 dpi, more than 98% of the EV exhibited obvious no GFP signaling in systemic leaves, whereas both PSR1 and CMV2b efficiently inhibited the systemic spread of the silencing signal by observing GFP fluorescence in about 80% of co-infiltrated plants, and in the remaining 20% of infiltrated plants with only a few red veins appeared in newly emerged leaves (**Figure 3**).

FIGURE LEGENDS:

Figure 1: *Phytophthora sojae* RxLR effector PSR1 suppresses local RNA silencing in *Nicotiana benthamiana* (*N. benthamiana*) 16c plants. Fully developed leaves of 3−4-week-old *N. benthamiana* 16c plants (left panel) were co-infiltrated in patches with *Agrobacterium* mixtures carrying 35S-GFP and the constructs indicated above each image. GFP fluorescence of the infiltrated area was imaged under natural light (middle panel) and long–wave UV light (right panel) at 4 dpi. The experiment was repeated twice with similar results. Red arrows represent infiltrated leaves.

Figure 2: Accumulation of GFP mRNA in infiltrated *N. benthamiana* 16c leaves. CK and EV represent *N. benthamiana* 16c leaves alone and 16c leaves co-infiltrated with 35S-GFP plus EV. Samples from EV and CMV2b were used as a negative and positive controls, respectively. In addition, rRNA was used as a loading control.

Figure 3: *P. sojae* RxLR effector PSR1 suppresses systemic RNA silencing in *N. benthamiana* 16c plants. Three or four leaves of 2-week-old *N. benthamiana* 16c seedlings (left panel) were transiently co-infiltrated by *Agrobacterium* harboring 35S-GFP and either EV or vector expressing PSR1 or CMV2b from the 35S promoter. GFP fluorescence of newly grown leaves was imaged under natural light (upper panel) and long-wave UV light (lower panel) at 14 dpi. This experiment was repeated twice with similar results. Red arrows indicated infiltrated leaves.

DISCUSSION:

RNA silencing is a key defense mechanism employed by plants to combat viral, bacterial, oomycete, and fungal pathogens. In turn, these microbes have evolved silencing suppressor proteins to counteract antiviral silencing, and these RSSs interfere with different steps of the RNA silencing pathway[22,23]. Several screening assays have been developed to identify RSSs[10].

Here, we describe an improved protocol for screening effector proteins secreted by *P. sojae* into the host cell upon infection for their ability to suppress RNA silencing in host. This modified assay is based on a viral co-infiltration assay but differs in several important ways. First, both bacteria and *N. benthamiana* 16c plants should be vigorous and healthy; this is very important for stable expression of effectors from the bacteria and the use of fully developed *N. benthamiana* 16c ensures experimental reproducibility and reliability. We often use only two or three fully

developed leaves per planta at the vegetative growth stage, the old and newly emerged leaves are unsuitable. Secondly, OD_{600} of the bacterial culture must be adjusted to an optimal value, usually 0.75−1.0 for *Agrobacterium*. A lower OD_{600} (even as low as 0.2) can be used to screen VSRs but not PSRs[24]. Third, the ideal time point for investigating green fluorescence must be optimized for each effector. In virus, leaves co-injected with cultures containing GFP plus putative VSRs exhibit a marked increase in green fluorescence in the infiltrated area at 3 dpi, and the signal remains high until 9 dpi[22], but it was only exhibited at 4 or 5 dpi for PSRs in our present study. Therefore, it is also important to further confirm the RNA silencing activity by quantify the accumulation of GFP mRNA. Finally, it is essential to use the appropriate control. VSRs are not always the ideal positive controls for identifying effector proteins because these proteins have much stronger suppressive activity than PSRs. This could result in failure to detect weak suppressors of RNA silencing.

In this report, we demonstrate the use of our modified assay to screen for suppressors of RNA silencing in *Phytophthora* and *Puccinia graminis* pathogens. Thus, our method represents a useful tool for characterizing potential effectors that encode RSSs, which promote disease susceptibility by inhibiting small RNA accumulation[15-17]. Therefore, we believe that our protocol could be used broadly to screen effectors secreted by plant pathogens. Future work will focus on identifying more RSSs in other pathogens, and on elucidating the role of RNA silencing in plant defense against invading microbes.

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DISCLOSURES:

The authors have nothing to disclose.

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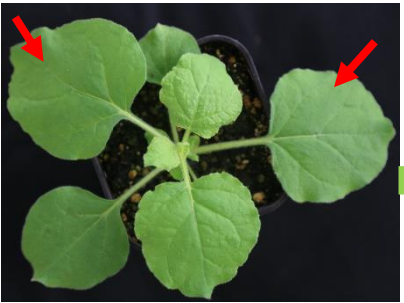
Figure 1



16c

4 dpi

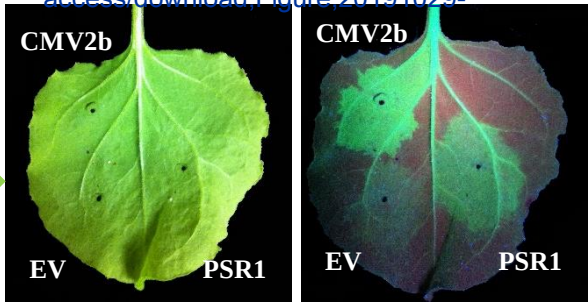


Figure 2

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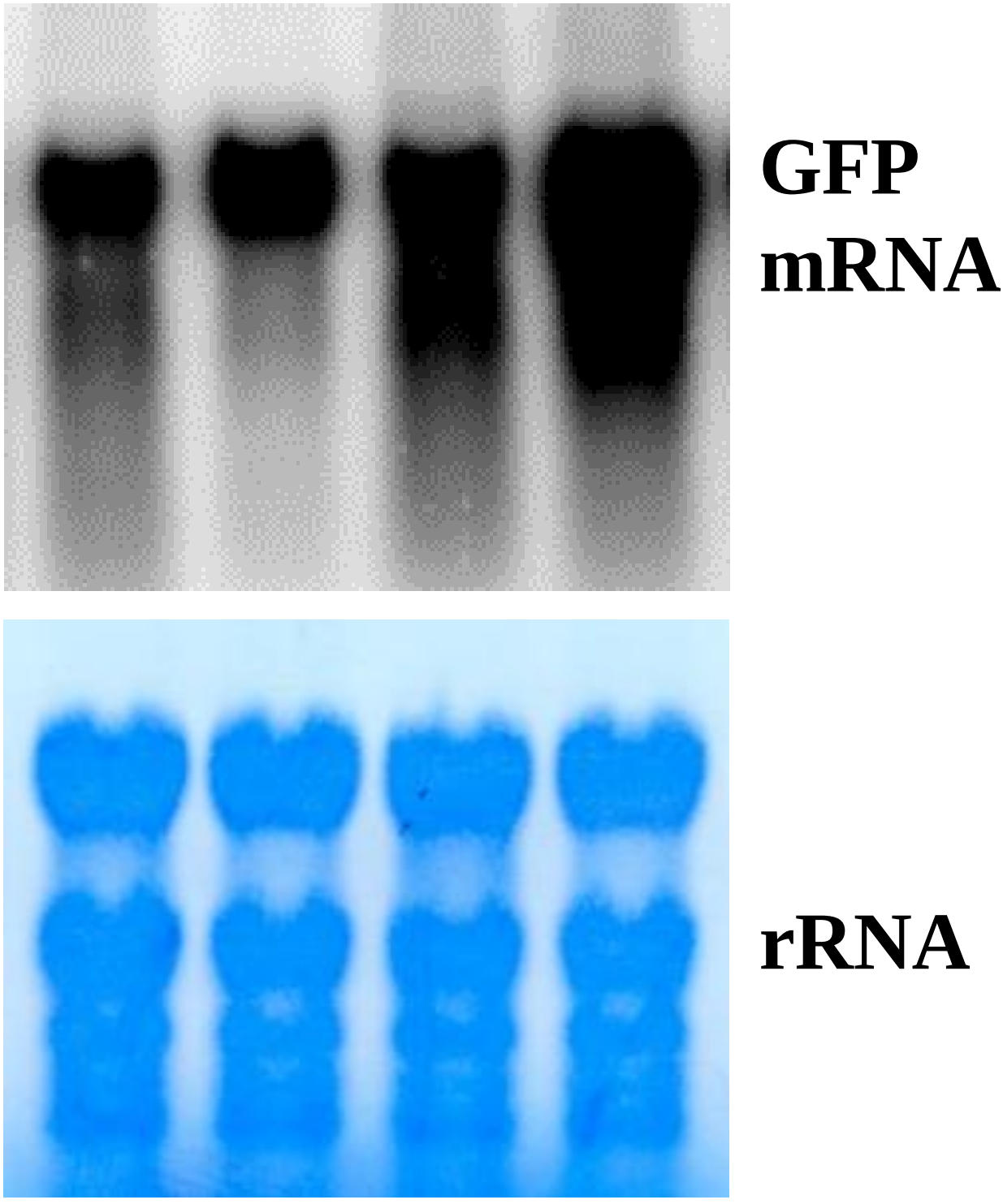

Figure 3

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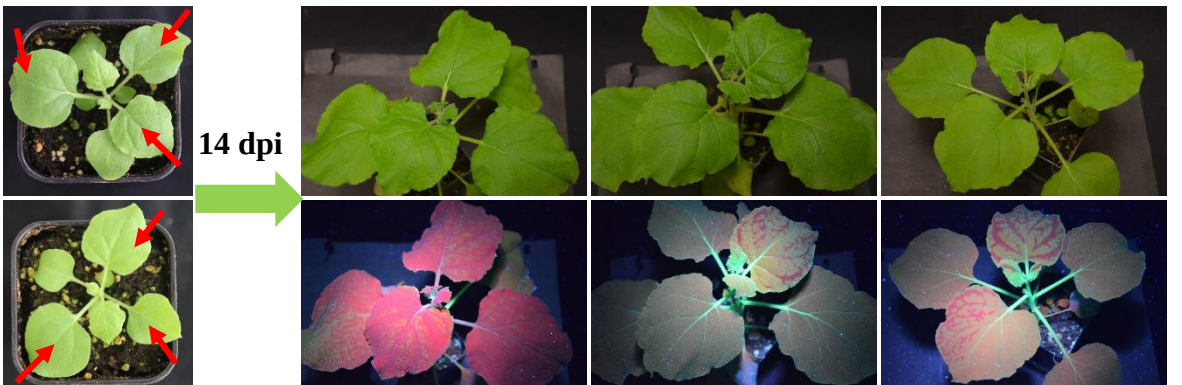

Table of Materials

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Name of Materials	Company	Catalog Number
2-Morpholinoethanesulfonic Acid (MES)	Biofroxx	1086GR500
2xTaq Master Mix	Vazyme Biotech	P112-AA
3-(N-morpholino) propanesulfonic acid (MOPS)	Amresco	0264C507-1KG
Acetosyringone (AS)	Sigma-Aldrich	D134406-5G
Agar	Sigma-Aldrich	A1296-1KG
Agarose	Biofroxx	1110GR100
Amersham Hybond -N+	GE Healthcare	RPN303 B
Bacto Tryptone	BD Biosciences	211705
Bacto Yeast Extraction	BD Biosciences	212750
Chemiluminescent detection module component of dafa kits	Thermo Fisher Scientific	89880
Chloramphenicol	Amresco	0230-100G
ClonExpress II One Step Cloning Kit	Vazyme Biotech	C112-01
DIG Easy Hyb	Sigma-aldrich	11603558001
Easypure Plasmid Miniprep kit	TransGen Biotech	EM101-02
EasyPure Quick Gel Extraction Kit	TransGen Biotech	EG101-02
EDTA disodium salt dihydrate	Amresco /VWR	0105-1KG
FastDigest EcoRV	Thermo Fisher Scientific	FD0304
Gentamycin	Amresco	0304-5G
Kanamycin Sulfate	Sigma-Aldrich	K1914
LR Clonase II enzyme	Invitrogen	11791020
NORTH2south biotin random prime dna labeling kit	Thermo Fisher Scientific	17075
Peat moss	PINDSTRUP	Dark Gold + clay
Peters Water-Soluble Fertilizer	ICE	Peter Professional 20-20-20
Phanta Max Super-Fidelity DNA Polymerase	Vazyme Biotech	P505-d1
Rifampicin	MP Biomedicals	219549005
RNA Gel Loading Dye (2X)	Thermofisher	R0641
Sodium Acetate Hydrate	Amresco /VWR	0530-1KG
Sodium Chloride	Amresco	0241-10KG
Tri-Sodium citrate	Amresco	0101-1KG
Trizol Reagent	Invitrogen	15596018

Name of Equipment:	Company	Catalog Number

Amersham Imager	GE Healthcare	Amersham Imager 600
Camera	Nikon	D5100
ChemiDoc MP Imaging System	Bio-Rad	
Electrophoresis Power Supply	LiuYi	DYY6D
Gel Image System	Tanon	Tanon3500
Nitrocellulose Blotting membrane 0.45um	GE Healthcare	10600002
PCR Thermal Cyclers	Bio-Rad	T100
UV lamp	Analytik Jena	UVP B-100AP
UVP Hybrilinker Oven	Analytik Jena	OV2000

Comments/Description
Buffer
PCR
MOPS Buffer
Induction of Agrobacterium
LB medium
Electrophoresis
Nothern blot
LB medium
LB medium
Luminescence detection
Antibiotics
Ligation
Hybridization buffer
Plasmid Extraction
Gel Extraction
MOPS Buffer
Vector digestion
Antibiotics
Antibiotics
LR reaction
Biotin labeling
Plants
Fertilizer
Enzyme
Antibiotics
RNA Gel Loading Dye
MOPS Buffer
LB medium
SSC Buffer
RNA isolation reagent

Comments/Description

Image
Photography

Nucleic acid electrophoresis.
Image
Northern
PCR
Observation
Northern

Click here to access/download;Rebuttal Letter;20191029 JoVE60697-Response to reviewers comments-R1.docx

Point by point response to the reviewers' reports (JoVE60697)

We greatly appreciate the careful review and highly constructive comments from the editor and the reviewers. All the comments were fully considered and appropriate revisions have been modified accordingly. Two revised versions with tracked changes and a clean copy were submitted. We believe that all the comments have been addressed sufficiently all the comments and the revised manuscript is greatly improved than the original submission.

Please find below detailed point-by-point responses to the reviewers' comments concerned by the editorial and the reviewers. The comments are indicated deep blue and normal fonts.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

>>> Thank you very much for giving us the opportunity to revise our manuscript! As suggested, we have thoroughly revised the main text and figure legends by two native English speakers and a Bioediting service (https://www.bioedit.com/)

2. Please revise lines 65-69, 252-255, and 308-309 to avoid textual overlap with previously published work.

>>> Thank you very much for the suggestions and reminders! We have modified all these three parts in our revised manuscript as following

"In recent years, putative effectors from plant pathogens have been shown to disrupt plant innate immunity by impeding host cellular processes to benefit the microbes in a variety of ways, including dysregulation of signaling pathways, transcription, intracellular transport, cytoskeleton stability, vesicle trafficking, and RNA silencing 3-5. However, the vast majority of pathogen effectors, particularly those from filamentous pathogens, have remained enigmatic." In line 56-61;

"Northern blot revealed that GFP mRNA accumulated higher in the leaves expressing 35S-GFP plus 35S-CMV2b or 35S-GFP plus 35S-PSR1 than in the leaves expressing 35S-GFP plus EV (Figure 2). Thus, transcriptional expression of PSR1 as well as CMV2b contributed to the stabilization of GFP mRNA, which resulted in elevated GFP fluorescence" in line 271-274.

"In virus, leaves co-injected with cultures containing GFP plus putative VSRs exhibit a marked increase in green fluorescence in the infiltrated area at 3 dpi" in line 333-334. And also previous publications were recited as references.

3. Please provide an institutional email for each author if possible.

>>> As suggested, we provided institutional email addresses for three of six authors.

4. Please define acronyms/abbreviations upon first use in the main text.

>>> Thank you for the suggestions! We have thoroughly defined acronyms/abbreviations upon first use in the main text, abstract and figure legend.

5. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u and abbreviate liters to L (L, mL, µL) to avoid confusion.

>>> Thank you for the comments! We have carefully corrected all units according to your suggestions.

6. Please include a space between all numbers and the corresponding unit: 15 mL, 5 g, 7 cm, 37 °C, 60 s, 24 h, etc.

>>> Thanks a lot for your suggestions! We have thoroughly included a space between numbers and units.

7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: ClonExpress II One Step, Vazyme Biotech Co., Ltd., Invitrogen, Kim wipes, Black Ray, Nikon, Eppendorf, TRIzol, HybondTM, ChemiDoc™, GE Healthcare, etc.

>>> As suggested, we have removed all the commercial language in our revised manuscript.

8. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

>>> Thank you very much for your careful review ！We have mended personal pronouns in our main text as your suggestion,.

9. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

>>> As suggested, we have separated the tense in protocol and notes and also moved the discussion

about the protocol to the Discussion.

10. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

>>> Thanks for your advice! We have fully modified our manuscript including the protocol steps and added more details and references in protocol steps.

11. 1.3: Please write the text in the imperative tense or include it as a "NOTE".

>>> Thank you for the constructive comments! As suggested, we have thoroughly modified our main text.

12. 1.4: Please describe how to identify positive transformants or provide a relevant reference here.

>>> Thank you for the suggestions! Colony PCR is a basic technique for identifying the positive transformants. Therefore, we also provided a relevant reference as below in the main text.

Woodman, M. E., Savage, C. R., Arnold, W. K. & Stevenson, B. Direct PCR of Intact Bacteria (Colony PCR). Current Protocols in Microbiology. 42 A 3D 1-7, doi:10.1002/cpmc.14, (2016)

13. 1.5: Please describe how the heat shock approach is done.

>>> As suggested, we have described the heat shock approach in protocol step 1.5 in details as" Introduce 50-100 ng recombinant plasmids carrying effector protein into 100 µL chemically competent A. tumefaciens cells (e.g., GV3101 or C58C1), mix gently, and then freeze in liquid nitrogen for 1 min, thaw the cells in a 37 °C water bath for 5 min" in line 124-126.

14. 2.1: How much soil is needed?

>>> We do not use soil for planting. The potting mixes include 30% vermiculite, 20% perlite, and 50% Pindstrup peat moss, which were purchased from commercial accompany (https://www.pindstrup.com/professional/product-details/pindstrup-dark-gold-+-clay).

15. 2.2: Please specify the composition of the plant fertilizer solution. If it is purchased, include the product in the Table of Materials. Specify the size/mass of the smaller pots and the size of the tray.

>>> We purchased the Peters Water-Soluble Fertilizer from the ICL Company as following link below. This link was also added in the revised manuscript.

https://icl-sf.com/us-en/products/ornamental_horticulture/peters-professional-20-20-20-general-purpose-g99290/

The size of smaller pots and tray were descripted in revised manuscript as "smaller pots (80 mm*80

mm*75 mm) stored in a larger tray (540 mm*285 mm*60 mm)" in line 143-144.

16. 2.5: How long does it take for the seeds to geminate?

>>> Usually it will take 3-4 days for seeds germination. We also put it in the revised manuscript in protocol 2.5..

17. 2.6: What amount of water is considered to be proper?

>>> Thank you for the comments! We have indicated how to water the plants in our revised manuscript as: "Add an appropriate amount of water, keeping soil moist but not soaking, every 2–3 days" in line 152. Actually, it is hard to say the exact amount of water. It is based on the growth environment and growth stage of plants. Normally, plants are watered only when the soil is kind of dry by touching the soil surface.

18. 3.3: What volume of buffer is used to suspend the pellet?

>>> As suggested, we have changed the sentence into "re-suspend the pellet in 2 mL of 10 mM MgCl2 buffer" in line 170-171.

19. 3.4: What amount is considered to be appropriate?

>>> As suggested, we have changed it into "by adding 2 mL of 10 mM MgCl2 buffer" in line 172-173.

20. 4.1: Please specify the volume used.

>>> Here it is hard to specify the exact volume of agrobacterium suspensions for infiltration since it is based whether the leaves are well-developed, are watered and so on, Normally, we will prepare excess cell suspension. To answer this question, Note 3 was added in line 194-195 as "Normally 1 mL agrobacterium suspensions per leaf is at least needed for infiltration. For systemic silencing, at least two leaves will be required for infiltration."

21. 4.2: Please provide specific details. For instance, what volume of the suspension is used to infiltrate?

>>> Thank you for the constructive comments! We have put the description about infiltration volume in note 3 in line 194-195.

22. 4.3: How to label the margin and with what?

>>> Thank you for the constructive comments! We have modified text in our revised manuscript as "circle the margins of the infiltrated patches with a maker pen." in line 187.

23. 5.1: Please describe how this is done. Are leaves collected or is the plant directly observed under a lamp?

>>> Thank you for the constructive comments! We have carefully modified text as "Visually detect GFP fluorescence in newly grown leaves of whole plants 2 weeks post-infiltration (for systemic RNA

silencing) or infiltrated patches of leaves 3-4 dpi using a long-wave UV lamp without leaves collection." In line 198-200.

24. 6.2.6: Please describe how this is actually done.

>>> Thank you for your suggestion! We have carefully modified text as "Put the membrane into hybridization tube and incubate for 60 min at 60 °C. Dilute a 5'Dig-labeled DNA probe (final concentration, 50 ng/ml) into the hybridization solution. Put the membrane into hybridization tube and incubate for 60 min at 60 °C. Dilute a 5'Dig-labeled DNA probe (final concentration, 50 ng/ml) into the hybridization solution with prewarmed DIG Easy Hyb." in line 240-242.

25. 6.1.3, 6.1.5, etc.: Please list all centrifugation speeds in terms of centrifugal g-force instead of rpm: 100 x g.

>>> Thank you for your suggestion! We have changed "rpm" to "g" in the revised text.

26. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

>>> Thank you for your suggestion! We have combined some of the shorter Protocol steps in the revised manuscript.

27. Please include single line spacing between each numbered step or note in the protocol.

>>> Thank you for the constructive comments! We have carefully checked and modified line spacing of main text in our revised manuscript.

28. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

>>> Thank you very much for the suggestions and reminders! Please see another file for highlighted text.

29. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

>>> Thank you very much for the suggestions and reminders! Please see another file for highlighted text.

30. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in

steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

>>> Thank you very much for the suggestions and reminders! We have highlighted the step as your requirement.

31. References: Please do not abbreviate journal titles; use full journal name.

>>> As suggested, we have corrected abbreviate journal titles into full journal titles in our revised manuscript.

32. Figure 2: Please mark the fragment sizes.

>>> This is Northern blot result. Normally it is not necessary to use marker to label the size of RNAs in Northern blot since only the bands that is relevant with probes are shown on the membrane.

33. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

>>> Thank you very much for the suggestions and reminders! We have put all relevant materials in Table of Materials.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this work, the authors described an optimized protocol that using the co-agroinfiltration assays to observe RNA silencing and identify plant pathogen effectors with RNA silencing ability. The co-infiltration of empty vector (EV) and cucumber mosaic virus suppressor 2b (CMV2b) in the Nicotiana benthamiana 16c leaves with reporter gene GFP were used as negative and positive controls. The experiments are well established and the conclusions are justified by the data provided. The MS is well written, and the protocol is clear and easy to follow. The key steps of the protocol are clear and useful tips are provided.

>>> Thank you very much for the careful review! We really appreciate your encouraging and excellent suggestion.

Reviewer #2:

Manuscript Summary:

The manuscript entitled 'Screening and identification of RNA silencing suppressor in plant pathogens secreted effectors' describes a method to screen for effector proteins that have an inhibitory effect on host RNA silencing. The method that is used is like the one described by Qiao et al (2013) (cited in this submission as reference number 15).

Major Concerns:

Because the method and results that are described in this submission look very similar to the results and methods described by Qiao et al (2013), I believe that the main value of the manuscript is the step-by-step explanation of how to carry out the experiment. However, I feel that important details are missing from the steps. If the authors think that these steps are obvious and that it is not necessary to include these details, then I think that these steps should be briefly explained in the introduction and not be included as individual steps of the protocol. Just to give some examples from the steps from start of the protocol description (the rest of the protocol should also be checked):

>>> Thank you very much for the highly constructive comments! We really appreciate your encouraging and careful review. We have carefully addressed your concerns and thoroughly revised our manuscript, which greatly improved our manuscript. Hope you will satisfy the revised manuscript!

Steps:

1.1: This is a general description of how effectors can be identified and not a detailed description of something that can be repeated following the explanation that is given.

>>> Thank you very much for the careful review! The objective of this protocol is to screen the effectors with RNA silencing suppression activity in different plant pathogens. Therefore, we showed the general description here. Actually, we also modified the main text in the revised manuscript to make this step more specific.

1.3: No details on how the cDNA is cloned in these vectors are given. I suppose it is according to the instruction by the manufacturer of the kit, but I think the point of this protocol is to explain the individual steps (how was cDNA made, how much cDNA was used as a template in the subsequent PCR, how was the PCR performed, how were the PCR fragments cloned in the vector, how was the vector transformed to E. coli (which cells were used) etc.)

>>> Thank you for this excellent suggestion! Since we have put the manufacturer and code number of PCR and cloning kit in Table of Materials, it is convenient to find more details about PCR and cloning steps in the instruction from manufacturer's website.

1.4.: The list of material does not specify how much of each of the ingredients I should use in Luria-Bertani broth. How was the colony-PCR done?

>>> Thank you for your suggestions! The ingredients of Luria-Bertani broth have been put into the list of material. And Colony PCR is a basic technique in the lab and we also cited one relevant reference for the colony PCR as following:

Woodman, M. E., Savage, C. R., Arnold, W. K. & Stevenson, B. Direct PCR of Intact Bacteria (Colony PCR). Current Protocols in Microbiology. 42 A 3D 1-7, doi:10.1002/cpmc.14, (2016)

1.5: How was the heat-shock performed (how much cells/DNA were used, what were the settings of the machine). Should I not let the transformed cells recover before plating them out? (step 1.6)

>>> Thank you very much for the careful review！ As suggested, we have carefully modified the main text in the revised manuscript (step 1.5 and 1.6).

2.6: How do I know when the plants are ready for use? In line 316-318, the authors stress the fact that the selection of plants and leaves is critical for the assay. The authors could give a better description of what the plants should look like in a note or include some pictures.

>>> Thank you for your excellent suggestions! As suggested, we have carefully modified the main text and provided one note in line 160-161 as" Plant growth conditions and facilities vary across laboratories; please choose healthy, well-developed and fully expanded leaves for infiltration." in the revised manuscript

2.7: Are the authors referring to the age of the leaf or the age of the plant (if it is the age of the leaf, how old should the plant be?)

>>> Thank you very much for the careful review! Our protocol indicated the age of plant. For local RNA silencing, 3-4 week old plants were used; for systemic silencing, 2 week plants were used.

3.4: What is an appropriate amount of buffer?

>>> Thank you for your suggestions! We have carefully modified the main text as "2 mL of 10 mM $MgCl_2$ buffer" in line 172-173.

3.5: How much is the culture diluted before measuring the OD600?

>>> Thank you for your suggestions! We have carefully modified the main text as "with 2 mL of 10mM $MgCl_2$ buffer-diluted agrobacterium cultures" in line 172-173.

4.2: I suppose the abaxial side of the leaf is meant

>>> Yes, we have modified it in line 184.

4.4: Put back? It was never mentioned that the plants were removed from the growth chamber

>>> Thank you very much for the careful review. We have carefully modified it into "Leave the infiltrated plants in the growth chamber under the same growth conditions" in line 188.

The writing needs to be improved. While it is most of the time clear to me what the authors mean, many sentences contain inaccuracies, important words are sometimes omitted, and singular/plural are often used incorrectly. To give a few examples, I include some remarks on only the first page, but I think the whole manuscript should be checked critically:

>>> We agreed with reviewer's comment and suggestion, and modified the description in the revised manuscript. The writing was also been improved by two native English speakers and a Bioediting service (https://www.bioedit.com/).

- 28: I think the authors meant to use plural: suppressors (also in the title)

>>> Thank you very much for the careful review! We have corrected "suppressor" into "suppressors" in the revised text.

- 32: 'Counter-defense'?: Why use counter? It was not mentioned in the previous sentence that RNA silencing is a defense strategy

>>> Thank you very much for the careful review! We have modified the main text in the revised manuscript as "Several plant pathogens have evolved proteins with the ability to inhibit the host plant RNA silencing pathway." in line 7-8.

- 34: ' unlike virus' : this is not a complete sub sentence and a new sentences should start with a capital

>>> Thank you very much for the careful review! We have modified into "Unlike virus-expressed proteins "in line 8 in the revised manuscript.

- 36: Use of 'nevertheless': I do not see a contrast with the previous sentence

>>> Thank you very much for the careful review! We have changed it into "and" in line 10.

- 38: use of 'would' is not appropriate

>>> Thank you very much for the careful review! We have changed it into "could" in line 11.

- 39: 'secreted' should be 'are secreted'. What is the function of the word respectively here? 'the healthy' should be 'healthy'

>>> Thank you very much for the careful review! We have fully modified this part in line 11-14.

- 40: 'full-developed' should be 'fully developed'

>>> Thank you very much for the careful review! We have corrected into "fully developed "..

- 44: The authors should clarify as a starting point for what. (For investigating the role of candidate effectors in suppressing RNA silencing?)

>>> Thank you very much for the careful review! We have modified the main text in the revised manuscript.

Minor Concerns:

In the introduction, I miss some background on the mechanism through which GFP-silencing is induced upon agroinfiltration. I understand that it is based on (transgenic GFP) N. benthamiana 16c,

which constitutively expresses GFP under control of the 35S promoter. I believe that these plants were first used by Ruiz et al (1998) to investigate VIGS and that GFP is silenced because a double stranded GFP-RNA is formed as a viral replication intermediate. 16c plants are also used by Johansen et al 2001 (referenced in the introduction), but here GFP-silencing is induced by an agrobacterium strain that carries a hairpin-GFP construct. Qiao et al (2013) used a construct for overexpression of GFP, like the authors from this protocol. Could the authors explain how this overexpression leads to silencing and why they use this system? Some of this background is given in the first paragraph of the discussion (lines 300-311), but it would be more appropriate to move this to the introduction.

>>> Thank you very much for the careful review!

Firstly, hairpin-GFP construct was NOT used in both this protocol and Qiao *et al* (2013). The constitutively expression of GFP protein under the control of 35S promoter was applied in (transgenic GFP) *N. benthamiana* 16c plants.

Secondly, actually specific mechanism of GFP silencing in 16c plants is not clear right now. We inferred that the mechanism might be similar to RNA silencing mechanism in virus, which suggested that a double strand GFP fragment might be formed by endogenous and exogenous GFP and then triggered GFP gene silencing. Nevertheless, we are not sure whether it is true or not.

Thirdly, as suggested, we have moved some of the background (line 77-86) in the discussion section to the introduction in the revised manuscript.

The inhibitory effect of CMV2b and PSR1, used as 'representative results' of the current manuscript, is also tested in the publication by Qiao et al (2013). There is some overlap between the authors from that publication and the authors that submitted the current manuscript, so I do not expect there to be a problem with publishing similar results. However, it would still be good to clearly state that the inhibitory effect of PSR1 on host RNA silencing was already previously tested using a similar method by Qiao et al (2013). The authors speak of an improved or optimized protocol (line 42, 87, 234 and 313), but it is not clear to me what improvements were made to the method described by Qiao et al. (or another method). This needs further explanation.

>>> Thank you very much for the careful review!

OD value, infiltrated way and plants conditions have been improved in this assay.

Firstly, the final OD value for infiltration has changed from 0.5 to 0.8-0.9, the higher OD shows much more obvious phenotype under UV light;

Secondly, all combinations including negative control, positive control and tested proteins were infiltrated in one leaf as different patches, which can clearly compare the florescence of GFP protein in the (transgenic GFP) *N. benthamiana* 16c leaves.

Thirdly, the conditions of plant and leaf are very important. Normally, plants with at least five fully

developed true leaves and no visible axillary and flower buds showing a healthy green appearance is good for infiltration, The fully development leaves is always ideal for silencing suppressor screening. Otherwise, it is hard to get the consistent results for biological replicates.

Nevertheless, screening assay in previous publication by Qiao et al. (2013) just simply followed the protocol for VSR.

Some comments on the notes:

- The first note starting on line 97 mentions that all materials and reagents should be sterile unless indicated, but I find no such indications in the protocol. I am sure that many of the materials and reagents used do not need to be sterile.

>>> Thank you for your suggestions! We have removed this description in the revised manuscript.

- The note on line 122 states that all effectors are cloned from genomic DNA and not cDNA. Then why does the protocol mention that effectors should be cloned from cDNA? Why is the gateway vector without tag recommended?

>>> Thank you for your suggestions! Since most of effector genes in plant pathogens do not include intron which is the characteristic of effectors, we can use DNA as PCR template. Otherwise, we need use cDNA as PCR template.

Sometimes, the vector with tags showed weaker phenotype in screening assay. We guess that the tag might influence protein expression in plants (our experience). Therefore, the vector with tag was not recommended.

- line 178: The use of 'should have' implies that it was not done in this way. I do not think that is what the authors mean to say.

>>> Thank you very much for the careful review! We have corrected into “should be” in the revised manuscript.

The statements that are made in the discussion remain a little vague. On line 326, the authors make a remark about the 'present study', but they refer to other papers. The authors state that the suppressive effect of VSR on RNA silencing is stronger than the effect of PSR(1?) (line 324-330). This is based on a visual examination of GFP fluorescence and GFP mRNA accumulation. Would it be possible to quantify this effect? This would make the results more robust and I think this would be useful when this method is used for screening purposes.

>>> Thank you for your excellent suggestions! The reference in line 326 (line 335 in current text) have been deleted. The stronger RNA silencing is based on a visual examination of GFP florescence. We completely agreed with reviewer’s advice, this would be useful when this method is used for

screening purposes.

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