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Full Length Article

Identification of an RNA Silencing Suppressor Encoded by *Lily Symptomless Virus*

Pinsan Xu[1,2], Changwei Zhao[3] and Zhengyao Zhang[2*]
[1]*School of Life Science and Biotechnology, Dalian University of Technology, Dalian Liaoning 116024, China*
[2]*School of Life Science and Medicine, Dalian University of Technology, Panjin Liaoning 124221, China*
[3]*Department of Orthopedics, Changchun University of Chinese Medicine, Changchen Jilin 130021, China*
[*]For correspondence: zhengyaozhang@dlut.edu.cn; abs@academicconf.com

Abstract

Lily symptomless virus (LSV) is one of the major viral diseases affecting lilies. Many plants have an innate antiviral response that includes viral RNA silencing. To counter this pathway, plant viruses have developed RNA silencing suppressor proteins. However, it is not known whether LSV encodes an RNA silencing suppressor. This study was undertaken to characterize the function of LSV movement protein TGB1 and unknown protein 16kDa and to determine whether LSV encodes an RNA silencing suppressor protein. A viral GFP expression system was used to determine whether16 kDa was able to inhibit RNA silencing, inhibit local and systemic GFP silencing and reverse systemic RNA silencing. However, 16 kDa did not inhibit local GFP silencing triggered by dsRNA in an agrobacterium co-infection assay in transgenic *Nicotiana benthamiana* (16c) carrying GFP. TGB1 did not inhibit gene silencing and appeared to function only as a movement protein. Neither 16 kDa nor TGB1 acted as a virulence factor in N. *benthamiana*is infected with PVX. Results further showed that 16 kDa and TGB1 localized to the nucleus and cytomembrane. This is the first report to show that LSV 16 kDa plays a critical role in RNA silencing suppression is not a virulence factor and has a clear subcellularlocalization pattern. Future studies are required to fully elucidate the function of 16 kDa in antiviral response in plants. © 2017 Friends Science Publishers

Keywords: Lily symptomless virus; TGB1; RNA silencing suppressor; Subcellular localization

Introduction

RNA silencing that selectively silences viral RNA is an innate host response in higher plants. Double-stranded RNAs (dsRNA) produced during viral replication potentially trigger virus-induced RNA silencing. The dsRNA molecules are cleaved into 2125 nucleotide double-stranded fragments called small interfering RNAs (siRNAs) by Dicer an RNase III-type endonuclease. The siRNAs are incorporated into RNA-induced silencing complex (RISC), which contains the protein AGO and provides a template for identifying viral mRNA with the same sequence as the incorporated siRNA (Baulcombe, 2004). Plant viruses have evolved numerous proteins to counter the RISC complex and suppress RNA silencing at different stages. The first RNA silencing suppressor was first identified in a plant virus in 1998 (Anandalakshmi *et al.*, 1998). Since then, almost 40 different RNA silencing suppressors have been found in a wide array of viruses (Guo *et al.*, 2013; Mann *et al.*, 2016). While many plant viruses contain one or more RNA silencing suppressors the structure and mechanism of action are not widely conserved amongst viruses (Zhao *et al.*, 2016). For example, viral RNA silencing suppressors have been shown to prevent accumulation of siRNA (*Potato virus Y* [PVY] (Wu *et al.*, 2010) suppress transfer of the silencing signal (*Cucumber mosaic virus* [CMV] (Xu *et al.*, 2013) interact with Dicer to disrupt shearing of the dsRNA (*Rice stripe virus* [RSV] (Park *et al.*, 2012) and interact with small RNAs that bind to AGO4 thereby inhibiting AGO4 activity (CMV; Hamera *et al.*, 2012). One commonality required is that the viral RNA suppressor proteins must be localized to the nucleus (Mayers *et al.*, 2000). Therefore, describing the subcellular localization of a putative RNA silencing suppressor protein can inform our understanding of how it interacts with the host.

Lily symptomless virus (LSV) a member of the carnation latent virus genus is one of the primary pathogens that infect lilies. It was first isolated in Oregon (USA) from a (*Lilium longiflorum*) that contained connecrotic-flecks (Brierley and Smith, 1944). LSV encodes six proteins in open reading frames (ORFs) 1–6. ORF1 encodes an RNA-dependent RNA polymerase (220 kDa) ORF4 encodes movement proteins (25, 12 and 7 kDa), which are related to the triple gene block 1–3 (TGB1–3) proteins that regulate the movement of virus in the host ORF5 encodes coat protein and ORF6 encodes an unknown 16 kDa protein. The 16kDa is a nucleotide-binding protein also referred to cysteine-rich protein (CRP), which belongs to the Carla-C_4 family is related to viral transcription (Cavileer *et al.*, 1994).

There is evidence to support two of the LSV encoded proteins TGB1 and 16 kDa act as viral RNA silencing

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suppressors. TGB1 is a multifunctional protein that has RNA helicase and nucleic acid hydrolase activities and contains both ATP-binding and nucleic acid binding domains. TGB1 serves as a guide for virus movement within the host (Tilsner *et al.*, 2012). In the potato virus X (PVX) system P25 (25 kDa) is the primary protein in the triple gene block complex, which is an RNA silencing suppressor and TGB1 is located in cell nucleus (Aguilar *et al.*, 2015). These properties are similar to LSV TGB1 (25 kDa) suggesting similar functions. Many plant viruses including tobacco rattle virus (TRV) Chinese wheat mosaic virus (CWMV) and sweet potato virus contain RNA silencing suppressors that have cysteine residues (analogous to LSV 16 kDa), which may become zinc fingers and relate to nucleic acid binding (Liu *et al.*, 2002; Bragg and Jackson, 2004; Deng *et al.*, 2015). Thus, there is preliminary support for the hypothesis that LSV TGB1 and LSV 16 kDa may function as RNA silencing suppressors.

Given the importance of RNA silencing in plant host defense, there is a need to better understand whether LSV contains putative RNA silencing suppressor proteins. Therefore, this study was undertaken to understand the effects of LSV TGB1 and 16 kDa on RNA silencing in an Agrobacterium transformation system to understand the mechanism of action and to determine the effects of LSV TGB1 and 16 kDa on LSV pathogenicity.

Materials and Methods

Materials

Tobacco (*Nicotiana benthamiana* Domin; 16c) *Agrobacterium tumefaciens* GV3101 and PVX (pGR107) were kindly provided by Professor David Baulcombe from the University of Cambridge (United Kingdom). The plant expression vector carrying *Cymbidium ringspot virus* (CymRSV) P19 (35S-P19) the dsGFP vector containing a GFP inverted repeat sequence and pGR107 carrying *Rice stripe virus* (RSV) NS3 were kindly provided by Professor Zhou Xueping from Zhejiang University. The PTF101.1 binary vector carrying a 35S promoter was kindly provided by Professor Li Wenli from Dalian University of Technology. Collected lily strain carrying LSV in Dalian was used to amplify the LSV 16 kDa and TGB1 ORFs. The tobacco plants used in these studies were propagated via tissue culture system and then transplanted. Follow-up testing was performed when the tobacco plants had grown 5–7 leaves.

Generation of Expression Constructs

The ORFs for LSV TGB1 and 16kDa were each cloned into an pMD18^{TM}-T simple vector. Once the sequence was confirmed they were cloned into the 35S-pTF101.1 vector and used to construct recombinant expression vectors termed 35S-TGB1 and 35S-16kDa. The codon following the initiation codon was mutated to a termination codon to produce the 35S-16kDa△ construct which is transcribed normally but is not translated. Agrobacterium (GV3101) was transformed with previous recombination plasmid then infected with *N. benthamiana* 16c. To determine pathogenicity of the target gene TGB1 and 16kDa were each cloned into the binary expression vector pGR107, which contains the PVX infectious sequence and used to construct the recombinant expression vectors pGR107-16kDa and pGR107-TGB1. Agrobacterium (GV3101) was transformed with the above recombination plasmid then infected with *N. benthamiana*.

Agrobacterium Infection Method

A tumefaciens (GV3101) was transformed with the recombinant constructs in YEB culture medium containing the corresponding antibiotics. The cultures were grown in suspension at 28°C suspension culture until reaching A_{600} = 0.5. Then the bacteria cultures were centrifuged to collect the bacteria. The pellet was suspended in penetrating fluid containing acetosyringone and kept at room temperature for 3 h. *A. tumefaciens* (GV3101) at an optical density (OD) of 1.0 was mixed and used to infect tobacco plants. GFP fluorescence was observed under ultraviolet light and pictures were recorded using a Sony digital camera.

Real time PCR and Western Blot

Total RNA was extracted from the leaves of the infected plants and real time PCR was used to quantify the levels of GFP mRNA using SYBR Green (Karsai *et al.*, 2002). The PCR primer sequences and restriction enzyme cutting sites are given in Table 1. The experiment was performed in triplicate. Total protein was extracted from the infected leaves to quantify protein expression by Western blot as previously described (Xiong *et al.*, 2009). A rabbit-anti-GFP monoclonal antibody was used as the primary antibody and a goat anti-rabbit alkaline phosphatase-conjugated polyclonal antibody was used as the secondary antibody. Immuno-stained bands were detected using a chromogenic substrate.

Subcellular Localization

Subcellular localization was determined using GFP fused constructs of TGB1 and 16 kDa. The constructs for 16kDa-GFP and TGB1-GFP were each inserted into the 35S-pTF101.1 vector, which contains a 35S promoter and transferred into Agrobacterium (GV3101). Onion epidermal cells were infected with the following Agrobacterium constructs: pTF-GFP (negative control), pTF-16kDa-GFP, and pTF-TGB1-GFP. Subcellular localization of the GFP expressing protein was observed by fluorescence microscopy. The bacteria were activated overnight and then diluted 1:100 in 50 mL of fresh liquid medium and cultured for 3–4 days at 28℃ with agitation (180 rpm) until reaching an OD600 between 0.5−0.6. Under hypertonic pretreatment conditions chunks of onion were added to the Agrobacterium bacterium suspension and cultured at 28℃ with agitation for 1.52 h. The onion chunks were then transferred to MS solid

Table 1: PCR primer sequences and restriction enzyme cutting sites

Primer	Sequence	Restriction enzyme
pGR107-16kDa-F	CGCCCGGGATGAGCGTCTGGGGAGTCT	Sma I
pGR107-16kDa-R	GCGTCGACTTATTTAGGTCTTAGGTAGAACTGGC	Sal I
pGR107-TGB1-F	CCATCGATATGGATGTTTTACTAAGTTTG	Cla I
pGR107-TGB1-R	TCCCCCGGGTCAGGAGGTGGTGTAAGCGG	Sma I
PTF-GFP-F	CGCGGATCCATGGTGAGCAAGGGC	BamH I
PTF-GFP-R	CGAGCTCTTACTTGTACAGCTCGTC	Sac I
PTF-16kDa-F	CGCGGATCCATGAGCGTCTGGGGAGTCT	BamH I
PTF-16kDa-R	GACTAGTTTATTTAGGTCTTAGGTAGAACTGGC	Spe I
PTF-TGB1-F	CGGGATCCATGGATGTTTTACTAAGTTTG	BamH I
PTF-TGB1-R	CGAGCTCTCAGGAGGTGGTGTAAGCGG	Sac I
PTF-16kDaΔ-F	CGCGGATCCATGTAGGTCTGGGGAGTCT	BamH I
PTF-16kDaΔ-R	GACTAGTTTATTTAGGTCTTAGGTAGAACTGGC	Spe I

medium and cultured in the dark for 24−36 h. The onion skin was torn off and made into a temporary pack piece and observed by fluorescence microscopy. Concurrently the epidermis was placed in MS hypertonic medium (MS + 60 g/L mannitol) for 20 min. When the onion epidermal cells had separated from the wall they were made it into a temporary pack piece and observed under the microscope.

Results

LSV 16 kDa Suppresses Local RNA Silencing in *N.* Benthamiana Line 16c

N. benthamiana line 16c was infected with P19 (positive control), 16kDa, TGB1, pTF101 vector (negative control), or GFP. GFP fluorescence was observed in the infected leaves under UV light. Four (4) dpi strong GFP fluorescence was observed in the P19 and 16kDa infected groups. In contrast there was no GFP fluorescence in the TGB1, 16 kDaΔ and vector plus GFP groups. Similar results were observed at 6 dpi leaves infected with 16 kDa-GFP did not express GFP but the P19-GFP infected leaves were GFP+. These results indicated that LSV 16 kDa was able to suppress RNA silencing triggered by GFP RNA. However 16 kDa was only weakly active and suppression did not last long. No RNA silencing suppression was observed with TGB1. In leaves infected with the 16kDaΔ-GFP construct, which was transcribed but not translated no GFP was observed indicating that 16 kDa-mediated suppression or RNA silencing is mediated by the protein but not mRNA (Fig. 1A).

Total RNA collected from leaves 4 dpi was analyzed for GFP expression by real-time PCR. The expression level of GFP mRNA in the 16 kDa group was slightly lower than the positive control (P19) but the difference was not significant. The levels of GFP mRNA expression in the TGB1, 16kDaΔ and negative control were similar (Fig. 1B). Western blot analysis indicated that there was less GFP protein in the 16 kDa group than the P19 group consistent with the PCR results. There was no GFP protein in the TGB1, 16 kDaΔ and negative control groups (Fig. 1C). Taken together the results suggest that 16 kDa is an RNA silencing suppressor encoded in the LSV genomic RNA.

Suppress of Systemic RNA Silencing by LSV 16kDa

When local silencing is initiated, some of the silencing signals can spread throughout the leaves and induce systemic silencing. To understand whether 16 kDa suppresses systemic RNA silencing *N. benthamiana* line 16c was infected with P19 (positive control), 16kDa, or vector (negative control) plus GFP. Systemic GFP silencing was observed by 14 dpi and visualized with UV illumination. By 14 dpi the upper leaves of most of the plants infected with vector plus GFP became chlorotic. GFP fluorescence was also observed in the majority of the *N. benthamiana* line 16c cells infected with P19 plus GFP or 16kDa plus GFP .The proportion of systemic silencing suppressed by 16 kDa was lower than the positive control but higher than the negative control. Thus 16 kDa suppresses systemic RNA silencing more than the negative control (pTF101 vector) but is less potent than P19 (positive control) (Fig. 2).

Non-Suppression of Local RNA Silencing Triggered by dsRNA by 16kDa

The dsRNA is a potent inducer of RNA silencing and can quickly lead to RNA silencing. To determine whether 16 kDa can suppress RNA silencing at this level 16 kDa-mediated inhibition of RNA silencing in response to dsRNA was analyzed. *N. benthamiana* was infected with P19 (positive control), 16 kDa or the pTF101 vector encoding 16 kDaΔ plus 35S-GFP plus 35S-dsGFP (inverted repeat GFP sequence). By 3–5 dpi, GFP was observed in leaves infected with P19 plus GFP but not in leaves infected with in contrast GFP was not observed in leaves infected with 16 kDa plus GFP, vector plus GFP or 16 kDaΔ plus GFP. Taken together these results suggest that 16 kDa does not suppress local RNA silencing triggered by dsRNA and may act upstream of the dsRNA trigger to inhibit RNA silencing (Fig. 3).

Reversal of RNA Silencing by pGR107-16 kDa

Next we assessed whether 16 kDa was able to reverse

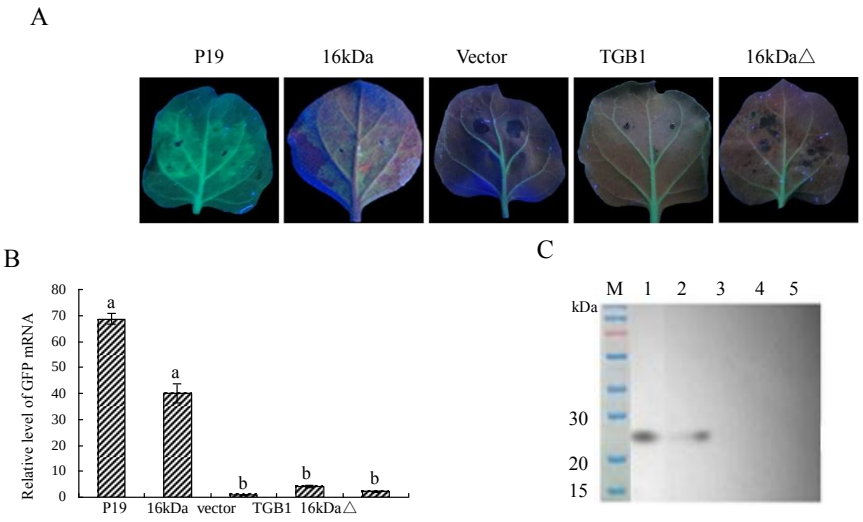

Fig. 1A: Green fluorescent protein (GFP) images of agrobacterium infected leaves from *N. benthamiana (*16c) under UV illumination at 4 days post-infection (dpi). The constructs used for agro-infection are indicated above the images. **(B)** Total RNA was extracted from leaves infected with the indicated agrobacterium. The levels of GFP mRNA expression was assess by real-time PCR analysis at 4 dpi. **(C)** GFP protein levels were assessed by Western blot in leaves triple infected with agrobacterium carrying the constructs indicated at 4dpi. 1:P19, 2:16 kDa, 3: vector, 4:TGB1, 5:16 kDa

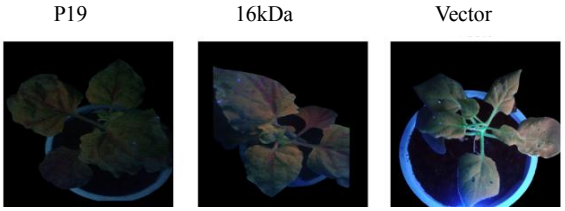

Fig. 2: Systemic RNA silencing suppression activities of Lily symptomless virus (LSV) nucleotide-binding protein (16 kDa). Green fluorescent protein (GFP) images from the upper leaves of *N. benthamiana (*16c) plants infected with 35S-GFP and the indicated constructs at 20 dpi

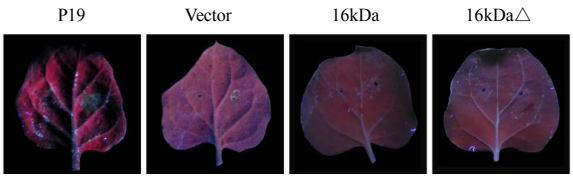

Fig. 3: Lily symptomless virus (LSV) nucleotide-binding protein (16 kDa) does not suppress local RNA silencing triggered by double-stranded RNA (dsRNA). Green fluorescent protein (GFP) images of *N. benthamiana* (16c) leaves infiltrated with 35S-GFP plus 35S-dsGFP plus P19, empty vector, or vector expressing 16 kDa at 4 dpi

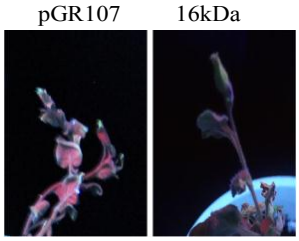

Fig. 4: Lily symptomless virus (LSV) nucleotide-binding protein (16 kDa) can reverse RNA silencing. GFP images from *N. benthamiana (*16c) leaves infected with 35S-GFP plus the pGR107 vector expressing 16 kDa at 40 dpi

Fig. 5: Effects of Lily symptomless virus (LSV) nucleotide-binding protein (16 kDa) and TGB1 on *Potato virus X* (PVX) pathogenicity. Symptoms of PVX infection on the systemic leaves of *N. benthamiana* plants infected with recombinant PVX were photographed at 25 dpi

systemic RNA. RNA silencing was induced in *N. benthamiana* (16c) by 35S-GFP until all of the cells turned red. At that point *N. benthamiana* (16c) cells were infected with pGR107 (negative control) or pGR107-16 kDa and the resulting green fluorescence was observed using a UV lamp. By 30 dpi green fluorescence was observed in new leaves and flower buds at the top of the 16c plants infected with agrobacterium carrying pGR107-16kDa. In contrast, new leaves and flower buds at the top of 16c plants infected with

the negative control agrobacterium remained red under UV illumination. By 40 dpi16 kDa was able to reverse GFP expression in the new leaves and flower buds at the top of the 16c plants, but the rest of the blades remained red. These results suggested that 16 kDa was able to reverse GFP silencing in flower buds but is a relatively weak suppressor and cannot reverse GFP silencing in whole plant (Fig. 4).

Non-Enhancement of Pathogenicity of PVX by 16 kDa and TGB1

To understand whether LSV 16 kDa or TGB1 were virulence factors *N. benthamiana* was infected with pGR107-NS3 (positive control), pGR107 (negative control), pGR107-16 kDa, or pGR107-TGB1 and observed for symptoms of PVX infection. Plants infected with pGR107-NS3 had displayed necrosis by 15 dpi. However, leaves infected with pGR107-16 kDa, pGR107-TGB1 or the pGR107 vector did not develop severe necrosis. These results suggested that 16 kDa and TGB1 are not virulence factors in PVX (Fig. 5).

Subcellular Localization of 16 kDa and TGB1

The subcellular localization of 16 kDa and TGB1 was investigated using confocal laser scanning microscopy to monitor GFP expression from fusion proteins. Onion epidermal cells were transformed with pTF-GFP (negative control), pTF-16 kDa-GFP or pTF-TGB1-GFP and the expression and localization of the fusion protein was observed. GFP was primarily distributed throughout the cytoderm or cytomembrane. In contrast, 16 kDa was distributed throughout the cytoderm and cell nucleus. TGB1 was distributed along the edge of the cytoderm, with some fluorescence embedded in cytoderm, and a few foci distributed in the cytoplasm (Fig. 6).

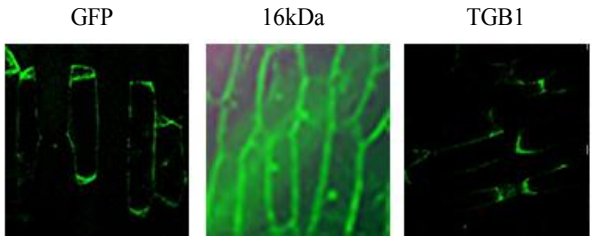

Fig. 6: Subcellular distribution of nucleotide-binding protein (16 kDa) and TGB1. Onion epidermal cells were scanned using confocal laser scanning microscopy to determine the localization of 16 kDa and TGB1 GFP fusion proteins at 48 h post-infection

Discussion

In other models, 16 kDa is usually described as a cysteine-rich protein that functions as an RNA silencing suppressor (Ghazala *et al.*, 2008). The results of this study are consistent with previous descriptions and found that LSV 16 kDa functions as an RNA silencing suppressor. The hypothesized structure and function of 16 kDa encoded by LSV are also consistent with previous reports. LSV TGB1 was not found to have any RNA silencing suppressor function. These results are contradictory to TGB1 encoded by P25, which is an RNA silencing suppressor (Aguilar *et al.*, 2015). Based on our results 16 kDa is the only RNA silencing suppressor in LSV and requires further study.

Agrobacterium-mediated transformation is a quick and effective method of identifying RNA silencing suppressor proteins (Voinnet *et al.*, 2000). The agrobacterium technique was used here to verify that LSV 16 kDa is an RNA silencing suppressor. Transient expression of GFP was slightly lower than the positive control (P19) showing 16 kDa inhibited GFP expression locally. It is possible that 16c may be insensitive to LSV or that 16 kDa is a weak inhibitor of gene silencing. 16 kDa not only inhibited GFP expression, it also able to inhibit systemic silencing and reverse gene silencing. This is consistent with the function of other gene silencing suppressors (Brigneti *et al.*, 1998). In addition, 16 kDa did not inhibit RNA silencing triggered by dsRNA suggesting it may act upstream of dsRNA in the RNA silencing pathways.

Often, a viral RNA silencing suppressor is a virulence factor that contributes to disease symptoms, (e.g., NS3 encoded by RSV and 2b encoded by CMV). In the PVX model, one hypothesis is that a viral RNA silencing suppressor might enhance accumulation of viral RNA by inhibiting RNA silencing and thereby aggravate the symptoms of disease (Díaz-Pendón and Ding, 2008; Xiong *et al.*, 2009). However, outside the system 16 kDa and TGB1 do not enhance the pathogenicity of a heterogeneous expression system suggesting they may not be virulence factors. This result verifies that lily infected with lily symptomless virus does not have obvious symptoms.

The subcellular localization of viral proteins can improve our understanding of viral pathogenesis. RNA silencing suppressors have a nuclear localization signal and localize to the cell nucleus (Dong *et al.*, 2003). Viral RNA suppressors, such as RSV NS3 play important roles in virus infection and spread and localize to the cell nucleus (Mori *et al.*, 2005). Here, 16 kDa localized to the cell nucleus and encoded an RNA silencing suppressor. Movement proteins primarily localize to the plasmodesmata dilate the endoplasm and mediate transfer of viral RNAs (Carrington *et al.*, 1996). TGB1 is located in the cytoderm or cytomembrane in this study, suggesting that TGB1 may be located in the plasmodesmata of the cytoderm.

We are the first to show that LSV 16 kDa has RNA silencing suppressor function and localizes to the cell nucleus. Studying the LSV encoded RNA silencing suppressor can help us to understand the virus host interactions in LSV and how they contributed to pathogenesis. The identification of an RNA silencing suppressor in LSV is also significant for antiviral studies. It is possible that an LSV resistant lily could be obtained by

transforming an RNAi gene construct targeting the RNA silencing suppressor into the lily.

Conclusion

We provide evidence that 16 kDa does not inhibit local RNA silencing triggered by dsRNA suggesting it may act early in RNA silencing. The 16 kDa was also able to reverse systemic RNA silencing. However, 16 kDa was not thought to be a virulence factor using a PVX infection model. Both 16 kDa and TGB1 were separately distributed in the cell nucleus and the cytoderm. These results improved our understanding of LSV/host interactions provided evidence for the structure and function of LSV 16kDa and TGB1 and contributed to understand the mechanisms of LSV infection.

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