In the Search of Viral Suppressors of *Prunus* Infecting Viruses

Prunus fajokat fertőző vírusok géncsendesítést gátló fehérjéinek keresése

Vivien Fákó*, Nikoletta Jaksa-Czotter and Éva Várallyay

Hungarian University of Agriculture and Life Sciences, Institute of Plant Protection, Department of Plant Pathology, Genomics Research Group; jaksa-czotter.nikoletta@uni-mate.hu, varallyay.eva@uni-mate.hu

**Correspondence: fako.vivien@phd.uni-mate.hu*

Abstract: Perennial woody plants can be infected by a wide range of viruses and viroids. During infection, the plant's defence system, the RNA interference (RNAi), the highly effective and specific defence reaction of the host, is induced. Throughout their evolution, viruses have developed strategies to block this defence process in various ways. One of these strategies involves the encoding of proteins that function as viral suppressors of RNA interference (VSR). In our study, we examined the potential RNAi silencing suppressor activity of P21 proteins of little cherry virus-1 (LChV-1) from different host plants (cherry and apricot) and of P4 of peach-associated luteovirus (PaLV). Our results revealed that the P21 protein of LChV-1apricot strain displayed systemic VSR activity, whereas the P4 protein of PaLV showed only weak local suppressor activity.

***Keywords**: Velarivirus; Luteovirus; LChV-1; PaLV; viral suppressor; agroinfiltration*

Összefoglalás: Az évelő fásszárú növényeket sokféle vírus és viroid fertőzi. A fertőzés során a növény védekezőrendszere, az RNS interferencia indukálódik, mely igen hatékony és specifikus. A vírusok evolúciójuk során olyan stratégiákat fejlesztettek ki, melyekkel ezt a védekező folyamatot különböző módon képesek blokkolni. Ilyenek, a legtöbb növényt fertőző vírus által kódolt, virális RNS csendesítést gátló, szupresszor fehérjék (VSR.). Kutatásunk célja VSR fehérjék keresése és jellemzése a cseresznye aprógyümölcsűség vírus 1 (little cherry virus-1 - LChV-1) és az őszibarack-asszociált luteovírus (peach-associated luteovirus - PaLV) különböző gazdanövényeket fertőző variánsaiban. Eredményeink alapján megállapítható, hogy a LChV-1 P21 fehérje kajsziból izolált variánsa szisztemikus VSR aktivitást mutatott, míg a PaLV P4 fehérjéje gyengén és csak lokálisan volt képes gátolni az RNS interferenciát.

***Kulcsszavak**: Velarivirus; Luteovirus; LChV-1; PaLV; virális szuppresszor; agroinfiltrálás*

1. Introduction

During viral infection, the plant's defence system, the RNA interference, is induced. It is a conserved, sequence-specific eukaryotic gene regulation mechanism (Covey et al., 1997). During evolution, viruses have evolved different VSR proteins that can block this defence reaction (Csorba et al., 2015). VSRs can inhibit the production or activity of siRNAs in the infected tissue (local silencing), and/or their spread to systemic leaves (systemic silencing) (Burgyán and Havelda, 2011).

LChV-1 is a member of the *Velarivirus* genus in *Closteroviridae* family of plant viruses. It has a single-stranded positive-sense RNA genome of 16–17 kb and includes eight open reading

frames (ORFs). Together with little cherry virus 2 and X-disease phytoplasma, it is associated with the little cherry disease. LChV-1 can infect sweet and sour cherry and other *Prunus* species such as almond, peach, plum and apricot (Baráth et al, 2018). VSR activity of ORF7 of a Greek sweet cherry strain encoding P21 was tested positive previously (Katsiani et al., 2017).

PaLV is a member of the genus *Luteovirus,* formerly belonging to the family *Luteoviridae*, and has recently been re-assigned to the family *Tombusviridae* (Miller & Lozier, 2022). The virus was detected in peach, flat peach and nectarine and described from Hungary (Barath et al., 2018). The PaLV genome is single-stranded positive-sense RNA of 5-6 kb and includes six ORFs (Khalili et al., 2023). Based on previous studies, the P4 protein of barley yellow dwarf virus-PAV (BYDV-PAV), a member of the same genus to which PaLV belongs, acts as a VSR (Fusaro et al., 2017).

The aim of this study was to investigate the possible local and systemic silencing suppressor activity of P21 protein of LChV-1 (apricot and sour sweet cherry strain) and P4 protein of PaLV.

2. Materials and Methods

The transient gene expression system is a well-established method for characterizing VSRs (Voinnet & Baulcombe, 1997). The potential VSR coding regions of LChV-1 and PaLV isolates were cloned into a BinHA binary plasmid using In-fusion method. The resulting recombinant BinHA constructs were transformed into the *Agrobacterium tumefaciens* (strain C58C1) by triparental mating and then infiltrated into 3-week-old *Nicotiana benthamiana* leaves together with a GFP expressing construct in 0.4 :0.6 ratio (Hamilton, 2002). The GFP fluorescence signal of the infiltrated leaves (local silencing, 3,5 days post inoculation (dpi)) and whole plants (systemic silencing, 20 dpi) were examined visually under UV light. The level of GFP and tested protein expression was determined by Western blotting while the level of GFP mRNA expression was determined by real-time PCR.

3. Results and Discussion

3.1. Assaying LChV-1 encoded P21 for silencing suppressor activity

The ORF7 of LChV-1 has a possibility to encode a shorter and a longer protein. In the sweet cherry strain investigated before only a short P21 protein is translated and found to act as a VSR (Katsiani et al., 2017). In the reference genome of LChV-1, an ORF7 encodes a P21 protein that is 181 amino acids long. In contrast, our isolates, specifically the sweet cherry strain 'Alex' and apricot strain 'Magyar kajszi,' exhibit a longer version of P21, with an additional 50 amino acids, alongside the shorter variant. Moreover, the short P21 version in the apricot strain is 6 amino acids longer than its counterpart in the cherry variant.

In this research, we amplified both a short (S) and a long (L) form of P21 from sweet cherry (*Prunus avium* 'Alex') (Ch) and apricot (*Prunus armeniaca* 'Magyar kajszi') (Ap) infected by LChV-1 strains to compare their possible VSR in both local and systemic silencing suppression assays. We used the well-characterized VSR, P19 from Tomato bushy stunt virus (TBSV) as a positive control, and BinHA empty plasmid for negative control.

The LChV1-P21 variants showed a weak, transient local VSR activity that could be detected only visually (Figure 1A) through the mild increase in GFP fluorescence when compared to the control empty vector. However, these differences were not detected in molecular analysis (Figure 1B, C).

This contradicts with a previous report that P21 from LChV-1 has local VSR activity (Katsiani et al., 2017), but could be explained by differences of the P21 proteins originating other LChV-1 isolates (Figure 1.).

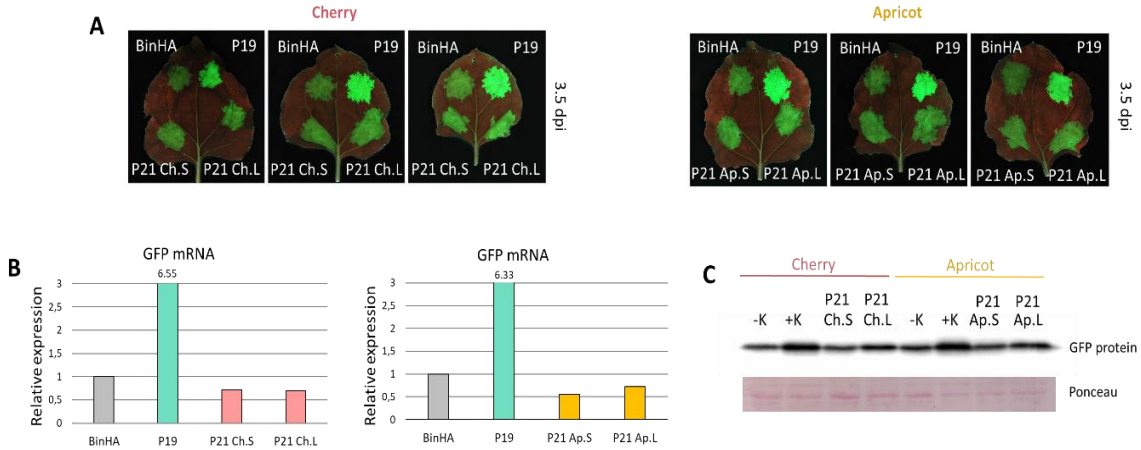

Figure 1. *(A) Local silencing suppression assays of LChV-1 short and long P21 protein of cherry and apricot isolates. (B) Quantitative real-time PCR for GFP mRNA expression measurement. (**C**) Western blot analysis of GFP protein*

Some VSRs, such as the apple chlorotic leaf spot virus (ACLSV) P50, suppress systemic RNA silencing without interfering with local silencing (Yaegashi et al., 2008). To find out whether P21 of LChV-1 can inhibit systemic RNA silencing, we tested its activity in a systemic silencing suppression assay (Figure 2).

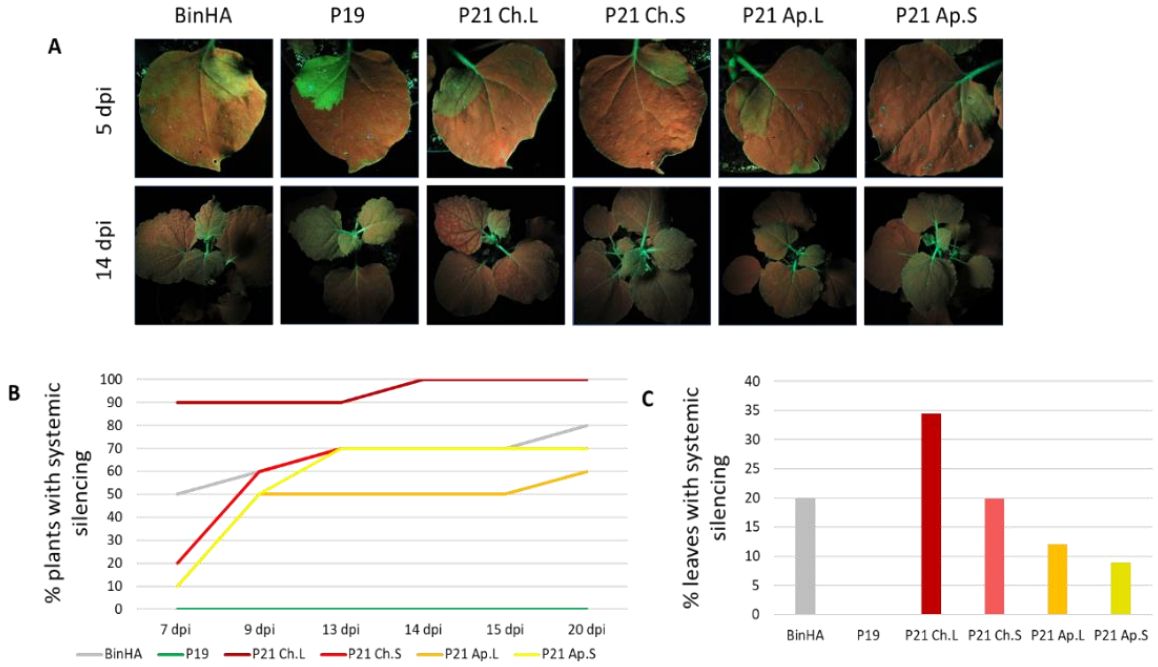

Figure 2. *(A) Systemic silencing suppression assays of LChV-1 short and long P21 protein of cherry and apricot isolates.(B) Percentage of plants with systemic silencing. (C) Percentage of leaves with systemic silencing*

Plants (n=10) and leaves were scored for the appearance of systemic silencing daily till 20dpi. The spread of the silencing signal was detected through the emission of red light resulting from chlorophyll fluorescence. Absence or minimal presence of red veins under UV light indicates a suppressor effect of the protein. In contrast to the negative result in the local silencing assay P21 apricot variants, both short and long proteins, showed mild suppression activity in the systemic silencing assay delaying the movement of the silencing signal. This activity was detected not only in decrease of the number of the plants showing the silencing signal (Figure 2B), but also in decrease in the number of the leaves with red veins (Figure 2C).

3.2 Assaying PaLV encoded P4 for silencing suppressor activity

Based on previous studies on BYDV-PAV (Fusaro et al., 2017), a close relative to PaLV, we investigated VSR activity of two variants of P4 proteins, differing in 11 amino acid, encoded by different strains of PaLV identified in 'Aranycsillag3' (Acs3) and 'Elvira' (Elv) cultivars.

In the local silencing suppression assay (Figure 3), both P4 'Acs3' and 'Elv' proteins showed a slightly stronger GFP fluorescent signal than the negative control (empty vector BinHA) (Figure 3A). The increase in the GFP signal could be detected, both by RT-qPCR and western blot analysis (Figure 3B, C).

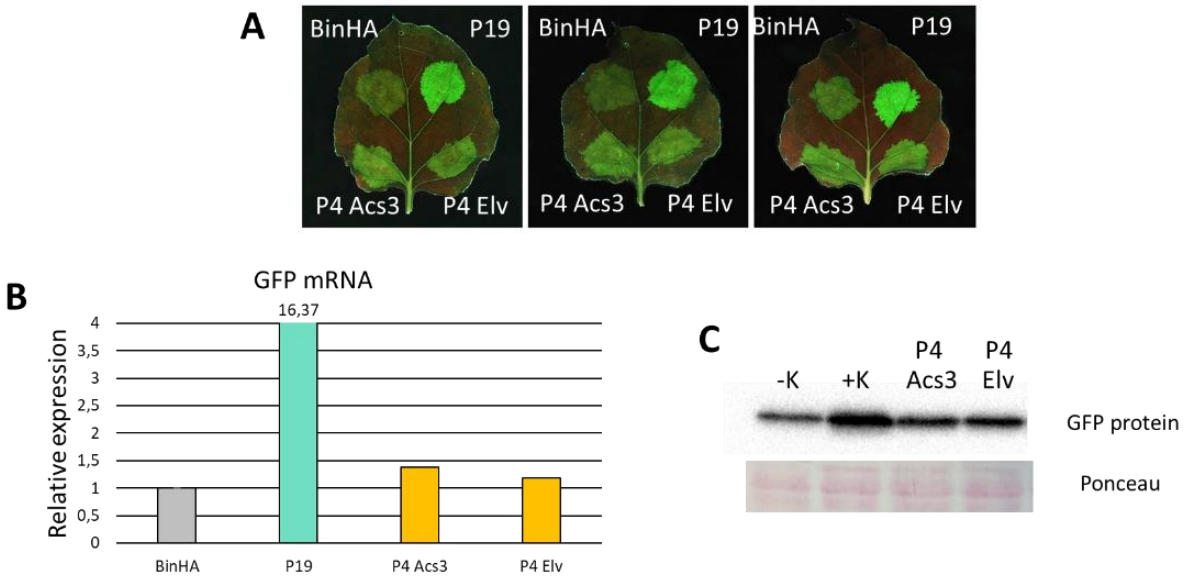

***Figure 3**. (A) Local silencing suppression assays of PaLV P4 isolates Acs3 and Elv. (B) Quantitative real-time PCR for GFP mRNA expression measurement. (C) Western blot analysis of GFP protein*

In the systemic silencing suppressor activity assays, P4 'Acs3' and 'Elv' variants failed to show VSR activity compared to the controls in the percentage of plants showing systemic silencing (data not shown).

4. Conclusions

In our study, we aimed to identify proteins with potential viral suppressor activity in two different *Prunus* infecting viruses. Based on our results, the P21 of LChV-1 isolate from apricot displayed mild systemic VSR activity, in both case of short and long variants. However, local silencing activity was not observed in either P21 variants. In the future, we plan to repeat the study using another GFP variant as silencing inducer, suited for detecting weak RNA silencing

suppressors (Mann & Dietzgen, 2017). P4 of luteoviruses can act as a viral suppressor, what was found also in case of PaLV: P4 protein showed only weak local but no systemic silencing activity. PaLV is usually present in asymptomatic trees which are frequently infected with other viruses. Its weak potential VSR activity can explain its latent presence. Its effect on the host and its role in co-infection with other viruses in terms of synergetic effects, is still unclear and needs further investigation in the future.

Acknowledgements

Our research was funded by the NKFIH K134895 OTKA project. Vivien Fákó is a PhD student of the Doctoral School of Biological Sciences at MATE.

References

Baráth, D., Jaksa-Czotter, N., Molnár, J., Varga, T., Balássy, J., Szabó, L. K., Kirilla, Z., Tusnády, G. E., Preininger, É. and Várallyay, É. 2018. Small RNA NGS Revealed the Presence of Cherry Virus A and Little Cherry Virus 1 on Apricots in Hungary. *Viruses*. **10** (6) 318. https://doi.org/10.3390/v10060318

Burgyán, J. and Havelda, Z. 2011. Viral suppressors of RNA silencing. *Trends in Plant Science*. **16** (5) 265–272. https://doi.org/10.1016/j.tplants.2011.02.010

Covey, S. N., Al-Kaff, N. S., Lángara, A., and Turner, D. S. 1997. Plants combat infection by gene silencing. *Nature*. **385** (6619) 781–782. https://doi.org/10.1038/385781a0

Csorba, T., Kontra, L. and Burgyán, J. 2015. Viral silencing suppressors: Tools forged to fine-tune host-pathogen coexistence. *Virology*. **479-480** 85–103. https://doi.org/10.1016/j.virol.2015.02.028

Fusaro, A., Barton, D., Nakasugi, K., Jackson, C., Kalischuk, M., Kawchuk, L., Vaslin, M., Correa, R. and Waterhouse, P. 2017. The Luteovirus P4 Movement Protein Is a Suppressor of Systemic RNA Silencing. *Viruses.* **9** (10) 294. https://doi.org/10.3390/v9100294

Hamilton, A. 2002. Two classes of short interfering RNA in RNA silencing. *The EMBO Journal*. **21** (17) 4671–4679. https://doi.org/10.1093/emboj/cdf464

Katsiani, A. T., Katsarou, K., Kalantidis, N. I. and Maliogka, V. I. 2017. Identification of an RNA silencing suppressor encoded by the genome of Little cherry virus 1 5–9.

Khalili, M., Candresse, T., Koloniuk, I., Safarova, D., Brans, Y., Faure, C., Delmas, M., Massart, S., Aranda, M. A., Caglayan, K., Decroocq, V., Drogoudi, P., Glasa, M., Pantelidis, G., Navratil, M., Latour, F., Spak, J., Pribylova, J., Mihalik, D. and Marais, A. 2023. The Expanding Menagerie of *Prunus* -Infecting Luteoviruses. *Phytopathology*. **113** (2) 345–354. https://doi.org/10.1094/PHYTO-06-22-0203-R

Mann, K. S. and Dietzgen, R. G. 2017. Functional analysis of a weak viral RNA silencing suppressor using two GFP variants as silencing inducers. *Journal of Virological Methods.* **239** 50–57. https://doi.org/10.1016/j.jviromet.2016.10.015

Miller, W. A. and Lozier, Z. 2022. Yellow Dwarf Viruses of Cereals: Taxonomy and Molecular Mechanisms. *Annual Review of Phytopathology*. **60** (1) 121–141. https://doi.org/10.1146/annurev-phyto-121421-125135

Voinnet, O. and Baulcombe, D. C. 1997. Systemic signalling in gene silencing. *Nature.* **389** (6651) 553–553. https://doi.org/10.1038/39215

Yaegashi, H., Tamura, A., Isogai, M. and Yoshikawa, N. 2008. Inhibition of long-distance movement of RNA silencing signals in Nicotiana benthamiana by Apple chlorotic leaf spot virus 50 kDa movement protein. *Virology*. **382** (2) 199–206. https://doi.org/10.1016/j.virol.2008.09.024