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Master in Plant Breeding

Investigation of possible viral silencing suppressor activity of cherry virus A

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Title: Investigation of possible viral silencing suppressor activity of cherry virus A

Abstract:

Plants have a highly conserved evolutionary defense mechanism known as RNA silencing, which involves sequence-specific gene regulation mediated by RNA interference (RNAi). This mechanism is activated during viral infections. Viruses, as effective pathogens, can evade plant defenses by expressing proteins called viral suppressors of RNA silencing (VSRs), which interfere with the silencing pathway through various mechanisms. Cherry virus A (CVA), a member of the genus *Capillovirus* in the family *Betaflexiviridae*, infects several plant species within the *Prunus* genus. These species (sweet cherry, sour cherry, apricot, and plum) are economically significant worldwide, and in Hungary, they are particularly valued for fresh consumption and the production of palinka or jams. Previous studies have demonstrated that either movement or coat proteins of *Capilloviruses* can act as VSR. This study aimed to compare CVA encoded movement protein (MP) and coat protein (CP) in different strains originating from different Hungarian host species, and to evaluate the VSR activity of CVA encoded MP. Sequence analysis of MP encoded by the different strains exhibit differences in the amino acid level, while the CP sequences vary only at the nucleotide level.

Utilizing Agrobacterium-mediated transient assays on wild-type and transgenic *Nicotiana benthamiana* line 16c, the research revealed that MPs encoded by sour cherry and apricot CVA strains function as weak local VSRs. The MP from the apricot strain is a stronger suppressor of systemic silencing than the sour cherry strain, although it is not as strong as p19, a well-known strong VSR. The MP from the sour cherry strain is a weak suppressor of the systemic response, capable of delaying but not completely inhibiting the silencing signal. This study provides valuable insights into the role of MP proteins encoded by CVA strains in the suppression of RNA silencing in plants, enhancing our understanding of RNA silencing mechanisms and paving the way for further research in this field.

Keywords: RNA silencing, viral suppressors of RNA silencing, VSRs, cherry virus A, CVA, movement protein, coat protein, Prunus, sweet cherry, sour cherry, apricot, plum

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I dedicate this work to my family, my dad Roberto, my aunt Zulma, and mostly to my mom Elsa, who is always there for me, no matter what. Thank you, mom, for everything you have ever done for me. I am really blessed to have a mom like you. None of this would have been ever be possible without your support.

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Alignment of this work with Sustainable Development Goals of the 2030 Agenda

Sustainable Development Goals	High	Medium	Low	Not applicable
SDG 1. End poverty.			X	
SDG 2. Zero hunger.		X		
SDG 3. Good health and well-being.			X	
SDG 4. Quality education.		X		
SDG 5. Gender equality.				X
SDG 6. Clean water and sanitation.				X
SDG 7. Affordable and clean energy.				X
SDG 8. Decent work and economic growth.			X	
SDG 9. Industry, innovation, and infrastructure.	X			
SDG 10. Reduced inequalities.				X
SDG 11. Sustainable cities and communities.				X
SDG 12. Responsible consumption and production.		X		
SDG 13. Climate action.				X
SDG 14. Life below water.				X
SDG 15. Life on land.	X			
SDG 16. Peace, justice, and strong institutions.				X
SDG 17. Partnerships for the goals.		X		

Description of the alignment of the TFM with the SDGs with a higher degree of relationship:

SDG 9: Industry, Innovation, and Infrastructure: The study is directly related to innovation in agricultural science. Understanding RNA silencing mechanisms can lead to new biotechnological applications and improvements in agricultural practices.

SDG 15: Life on Land: The research directly improves our understanding of plant biology and can contribute to the protection and sustainability of terrestrial ecosystems, particularly in agriculture.

1. Introduction

Plant diseases pose significant threats to humans as they cause harm to plants and products obtained from them, which are essential for human sustenance, including food, clothing, furniture, and the overall environment. Both cultivated and wild plants are susceptible to a high amount of diseases. On average, each type of crop plant can suffer from various plant diseases. Some pathogens target specific plant varieties, while others can affect numerous plant species, ranging from several dozen to hundreds. These diseases are caused by microorganisms, including nematodes, protozoans, bacteria, phytoplasmas, viruses, and viroids (Agrios, 2005). Of all these microorganisms, viruses are one of the most important since they cause a great economic loss since they reduce the quality and yield of crops, and their management includes only strategies as eradication and transmission prevention (Koeppe *et al.*, 2023; Barrientos *et al.*, 2021; Garcia-Ruiz *et al.*, 2016; Boualem *et al.*, 2016; Atencio, 2005, Hull, 2000).

Viruses are strictly intracellular obligate parasites because they depend on the host's protein synthesizing machinery (Atencio, 2005; Mukhopadhyay, 2018; Barrientos *et al.*, 2021). These pathogens are constituted by a nucleic acid (which can be either DNA or RNA), that may be single or double stranded, positive- or negative-sense, and normally is encapsulated by a protein. The virus genome encodes the necessary proteins for various stages of its life cycle, including replication and multiplication. These proteins can include enzymes involved in processes such as replication of the viral genome, assembly of new virus particles, and evasion of host immune responses (Hull, 2000; Mukhopadhyay, 2018). When a plant is infected by a virus, the infection can proliferate by the transportation of the pathogen through plasmodesmata from one cell to another (short distance) or through the phloem (systemic movement) (Csorba *et al.*, 2015)

1.1. Importance of Prunus species

The genus *Prunus* belongs to the family Rosaceae, order Rosales, and groups 230 species that are divided in four subgenera: *Amygdalus, Cerasus, Prunus* and *Eplectocladus*. The main attribute of these species is that they produce edible drupes and seeds that are highly important economically. Although it was originated in central Asia, now its cultivation is distributed worldwide, primarily centered in Asia but followed by Europe, North America, South America, Australia, and South Africa. In 2015, there was an annual production of more than 43 million metric tons for edible fruits and seeds (Rubio *et al.*, 2017).

Viral infections in plants result in considerable economic setbacks as they diminish both the quality and productivity of crops, posing a threat to food security in certain nations (Garcia-

Ruiz *et al.*, 2016). Some of the viruses that infect plants from *Prunus* genus are: prune dwarf virus (PDV), prunus necrotic ringspot virus (PNRSV), plum pox virus (PPV), plum bark necrotic stem pitting-associated virus (PBNSPaV), peach latent mosaic viroid (PLMVd), prunus necrotic ringspot virus (PNRSV), apple mosaic virus (ApMV), american plum line pattern virus (APLPV), prune dwarf virus (PDV), cherry leaf roll virus (CLRV), little cherry virus 1 (LChV-1), and cherry virus A (CVA), among others (Rubio *et al.*, 2017).

One of the main important *Prunus* species that are cultivated and used in Central Europe (particularly in Hungary) is the apricot. The fruit of this plant is not only consumed fresh, but also as an ingredient for the popular drink in Hungary called "palinka" or for production of jam (Baráth *et al*., 2018). In addition, in this region is also important the production of other species of this genus like sour cherry, peach, plum and sweet cherry (Papp *et al*., 2004).

1.2. Cherry virus A

Cherry Virus A (CVA) is a virus that belongs to the genus *Capillovirus*, in the family *Betaflexiviridae.* CVA is transmitted by grafting and infects species from the genus Prunus, mostly sweet cherry and sour cherry, and in less frequency other species such as apricot, plum and peach (Gao *et al*., 2017). It was first discovered in sweet cherry in Germany in 1995 (Jelkmann, 1995), but it has been reported to be detected in many other *Prunus* species all over the world, including China (Gao *et al.*, 2017; Xu *et al*., 2018), Hungary (Baráth *et al.*, 2018), Czech Republic (Ben Mansour *et al.*, 2023), Russia (Chirkov *et al.*, 2023), Japan (Isogai *et al.*, 2004), India (Noorani *et al.*, 2010), Poland (Komorowska *et al.*, 2020), South Korea (Lim *et al.*, 2017), Serbia (Mandic *et al.*, 2007), Canada (Simkovich *et al.*, 2021), Italy (Barone *et al*., 2006), France (Marais *et al*., 2008) and the United Kingdom (Kirby *et al*., 2001). Some of the *Prunus* species infected were: *P. avium* (sweet cherry), *P. cerasus* (sour cherry), *P. armeniaca* (apricot), *P. persica* (peach), *P. domestica* (plum), *P. cersasifera* (myrobalan), *P. salicina* (Japanese plum), *P. mume* (Japanese apricot), and *P. serrulata* (flowering cherry).

CVA is a virus of a single positive strand of RNA of about 7,383 nucleotides (nt) in length without include its 3' terminal poly (A) tail. It has two open reading frames (ORFs). While the first ORF encodes a polyprotein having RNA-dependent RNA polymerase (RdRp), methyltransferase (MT), and helicase (HEL), and coat protein (CP) functioning in frame, the second ORF encodes the movement protein (MP) **(Figure 1)** (Gao *et al.*, 2017; Kesanakurti *et al.*, 2017). The length of the 5'-UTR is of about 100 nt and its function appears to be associated with translation regulation (Wang *et al*., 2022).

Figure 1: Simplified illustration of genome of CVA. The first open reading frame encodes the polyprotein formed by polymerase (RdRp), methyltransferase (MT) and helicase (HEL), and the coat protein, while the second reading frame encodes the movement protein. Extracted and modified from ViralZone.

The detection of this virus posed significant challenges in the past, since it is not possible to detect it by traditional diagnostic methods like ELISA. However, the emerging next generation sequencing (NGS) tools have made its identification easier (Baráth *et al*., 2018). Nevertheless, it is often found in mixed infections together with one or more viruses like Little cherry virus 1 or 2 (LChV-1, LChV2), apple chlorotic leafspot virus (ACLSV), among others (Gao *et al*., 2017). This makes the correlation between the symptoms and the occurrence of a disease with its presence very difficult to establish. The virus can be latent but, in some cases, its presence can intensify the symptoms exhibit in the presence of other virus alone (Baráth *et al*., 2018).

1.3. RNA silencing

1.3.1 RNA silencing in plants

RNA interference (RNAi) is an evolutionary conserved process of eukaryotes that basically is a sequence-specific regulation of genes. Some roles of this mechanism include developmental regulation, stress responses and defense against strange nucleic acids, like transposons or viruses (Barrientos *et al*., 2021; Csorba *et al*., 2015; Ricaño-Rodríguez *et al*., 2014; Burgyán *et al*., 2011; Atencio, 2005). The molecules of RNAi are the ones in charge of the endonucleotic cut and degradation or repression of the translation of the genes that have to be inactivated (Garcia-Ruiz *et al*., 2016; Cuperus *et al*., 2010). In plants, there are two types of RNAi: the transcriptional genetic silencing (TGS) **(Figure 2)** and post-transcriptional genetic silencing (PTGS) **(Figure 3)**.

TGS is a process whose main objective is to regulate genes by blocking the transcription (Vaucheret & Fagard, 2001). The RNA polymerase IV produces single-stranded RNA (ssRNA), which later is transformed into double-stranded RNA (dsRNA) by the action of another polymerase called RNA polymerase dependent on RNA 2 (RDR2). The cellular machinery involved in transcriptional gene silencing (TGS) includes specialized proteins known as Dicer-like proteins (DCLs). These DCLs produce small interfering RNAs (siRNAs) approximately 24 nucleotides long. These siRNAs then associate with a protein called Argonauta 4 (AGO4), leading to the formation of the RNA-induced transcriptional silencing complex (RITS). The RITS complex acts as a guide, facilitating the assembly of heterochromatin and DNA methylation that

prevents transcriptional machinery from binding to the promoter region, effectively silencing the gene (Barrientos *et al*., 2021; El-Sappah *et al*., 2021; Muthamilarasan, 2013; Wang & Metzlaff, 2005).

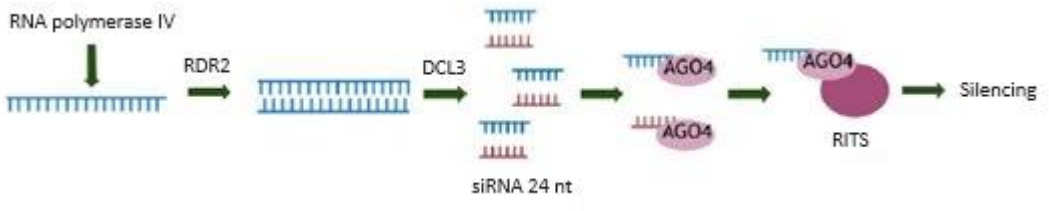


Figure 2: Mechanism of transcriptional gene silencing (TGS). RNA polymerase IV produces ssRNA, which is transformed into dsRNA by the action RNA polymerase dependent of RNA 2 (RDR2). Dicer-like proteins (DCLs) fragment the dsRNA into siRNAs approximately 24 nucleotides long, which then associate with Argonauta protein 4 (AGO4). This causes the formation of the RNA-induced transcriptional silencing complex (RITS) that acts as a guide and leads to DNA methylation, ultimately silencing the gene. Figure extracted and modified from Barrientos *et al*. (2021).

Post-transcriptional gene silencing (PTGS) is a crucial defense mechanism that occurs at the post-transcriptional level, and it involves the inactivation of genes through the cleavage of the RNA or by translational repression (Martinez de Alba *et al*., 2013; Csorba *et al*., 2016). This mechanism involves several steps. First, the RNA-dependent RNA polymerase (RDR) for RNA viruses or the cellular RNA polymerase II for DNA viruses synthesize the complemented strand and during this synthesis dsRNA or an RNA hairpin (hpRNA) are formed. The dsRNA serves as a substrate for the Dicer-like protein 4 (DCL4), which processes it and produces 21 nucleotides long virus derived siRNAs. These are then transported and bind to the AGO protein, one of the major components of the RNA-induced silencing complex (RISC), which role is to cleave the viral mRNA by binding complementary siRNA, resulting in the formation of the siRNA- viral RNA duplex. Consequently, viral RNA or viral DNA transcripts are effectively removed through PTGS (Moissiard & Voinnet, 2004; Barrientos *et al.*, 2021).

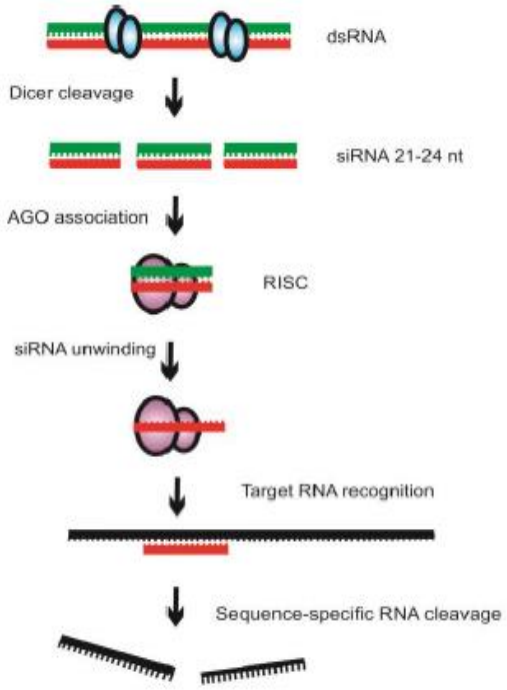


Figure 3: Mechanism of post-transcriptional gene silencing (PTGS). dsRNA is processed by the Dicer proteins into 21-24 nucleotides long siRNA. Together with the AGO protein they form the RNA-induced silencing complex (RISC) which recognize and cleave sequence-specific RNA. Extracted from Koeppe *et al.* (2023).

1.3.2 Antiviral RNA silencing

During viral infection, the RNAi based defense mechanism is induced in the plants. The mechanism of antiviral RNA silencing in plants consists of three phases: initiation, effector, and amplification. Most of the information available comes from the studying of this process in the model plant *Arabidopsis thaliana* (Csorba *et al.*, 2016).

The initiation phase involves two primary phases, the biogenesis of siRNAs and their incorporation into effector complexes. The process begins with the production of dsRNA that can be formed as a consequence of ssRNA that fold into hairpins secondary structures, intermolecular between partially reversed complementary ssRNAs, or ssRNAs that are transformed into dsRNAs by the action of RNA-dependent RNA polymerase. (Csorba *et al.*, 2016). DICER enzymes are in charge of the recognition of dsRNA molecules. These proteins belong to the RNase III type enzyme family, and are composed by a helicase, a PAZ, two RNase-III and two dsRNA-binding domains (Koeppe *et al.*, 2023). The PAZ and RNA-binding domains situate the dsRNA substrate, allowing the two RNase-III pseudo-dimers to catalyze the processing of dsRNA molecules into siRNA duplexes, which are typically 21–24 nucleotides long and have specific 3’ overhangs (with

5’-phosphate and 3’-hydroxyl ends) (Csorba *et al*., 2016; Csorba *et al*., 2015; Bernstein *et al*., 2001; Hutvagner *et al*., 2001; Hamilton & Baulcombe, 1999).

In the effector phase, the Argonaute proteins (AGOs), which are RNase-H type endonucleases, with the help of accessory proteins create the RNA-Induced Silencing Complex (RISC) or the RNA-Induced Transcriptional Silencing Complex (RITSC), that act during PTGS or TGS respectively (Castel & Martienssen, 2013; Mallory & Vaucheret 2010; Hutvagner & Simard, 2008; Ekwall 2004; Liu *et al*., 2004; Pham *et al*., 2004; Tomari *et al*., 2004; Hammond *et al*., 2001; Fagard *et al*., 2000). The sRNAs are loaded into AGOs depending their 50 terminal nucleotides, thermodynamical properties of sRNA duplex ends and duplex structure (Zhang *et al*., 2014; Schuck *et al*., 2013; Burgyán & Havelda, 2011; Mi *et al*., 2008; Schwarz *et al*., 2003). AGO-loading process requires cofactors such as heat shock protein 70 and 90 (HSP70, HSP90) and ATP hydrolysis to produce the conformational changes in AGO (Mallory & Vaucheret, 2010). Within the double-stranded small RNA (ds-sRNA) molecule, the strand with less stable 5’-end pairing is retained by AGO (Argonaute), while the complementary “star” strand is discarded (Khvorova *et al*., 2003; Schwarz *et al*., 2003). Guided by the sRNA sequence, the RNA-induced silencing complex (RISC) either cleaves or represses translation of its target RNAs during post-transcriptional gene silencing (PTGS) in a sequence-specific manner (Brodersen & Voinnet, 2009). The cleavage products generated by RISC are subsequently eliminated by the cell’s mRNA decay and quality control machinery. Additionally, the RITSC complex induces histone and/or DNA methylation, leading to transcriptional gene silencing (TGS) of the homologous gene (Csorba *et al*., 2016; Martinez de Alba *et al*., 2015; Castel & Martienssen, 2013; Creamer & Partridge, 2011).

In the last phase, amplification, RNA-dependent RNA polymerases act to amplify the response and they are stimulated by the presence of RNAs without CAP-structure, lacking polyA tail, among other aberrant types, or fragments that have been endonucleolytically cleaved by RISC (Csorba *et al*., 2016; Csorba *et al*., 2015; Moreno *et al*., 2013; Wassenegger & Krczal, 2006; Gazzani *et al*., 2004). Once these fragments are transformed into dsRNAs, they can enter or re-enter the whole process of silencing, producing sRNA (Csorba *et al*., 2016; Branscheid *et al*., 2015; Martinez de Alba *et al*., 2015; Parent *et al*., 2015; Mourrain *et al*., 2000; Sijen *et al*., 2001; Vaistij *et al*., 2002; Voinnet *et al*., 1998)

One of the practical applications of this mechanism is Virus-induced gene silencing (VIGS), a technique extensively utilized in plant research to selectively down-regulate specific transcripts in different plant species. By taking advantage of the plant's natural defense mechanisms, this technique enables researchers to target and silence endogenous genes by inserting a foreign target gene derived from the virus (Dommes *et al*., 2019).

1.3.3 Viral suppressors of RNA silencing

In plant-virus interactions, plants utilize gene-silencing mechanisms to prevent, restrict, and eliminate viral infections. For a plant to be susceptible or resistant to a viral infection, the balance between the silencing of the virus from the plant, and the evasion or suppression of defenses from the virus is significantly important (Garcia-Ruiz *et al*., 2010; Garcia-Ruiz *et al*., 2015; Ma *et al*., 2015).

Plant viruses are effective pathogens that can infect plants and produce symptoms and diseases. To be able to replicate themselves and transport from cell to cell, viruses had to develop a strategy to surpass the plant defenses either by evasion or suppression of them. This counter-defensive strategy consists in proteins that are able to suppress the antiviral RNA silencing called viral suppressors of RNA silencing (VSRs), which is usually an additional function of coat proteins, replicases, movement proteins, among others. VSRs can suppress the host's defensive system at different stages of the mechanism, targeting different effectors like Dicer proteins or AGO proteins (Burgyán & Havelda, 2011; Garcia-Ruiz *et al.*, 2016; Moissiard & Voinnet, 2004). The points in which the VSR can suppress the pathway of the RNA silencing in plants are dsRNA binding, blocking DCL4, inactivating DRB4, ds-siRNA cleaving, sequestering the ds-siRNA and inhibiting the systemic silencing, AGO1 or AGO2 degradation, HEN1 binding, AGO1 or AGO2 inactivation, interaction with AGO1 in formed RISC complex, increasing the pre-miR168, inactivating SGS3, RDR6 inhibition, blocking DCL3, degradation of AGO4, and blocking the methylation, as we can see in **Figure 4**.

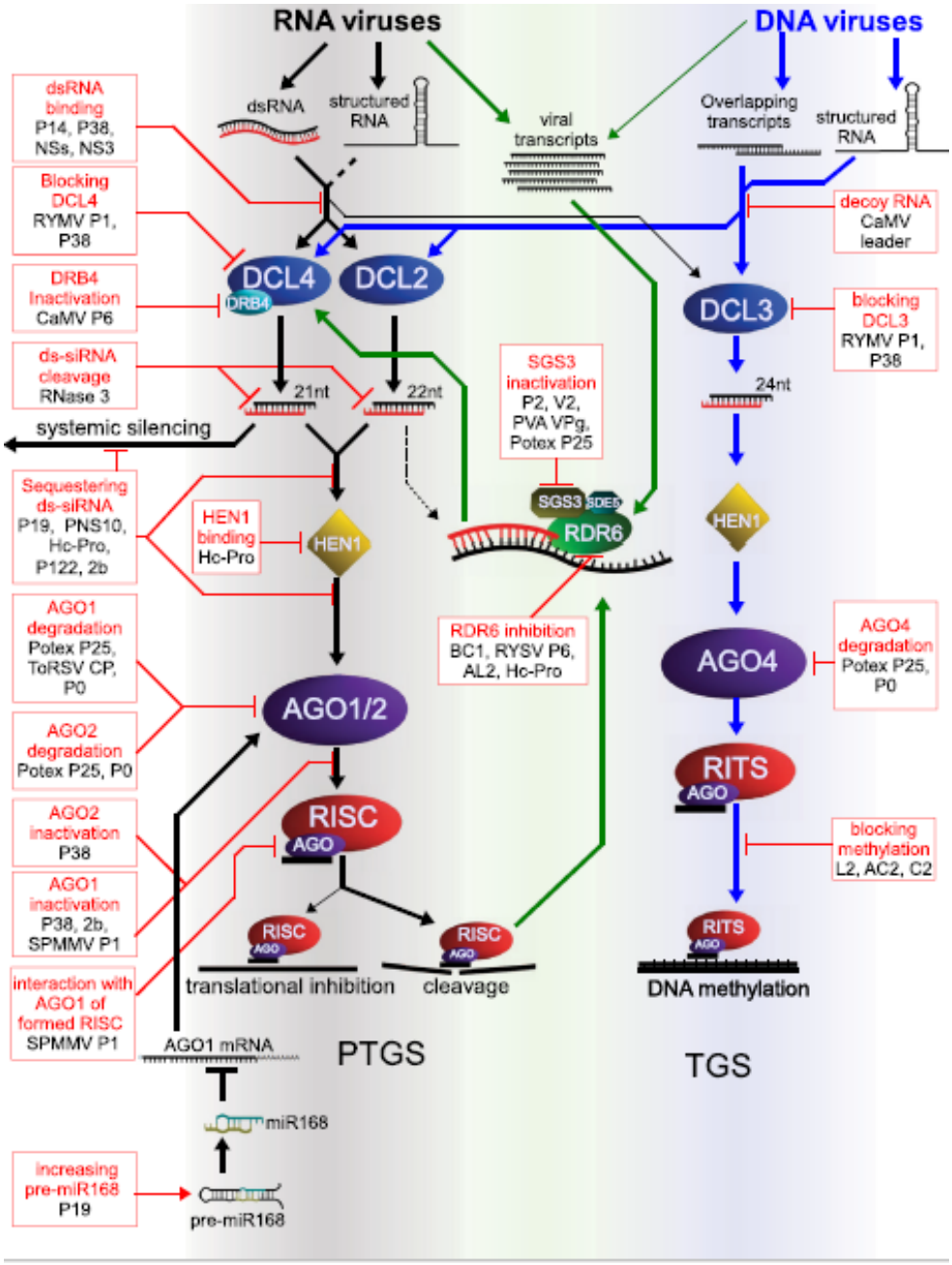


Figure 4: Model figure showing the pathways of RNA silencing for RNA or DNA viruses (PTGS and TGS). In red boxes are shown the different types of VSRs with examples of viral proteins that behave that way, and which point of the pathway they suppress. Extracted from Csorba *et al.* (2015).

As previously said, VSRs can act at different point of the defensive RNA silencing pathway. In the initiation step, it can be inhibited the DICER proteins or the activity of co-factors, the dsRNA or siRNA can be sequestered or the AGO proteins can be destabilized before the RISC complex forms, blocking this way the first phase of the viral RNA silencing pathway. Examples of VSRs that sequestrate ds-siRNA are P21, P15, P19, among others. (Csorba *et al.*, 2007; Csorba *et al.*, 2015; Hemmes *et al.*, 2007; Kubota *et al.*, 2003; Lakatos *et al.*, 2006; Mérai *et al.*, 2005, 2006; Silhavy *et al.*, 2002). Another kind of VSRs are the ones that can bind to AGO proteins,

preventing the assembly of the RISC complex, like the P0 protein, suppressor of Poleoviruses that increase the degradation of AGO 1, 2, 4, 5, 6 and 9 (Csorba *et al*.,2010; Csorba *et al.*, 2015).

Another way to interfere with the viral RNA silencing in plants is making the RISC complex inactive by imitating the cellular protein factors (Csorba *et al*., 2015; Eulalio *et al.*, 2009) or disrupt host defense mechanisms downstream of RNA-induced silencing complex (RISC) or RNA-induced transcriptional silencing (RITS). Some DNA viruses carry VSRs known to modify DNA/histone methylation, a critical step in transcriptional gene silencing (TGS). For example, the AL2 suppressor of Tomato Golden Mosaic Virus (TGMV) (Buchmann *et al*., 2009; Csorba *et al*., 2015; Wang *et al.*, 2005; 2003). Lastly, RNA-dependent RNA polymerases (RDRs) can also be affected by VSRs, reducing the amplification of the defense response and the systemic transmission of the signal to distant tissues. One particular example is the V2 protein from Tomato yellow leaf curl virus (TYLCV) that interacts with a cofactor of RDR6 blocking the amplification (Csorba *et al*., 2015; Glick *et al*., 2010).

Viral proteins such as the movement protein (MP) or the coat protein (CP) are very important for the replication and movement across cells in plants. The movement protein is crucial for the movement from cell to cell (Díaz-Pendón & Ding, 2008; Lucas, 2006), but also can act as a VSR. There has been reported that p25 of PVX, p69 of Turnip yellow mosaic virus, p7b of Melon necrotic spot virus, and p50 of Apple chlorotic leafspot virus are all movement proteins that act suppressing the RNA silencing (Díaz-Pendón & Ding, 2008; Bayne *et al*., 2005, Chen *et al*., 2004, Genovés *et al*., 2006). As for CP, it is a protein that is involved in long-distance movement (Díaz-Pendón & Ding, 2008; Saito *et al.*, 1990). However, it can also have VSR activity. The most known example is the protein p38 that can replace p19 in the suppression of the plant defense system (Díaz-Pendón & Ding, 2008) and works inhibiting the production of viRNAs by DCL4 (Díaz-Pendón & Ding, 2008; Deleris *et al*., 2006).

2. Objectives

CVA can infect several different *Prunus* hosts. Both MP and CP have been identified as VSR for other viruses in general before, but MP has been identified as VSR in viruses belonging to the same family as CVA (*Betaflexiviridae*), as apple chlorotic leafspot virus. For this work, the general objective was to amplify two proteins, MP and CP, from different strains of CVA infecting different host species that, considering the literature reviewed, might be acting as VSRs; and compare the potential activity of these slightly different variants. Taking into consideration this general objective, the following specific objectives were proposed:

1. Study the differences in the CVA encoding MP in different strains originating from different hosts.
2. Test and compare the possible local VSR activity of the MP of CVA encoded by different Hungarian strains originating from different hosts.
3. Determine if the MP protein can act as a VSR of the systemic signal of silencing.
4. Identify the position of the CP encoding part in the genome of CVA, and study the differences in the CP in different strains originating from different hosts.

2.1 Hypothesis

The encoded movement protein of CVA shows VSR activity at local and systemic levels, and the activity of MPs of different Hungarian strains encoded by four host species will differ from each other.

The encoded coat protein of CVA originating from four host species will show differences at the sequence level.

3. Materials and methods

3.1. Virus strains and primer design

The virus strains utilized in this work originated from four Hungarian *Prunus* host cultivars (sweet cherry - Szomolyai fekete; plum – Besztercei; sour cherry – Érdi bőtermő; and apricot – Pannónia).

The primers used in different experiments in this study are summarized in Table 1, including their sequences and the expected product size.

Table 1: Primers used in different experiments in this study.

Primer name	Sequence 5'-3'	Reference
CVA_MP_FW	ATGTCGATCATACCAGTYAAG	Barath *et al.*, 2018
CVA_MP_R	TTACCTTCTGCACCAACYAC	
CVA_CP_FW	ATGATAGATCCAAGGGCTC	Noorani *et al.*, 2013
CVA_CP_R	TTAAGCATCAAAACTTAAG	
CVA_MP_fusion_FW	TCGATCGACGGATCCATGT GATCATACCAGTTAAGAAG	This work
CVA_MP_fusion_R	TCACTGCAGTGGATCTTACCTTCTGCA CCAACTACACCC	This work
Bin61FW primer	TCCCACTATCCTTCGCAAGACCC	This work
GFP_Taq_Fw	GCCAACACTTGTCACTACTTTCTC	Gouveia *et al.*, 2012
GFP_Taq_Rv	GTAGTTCCCGTCGTCCTTGAAG	Gouveia *et al.*, 2012
Ubi3_Fw	GCCGACTACAACATCCAGAAGG	Tang *et al.*, 2017
Ubi_Rv	TGCAACACAGCGAGCTTAACC	Tang *et al.*, 2017

3.2. Plasmid constructions

3.2.1. BinHASanyi binary vector

For the preparation of the BinHASanyi binary vector, a purified vector was re-transformed into competent *Escherichia coli*. The cells were cultured in an agar plate to create a master plate for long-term storage, and LB-K liquid cultures for the plasmid isolation. For this objective, plasmids were purified from overnight liquid culture using the NucleoSpin® Plasmid DNA Isolation Kit (Macherey Nagel, Germany) according to the manufacturer's guidelines. The 30 µL DNA was precipitated overnight with 30 µL 4M Na-acetate and 1 mL 96% alcohol. The samples were washed with 1 ml of 70% alcohol and eluted in 50 µL of distilled water, and their concentration was measured with a Nanodrop ND-1000, showing a concentration of 178 ng/µL

in the first replica, and 237.4 ng/µL in the case of the second replica. The integrity of the plasmid was checked on Agarose gel 1.2%.

Digestion of the plasmid was made in 150 µL (28 µL purified plasmid in the first replica and 21 µL purified plasmid in the second replica), and 5µL of BamH1 (10U/µL), 15 µL of 10X Buffer BamH1 and the necessary volume of distilled water was added to complete until 150 µL in total. The mixture was incubated overnight in a water bath set at 30°C. The completeness of the digestion was checked by separation of aliquot of the digestion mixture on 1.2% agarose gel. The fully digested plasmid (the total digestion mixture) was cut from a gel and proceeded with DNA extraction and purification with the help of NucleoSpin® Gel and PCR Clean-Up kit (Macherey Nagel, Germany), which was used according to the manufacturer's guidelines. To check the final integrity and concentration of the digested plasmid aliquot was separated on 1.2% agarose gel and its concentration was measured with a Nanodrop ND-100.

3.2.2. Expression vector containing CVA MP coding sequences

The MP and CP coding regions of cherry virus A (CVA) originating from four Hungarian *Prunus* host cultivars (sweet cherry, plum, sour cherry, and apricot) were amplified individually by polymerase chain reaction (PCR). The DNA polymerase used for this amplification was Q5, a proofreading polymerase (New England Biolabs), the specific primers utilized were CVA_MP_FW (forward primer) and CVA_MP_R (reverse primer) in the case of MP, and CVA_CP_FW (forward primer) and CVA_CP_R (reverse primer) in the case of CP (see Virus strains and primer design). The reactions and programs used for the PCRs were as seen in **Table 2** and **Table 3**.

Table 2: PCR reaction mix and program conditions for amplifying MP coding regions of CVA.

1x PCR reaction mix		**Program**		
9 µL	Water	98°C	30 sec	
3 µL	5X Q5 Buffer	98°C	30 sec	40 times
1 µL	CVA_MP_FW 10 µM	62°C	30 sec	
1 µL	CVA_MP_R 10 µM	72°C	2 min	
0.3 µL	10 mM dNTPs	72°C	5 min	
0.2 µL	Q5 Polymerase (2U/µL)	4°C	Hold	
0.5 µL	10X template			

Table 3: PCR reaction mix and program conditions for amplifying CP coding regions of CVA.

1x PCR reaction mix		Program		
9 µL	Water	98°C	30 sec	
3 µL	5X Q5 Buffer	98°C	30 sec	40 times
1 µL	CVA_CP_FW 10 µM	57°C	30 sec	
1 µL	CVA_CP_R 10 µM	72°C	2 min	
0.3 µL	10 mM dNTPs	72°C	5 min	
0.2 µL	Q5 Polymerase (2U/µL)	4°C	Hold	
0.5 µL	10X template			

The PCR products were then separated in an 1.2% agarose gel, and then extracted and purified from the gel using the NucleoSpin® Gel and PCR Clean-Up kit (Macherey Nagel, Germany). The purified DNA product was cloned into a pJET1.2/blunt **(Figure 4)** vector using the CloneJET PCR Cloning Kit (Thermo Fisher, USA), and transformed into *E. coli* competent cells. For this, 5 µL of the ligation mixture was added to the competent cells. The cells were stored in ice for 20 minutes, then exposed to a heat shock of 42°C for 30 seconds, and after rapidly transferred again to ice. They were incubated for 40 minutes in 500 µL of SOC medium at 37°C in a shaking incubator so that the bacteria could recover and express the antibiotic resistance encoded by the plasmid. The bacteria were then cultured in plates containing LB+ampicillin medium and incubated at 37°C overnight.

Transformed colonies that showed antibiotic resistance were cultured on agar plates to generate a master plate for long-term storage, as well as in LB-K liquid culture for plasmid DNA extraction. Pure plasmid DNA isolation was performed using the NucleoSpin® Plasmid DNA Isolation Kit (Macherey Nagel, Germany) according to the manufacturer's guidelines. The plasmid was digested with XbaI and XhoI restriction enzymes: 2 µL of plasmid was mixed with 2 µL of 10X Tango buffer, 0.2 µL of XbaI (10U/ µL), 0.2 µL of XhoI (10U/ µL), and 5.6 µL of distilled water. The result of the digestion was analyzed by running it in 1.2% agarose gel to determine if it produces bands of the anticipated size. After confirming this, the clones were sequenced by the Sanger method.

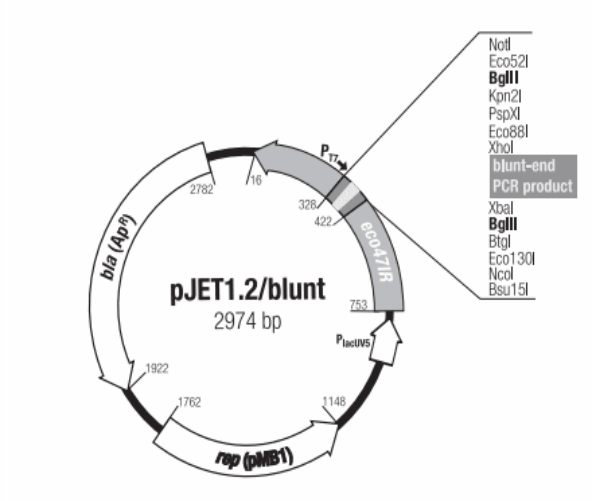


Figure 5: Vector pJET1.2/blunt. "rep (pMB1): Replicon from the pMBI plasmid responsible for the replication of pJET1.2; replication start: initiation of replication; bla (ApR): β-lactase gene conferring resistance to ampicillin, used for selection and maintenance of E. coli cells; eco47IR: lethal gene eco47IR enables positive selection of recombinant plasmid; PIacUV5: modified PIac promoter for expression of the eco47IR gene at a level sufficient to allow for positive selection; T7 promoter: T7 RNA polymerase promoter for in vitro transcription of the cloned insert; Multiple cloning site (MCS); Insertion site: blunt DNA ends for ligation with insert; pJET1.2 forward sequencing primer; pJET1.2 reverse sequencing primer". Extracted from CloneJET PCR Cloning Kit Guide (Thermo Fisher, USA).

3.2.3. Positive control plasmids

As positive controls, there were used three constructs that were already prepared previously to this work. These constructs consisted in a BinHASanyi plasmid containing seqeuences able to encode P14 of Pothos latent virus (PoLV), P19 of Cymbidium ringspot virus (CymRSV), and P21 of Beet yellows virus (BYV), previously characterized VSRs.

3.3. Bioinformatic analysis

Multiple sequence alignment was performed using the MUSCLE algorithm in Geneious Prime software 2023.0.4 (Biomatters Ltd., Auckland, New Zealand). Additionally, a phylogenetic tree was constructed, which was based on maximum-likelihood (Tamura-Nei model), using 100 bootstrap iterations (shown at the branches points) to assess the robustness of the inferred evolutionary relationships.

3.4. *Agrobacterium*-mediated transient assay

3.4.1. Plant material

Wild type or GFP expressing, 6-week-old *Nicotiana benthamiana* plants were used for the infiltration assays. The plants were grown in normal conditions of irrigation and temperature (temperature of 21°C and a photoperiod of 16 hours/8 hours light/dark).

3.4.2. In-fusion cloning method

3.4.2.1. Primer design

Primers used for the In-fusion reactions have specific requirements, that is why a special software has to be used for their design. For their design, it was used the online software: In-Fusion Cloning Primer Design Tool v1.0 (Takara Bio, USA), using CVA MP coding region from the Hungarian *Prunus* host cultivars and the BamHaI digested BinHASanyi vector, with BamH1 as the restriction enzyme. The primers obtained can be seen in **Table 1**.

3.4.2.2. Vector constructions

For the fast and directional cloning into the BinHASanyi binary vector guidelines of the In-Fusion® Snap Assembly Kit (Takara Bio, USA) were followed. The DNA for this assembly strategy was amplified by PCR with the In-Fusion primers able to amplify the MP coding region of CVA (see primer design). The volumes of the reagents and the program of the PCR were as seen in **Table 4**.

Table 4: PCR reaction mix and program conditions for amplification of DNA for later cloning into the BinHASanyi binary vector.

1x PCR reaction mix		**Program**		
10.75 µL	Water	98°C	1 min	
12.5 µL	2X PrimeSTAR Max Premix (reaction buffer, dNTPs, and PrimeSTAR Max DNA Polymerase)	98°C	10 sec	35 times
		55°C	15 sec	
0.75 µL	10X CVA_MP_fusion_FW	72°C	10 sec	
0.75 µL	10X CVA_MP_fusion_R	72°C	2 min	
0.5 µL	50X Template	4°C	Hold	

The PCR products were separated in 1,2% agarose gel, and the DNA was then cut and extracted from the gel using the NucleoSpin® Gel and PCR Clean-Up kit (Macherey Nagel,

Germany). To insert into the MP coding sequence into the vector BinHASanyi an In-fusion reaction was performed. The reaction and incubation temperatures (it was used a PCR machine to have the exact temperatures) used for the In-fusion reaction were as seen in **Table 5**.

Table 5: In-fusion reaction conditions

1x reaction mix		**Program**	
1 µL	5X Snap Assembly Master Mix	50°C	15 min
1.5 µL	BinHA vector		
0.5 µL	Insert		
2 µL	Water		

Competent *E. coli* cells were transformed with the In-fusion reaction mixture using the same protocol as previously described. Four of the transformed colonies/constructs were cultured in agar plates to generate a master plate for long-term storage and for colony PCR to verify that the insertion of the MP coding region into the expression plasmid happened and contains the full desired genetic insert. For the colony PCR, the forward primer for the BinHASanyi vector (Bin61FW primer), and the reverse primer for the construct (CVA_MP_fusion_R) were used. The reagents and PCR program utilized are seen in **Table 6**.

Table 6: Reaction mix and program conditions for colony PCR

1x PCR reaction mix		**Program**		
14.28 µL	Water	95°C	3 min	
2 µL	10X Taq Buffer	95°C	30 sec	40 times
1 µL	Forward primer	50°C	1.3 min	
1 µL	Reverse primer	72°C	1.3 min	
1.2 µL	10 mM MgCl2	72°C	10 min	
0.4 µL	10 mM dNTPs	4°C	Hold	
0.12 µL	Taq DNA Polymerase (5U/µL)			
One dot from a colony taken with a toothpick				

After the completion of the reaction, the products were separated in 1.2% agarose gel.

The transformed colonies were cultured in a liquid medium. The plasmids were purified utilizing the NucleoSpin® Plasmid DNA Isolation Kit (Macherey Nagel, Germany) according to the manufacturer's guidelines. The isolated plasmid was then separated in an 1.2% agarose gel,

and sequenced by the Sanger method. The sequences were compared (using BLAST algorithm) with the original CVA MP and with the vector sequences to check if our sequence of interest is in frame.

After checking that the sequence is in frame, a triparental mating was performed. In this technique, bacterial conjugation happens where a conjugative plasmid present in one bacterial strain assists the transfer of a mobilizable plasmid present in a second bacterial strain into a third bacterial strain. The donor that carried the mobilizable plasmid was DH5£ BinHA clone, the recipient wished to acquire the mobilizable plasmid into was C58C1 *Agrobacterium tumefaciens* strain, and the helper that was carrying the conjugative plasmid was *E. coli* pRK. For this, a colony from each component was picked up and cultured in a LB plate drawing a line with each of them, with a transacting point between them forming a "star". The bacteria were grown overnight at 30°C, and from each transacting point colonies were picked up and streaked into another plate containing YEB medium supplemented with rifampicin, kanamycin, and tetracycline (YEB+R+K+T) and grown at 30°C for 2 days. The best colony for each sample was chosen for the preparation of glycerin stock for archiving. To do this, the colony was picked from the plate with a toothpick and placed in 3 mL of YEB medium supplemented with kanamycin and tetracycline (YEB+K+T) liquid culture and left shaking at 30°C overnight. After that, 850 µL of liquid culture grown bacteria was mixed with 150 µL of glycerin, and stored at -70°C for storage. In addition, the same procedure to prepare a glycerin stock of the BinHA vector in different individual conditions (without insert, with an insert of P19, with an insert of P21, with an insert of P14, and with an insert of GFP).

3.4.3. Agroinfiltration of *N. benthamiana*.

The transient gene expression system is a well-established method for characterizing VSRs (Voinnet & Baulcombe, 1997). As previously said, the MP coding region of CVA Hungarian strains, a potential VSR, was cloned into a BinHA binary plasmid using the In-fusion method. The resulting recombinant BinHA constructs were transformed into *A. tumefaciens* (strain C58C1) by triparental mating and then infiltrated into 6-week-old *N. benthamiana* leaves with GFP as the inducer of the defense RNA silencing response. The infiltrated patch expresses high levels of GFP that glows bright green under ultraviolet (UV) light (Roth *et al.*, 2004). This fluorescence signal was examined at the 3.5 days post inoculation (dpi) in the leaves for local silencing, and until 20 dpi for systemic silencing in the whole plants under the UV light.

3.4.3.1. Local silencing signal analysis

3.4.3.1.1. Protein expression and sizing verification

The first step was preparing a liquid culture from *Agrobacterium* colonies obtained from the triparental mating. A colony from the plates containing the triparental transect growing in a YEB+R+K+T was picked up with a toothpick and placed in YEB liquid medium supplemented with Tetracycline, Kanamycin, 1 M MES buffer and 1 M acetosyringone (YEB + T + K + MES + Acetosyringone) and left shaking at 30°C overnight. Additionally, the same was done with Agrobacterium colonies containing the vector with the gene of GFP, and *Agrobacterium* colonies containing the vector with the gene of P14.

The grown *Agrobacterium* liquid cultures were placed in corex tubes and centrifuged in a Sorwall centrifuge for 10 minutes at 4000 rpm and room temperature. The pellet was then resuspended in the infiltration buffer previously prepared (200 mL of distilled water, 800 µL 2.5 M MgCl2 and 50 µL 1 M acetosyringone). The OD_{600} was measured in a 5X dilution of the sample (20 µL of the resuspended bacteria + 80 µL infiltration buffer), using 100 µL of infiltration buffer as the blank. The infiltration mixture was then diluted using the infiltration buffer to set the concentration to OD_{600}=1. After setting the concentration to this value, the tubes were incubated at room temperature for 3 hours in the dark.

The infiltration was done after the incubation period with syringes in the abaxial face of *N. benthamiana* plants. For checking if the protein was successfully expressed and that it has the expected size (HA-tag detection), the infiltration was made in the presence of P14 and GFP. The mix for the infiltration was prepared as follows: agrobacteria containing vector with P14 insert – agrobacteria containing vector with GFP insert – agrobacteria containing vector with MP insert in a 0.2:0.2:0.6 ratio.

3.4.3.1.2. Local silencing dynamics in transient infiltration

Plates with YEB+R+K+T medium were prepared from the glycerin stock previously done from the BinHA empty plasmid, the plasmid with the insert of GFP, the plasmid with the insert of P21, the plasmid with the insert of the CVA MP (from sour cherry and apricot). After two days of incubation at 30°C, liquid cultures were prepared from the bacteria grown in the plates and put on a shaker at 30°C. Once the *Agrobacterium* liquid cultures were grown, it was preceded the same way as for the protein expression verification. The final concentrations were set to OD_{600}=1, and after three hours of incubation in the dark, the mixes for the infiltration were prepared. In co-infiltration experiments, GFP-expressing Agrobacteria and the potential VSR coding ORFs were mixed 0.4:0.6 ratio. GFP functions as a reporter gene and induces the RNA silencing defense of

the plant. The different inserts mixed with GFP-expressing Agrobacteria were: BinHA empty vector (negative control), P21 (positive control with a strong VSR activity), MP from sour cherry CVA strain, and MP from apricot CVA strain. The infiltration was done with syringes in the abaxial face of *N. benthamiana* plants, injecting the four mixes in each leaf.

3.4.3.2. Systemic silencing signal analysis

From the glycerin stock previously done, plates with YEB+R+K+T medium were prepared for the BinHA empty plasmid, BinHA with the GFP insert, BinHA with the insert of P19 (a gene that shows a very strong systemic VSR activity), BinHA with the MP from sour cherry insert, and BinHA with the MP from apricot insert. These plates were incubated at 30°C for 2 days, and after that, liquid cultures were prepared from them and incubated at 30°C in a shaker. After the Agrobacteria in the liquid cultures were grown, it was proceeded as with the protein expression verification and the local silencing infiltration. The final concentrations were set to OD_{600}=1, and after three hours of incubation in the dark, the mixes for the infiltration were prepared. The mixing was prepared in a 0.4:0.6 ratio of GFP-expressing Agrobacterium and the potential VSR coding ORFs respectively. The infiltration was done with syringes in the abaxial face of *N. benthamiana* plants, injecting 2 leaves with one construct mix per plant. The plants used for this experiment were GFP transgenic *N. benthamiana* plants (line 16c).

3.5. Molecular analysis

3.5.1. Total nucleic acid extraction

Four discs of 10 mm per sample were cut from the infiltrated patches on the leaves of *N. benthamiana* plants and conserved at -70°C until the day of extraction. Extraction buffer was freshly prepared mixing 700 µL sterile water, 100 µL 10X EB (pH=9,5, 7,5g glycine, 20 mL 100 mM EDTA, 1M NaCl), and 200 µL 10% SDS. The plant material was placed in sterile mortars with a small amount of liquid nitrogen to start the homogenization. To continue with this homogenization, 650 µL of extraction buffer was added, and after the plant material was completely homogenized, it was transferred to a tube containing 600 µL of phenol, vortexed and put on ice. The sample was then again vortexed and centrifuged at 15000 rpm for 5 minutes at room temperature, and then the supernatant was transferred to a new tube containing 300 µL of phenol mixed with 300 µL of chloroform, vortexed and put on ice. The sample was then again vortexed and centrifuged at 15000 rpm for 5 minutes at room temperature, and the supernatant was placed in a new tube containing 600 µL of chloroform - isoamyl alcohol, vortexed and put on ice. This was followed by another vortexing and centrifugation at 15000 rpm for 5 minutes at room temperature, and then the supernatant was placed on a new tube containing 20 µL 4M Na-

acetate and 1 mL of 96% ethanol, the tube was inverted 6-8 times and put on ice. The samples were then stored at -4°C.

The next day, the samples were inverted 6-8 times and centrifuged at 15000 rpm for 15 minutes at 4°C. The ethanol was poured out by inverting the tubes, and it was added 1 mL of 70% ethanol. The following step was centrifugation at 15000 rpm for 3 minutes at room temperature, and again the ethanol was poured out by inverting the tubes. The pellet was then dried to evaporate the alcohol in a SpeedVac Vacuum for 10 minutes with open tubes. The pellet was then resuspended in 30 µL of sterile water, vortexed two times, centrifuged at 15000 rpm for 1 min at room temperature and put on ice. The concentration of the RNA was measured in a Nanodrop ND-1000. 2 µL of the sample was mixed with 5 µL of FDE and 3 µ of sterile water, incubated for 5 minutes in 65°C bath, and then run in a 1.2% agarose gel. The remaining of the samples were stored at -70°C.

3.5.2. Protein purification

Four discs of 10 mm per sample were cut from the infiltrated patches on the leaves of *N. benthamiana* plants. It was placed on sterile mortars where it was homogenized with 650 µL of extraction buffer (700 µL sterile water, 100 µL 10X EB [pH=9,5, 7,5g glycine, 20 mL 100 mM EDTA, 1M NaCl], and 200 µL 10% SDS). After homogenization, 60 µL of the homogenized sample were placed in Eppendorf tubes containing 60 µL of 2X Laemmli Sample Buffer, vortexed, and centrifuged at 13,5 rpm for 3 minutes. The supernatant was placed in another tube and stored at -70°C for later analysis.

3.5.3. Western Blot

For this assay, we performed SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis). Gels were cast in two steps, starting with the preparation of the 12% separating gel and after that the 5% stacking gel. For the separating 12% gel, for two gels it was mixed 4,9 mL of sterile water, 6 mL of 30% acrylamide mix, 3,8 mL of 1,5 M Tris buffer (pH 8.8), 150 µL of 10% Sodium Dodecyl Sulfate (SDS), 150 µL of 10% Ammonium Persulfate (APS), and 6µL of Tetramethylethylenediamine (TEMED). After setting up the glass and the gel case, the mixed solution was poured until reaching the 70% of the followed by the addition of 1 mL of butanol in order to smoothen the surface and prevent formation of bubbles. After 20 minutes, the butanol was poured out and the gel case was washed with 1 mL of water. The remaining water was dried with the help of Whatman sheets.

The 5% stacking gel was prepared by mixing 2,7 mL of sterile water, 670 µL of 30% acrylamide mix, 500 µL of 1 M Tris buffer (pH 6.8), 40 µL of 10% SDS, 40 µl of 10% APS, and

4 µl of TEMED. After its preparation, it was poured on the separating gel and incubated for 20 minutes. Later, 1 L of 1X running buffer was prepared (200 µL of 5X running buffer (15,1 g TRIS base, 94 g glycine, 900 mL distilled water, 50 mL 10% SDS) and 800 µL of sterile water). Before performing the SDS-PAGE for the Western Blot, the samples stored at -70°C were denaturalized in boiling water for 3 minutes. After loading the samples in the gel, initially it was run at 50 V for 30 minutes, and then at 85 V for 1.5 hours. Following the running, proteins that have been separated by gel electrophoresis were transfer to polyvinylidene difluoride (PVDF) by semi-dry blotting procedure and afterwards detected by incubation with specific antibodies.

After the electrophoresis, the gel soaked in transfer buffer and incubated for 15 minutes in a shaker. The polyvinylidene difluoride (PVDF) membrane (6 cm x 8,5 cm) was activated in methanol for 10 seconds and then incubated in transfer buffer for 10 minutes. Eight sheets of Whatman paper (6,5 cm x 8.5 cm) were also wet in transfer buffer. The transfer *sandwich* contained the following order: four pieces of Whatman sheets, the gel, the membrane, and four pieces more of Whatman sheets. The air bubbles were removed by gently applying pressure onto the stack with a blotting roller. Electroblotting was performed in Trans-Blot Turbo Transfer System (Bio-Rad) by administering SD Standard 1.0 A/blotted gel at 25V for 30 minutes.

For the hybridization, the membrane was washed in methanol for 30 seconds and then washed in 1X phosphate-buffered saline (PBST; 900 mL of sterile water, 100 mL of 10X PBS and 500 µL of Tween 20) for 5 minutes, two times. The membrane then was put in 50 mL of blocking buffer prepared with 2,5 g of nonfat milk powder and 50 mL 1X PBST, and incubated for 1-2 hours under gentle agitation, to prevent unspecific antibody binding. After the blocking step, the membrane was washed 2 times for 5 minutes with 1X PBST.

The blocked membrane was incubated with the respective antibody solution **(Table 7)**. After this, the blots were rinsed 3 times for 10 minutes with 1X PBST.

Table 7: Mix conditions for Western Blot at the washing with antibody solution step.

Mix of antibody solution for HA-tag detection		**Mix of antibody solution for GFP detection**	
2 mL	Blocking buffer	2 mL	Blocking buffer
8 mL	1X PBST	8 mL	1X PBST
5 µL	Anti-HA peroxidase High Affinity rat monoclonal antibody (20U) (Sigma, Germany)	5 µL	GFP antibody HRP (Miltenyi Biotec, USA)

The signal was detected using Clarity Western ECL Substrat. The membrane was covered with luminol/enhancer and peroxide solution (800 µl-800 µl) and incubated at room temperature for 30 seconds. After draining the excess ECL reagent, Bio-Rad ChemiDoc MP Imaging system with Chemi Hi-Resolution was performed for the imaging. The results were validated by Ponceau S staining to ensure that the loading was equal in all cases.

3.5.4. Quantitative PCR (q-PCR)

Quantification of the GFP mRNA level, was performed using q-PCR. mRNA was extracted as previously described, and then was transformed to complementary DNA (cDNA) by reverse transcription. For this, the Turbo DNase-Free Kit (Invitrogen by Thermo Fisher Scientific, USA) was used. To clarify the reagents and their volume used, see **Table 8**.

Table 8: Components and their volume used to complete digestion of DNA along with the removal of the enzyme and divalent cations post-digestion.

Component	Amount
RNA sample	5 µg
0.1 volume 10X DNase	5 µL
Turbo DNase enzyme	1 µL
Sterile water	X µL
Final volume of 50 µL	

After this, the samples were incubated at 37°C for 30 min, then 5 µL of 0.1 volume DNAse inactivation reagent was added, and incubated at room temperature for 5 minutes with occasional mixing. The samples were then centrifuged for 1.5 minutes at 13000 rpm, and then transferred to a new tube. After genomic DNA removal, the RT-PCR was carried out. In the **Table 9**, it can be seen the reagents and PCR program utilized.

Table 9: RT-PCR mix and program for reverse transcription.

1x RT-PCR mix		Program	
Sterile water	3.2 µL	25° C	10 min
10x RT buffer	2 µL	37°C	120 min
10X RT random primer mix	2 µL	85°C	5 min
25X dNTPs mix (100 mM)	0.8 µL	4°C	HOLD
DNA inhibitor (Ribolock 40 U/µL)	1 µL		
Transcript	1 µL		

A dilution of 5 times (5x) the RT-PCR product was done, mixing 32 µL of sterile water with 8 µL of the RT-PCR product. The q-PCR was then performed with two mixes: the GFP mix to measure the amount of cDNA containing the GFP gene, and the UBI mix, to measure the amount of cDNA containing the ubiquitin-ribosomal protein gene (ubi3). This last one is the housekeeping gene that is expressed in a stable way regardless of tissue type, developmental stage, cell cycle state, or external signal. The composition of both mixes can be seen in **Table 10**.

Table 10: Reagents and its volume used for q-PCR.

GFP mix		**UBI mix**	
5X HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia)	3 µL	5X HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia)	3 µL
Sterile water	7.6 µL	Sterile water	7.6 µL
Primer GFP_Taq_Fw 10X	0.7 µL	Primer Ubi3_Fw 10X	0.7 µL
Primer GFP_Taq_Rv 10X	0.7 µL	Primer Ubi3_Rv 10X	0.7 µL
Template	3 µL	Template	3 µL

3.6. Imaging and statistical analysis

For the analysis of fluorescence intensity, it was used ImageJ 1.54g software (Schneider *et al.*, 2012) as stated in Weligodage *et al.* (2023), and for the measuring of the intensity of the bands obtained after Western blotting, it was used Image Lab software 6.0.1 (Bio-Rad Laboratories, 2017).

The normality of the data was evaluated with the Shapiro-Wilk test and the equality of variances with Bartlett's test. After normality of the data and equality of variances were met, the ANOVA test was used to verify if there were significant differences between the different conditions. An α value of 0.05 was considered. GraphPad Prism 8.0.1 was used for data analysis.

4. Results

4.1. Amplification and comparison of MP encoded by different CVA strains

After performing a gradient PCR to define the best annealing temperature for the primers used **(Figure 6A)**, which resulted to be 62°C, the coding region of the movement protein of the CVA strains from four Hungarian *Prunus* host cultivars (sweet cherry, plum, sour cherry and apricot) were amplified. The size of the movement protein coding sequences was about 1300 bp **(Figure 6B)**.

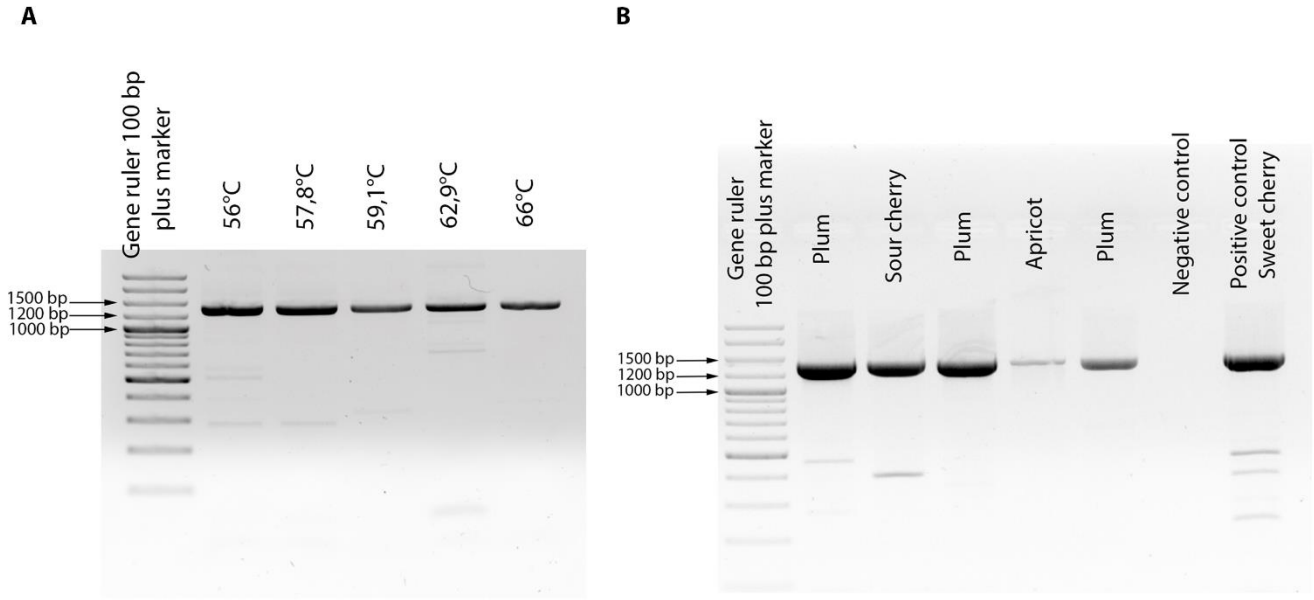


Figure 6: Amplification of MP coding region of CVA. **(A)** PCR products of PCR reactions using different annealing temperatures were separated on 1.2% Agarose gel in order to test which would be the best annealing temperature for the primers of the MP amplification using Q5 polymerase. The temperature chosen was 62°C. The marker utilized was Gene ruler 100 bp plus. **(B)** 1.2% Agarose gel separation of the PCR products corresponding to the movement protein of plum, sour cherry, apricot CVA strains, positive control (MP of a sweet cherry strain), and the negative control (water). The size of the movement protein is around 1300 bp.

After extraction and purification of the PCR products from the gel, the DNAs were cloned in a pJET1.2/blunt vector and transformed into competent cells of *E. coli*. After making liquid cultures from three transformed colonies, a miniprep was performed for isolation of the plasmid. The purified plasmid was digested with XbaI and XhoI restriction enzymes, in order to determine if the PCR products were successfully ligated into the plasmid or not. The isolation of the plasmids containing the inserts was successful and the digested products of the expected size for the MP construct appeared on the gel (2974 bp plasmid + ~1300 bp MP) **(Figure 7)**.

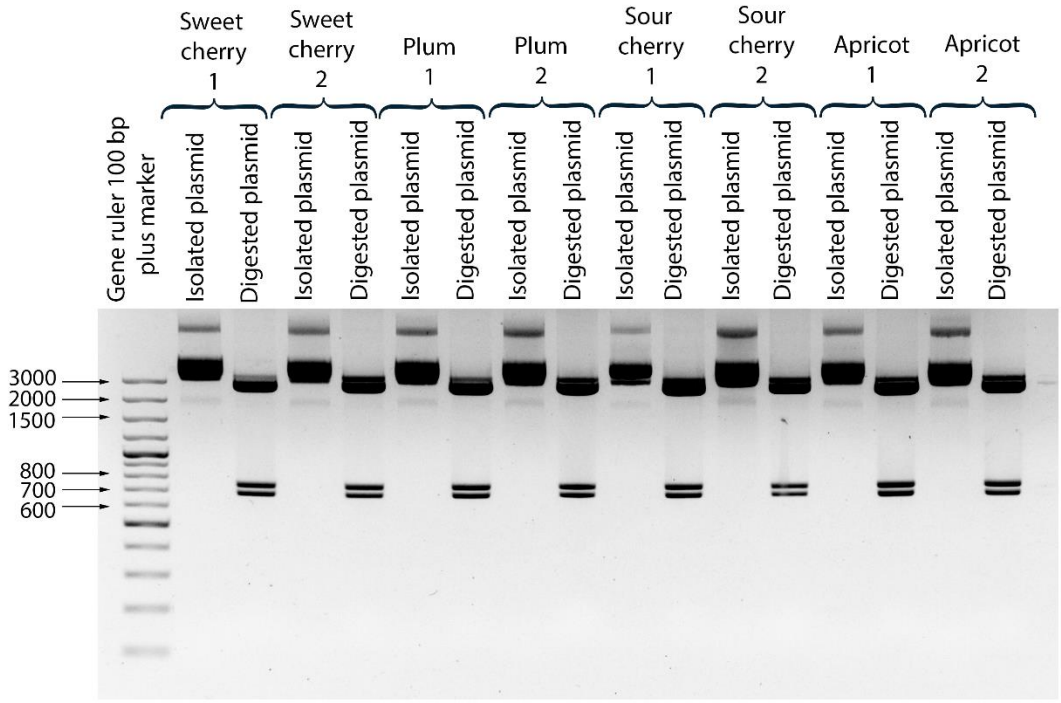


Figure 7: Separation of the purified plasmids (pJET1.2 blunt vector (2974 bp) with the insert of MP (~1300 bp) before and after digestion with two restriction enzymes (XbaI and XhoI). The marker utilized was Gene Ruler 100 bp plus.

The plasmids with the inserts originating from the MP of sweet cherry, plum, sour cherry, and apricot infecting CVA strains were Sanger sequenced (sequences can be found in **Annex 1**). The sequences were analyzed using bioinformatic methods and revealed that sequences of CVA MPs of sweet cherry and sour cherry strains were highly similar, and also plum and apricot were alike. Considering that the MP encoded by the highly similar sequences were identical at the amino acid level, the next experiments were carried out only with the MP encoded by the sour cherry (same protein as sweet cherry) and apricot (same protein as plum) CVA strains.

Based on the determined sequences in-fusion primers were designed, a PCR was conducted using these primers and its success was analyzed on a gel (**Figure 8A**). The amplified DNA was extracted and purified from a 1.2% Agarose gel. For efficient and direct cloning of the insert into BinHASanyi, an In-Fusion reaction was performed. The in-fusion product was transformed into of *E. coli* cells. The colonies obtained were then cultured on master plates with LB+K medium for storage, and also in LB+K liquid culture for Colony PCR to confirm the presence of the full desired genetic insert in the transformed vector. As seen in **Figure 8B**, it was successful for three colonies in the case of the MP from sour cherry and three colonies in the case of the MP of CVA from apricot strain. One colony bearing the constructs was chosen to carry out the VSR assays.

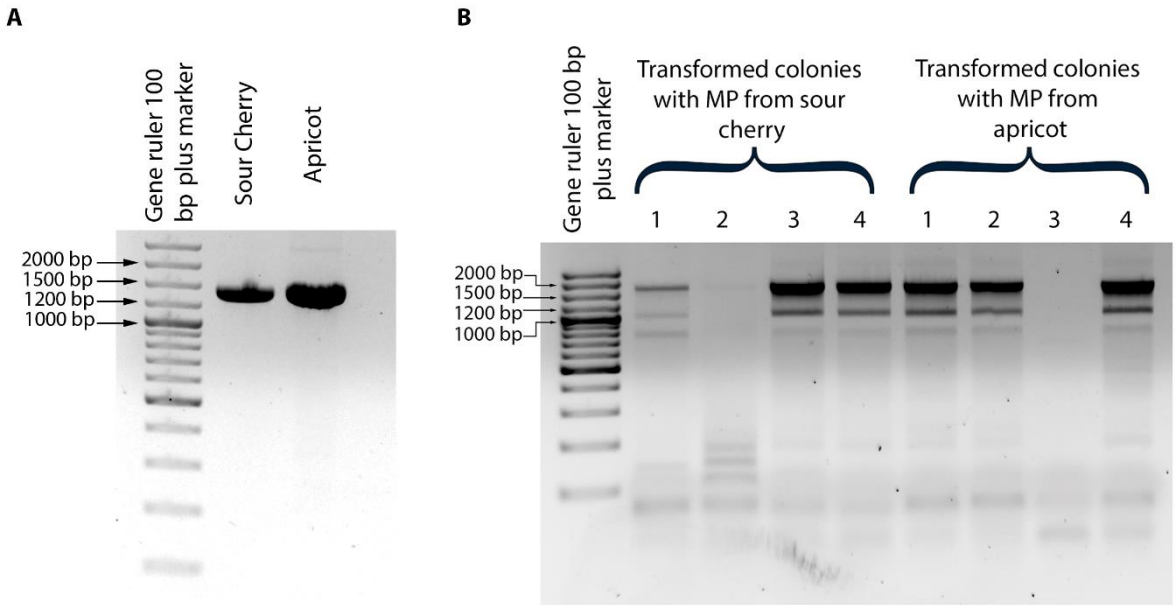


Figure 8: Successful cloning of insert into BinHASanyi plasmid. **(A)** 1.2% Agarose gel of in-fusion PCR of sour cherry and apricot strains. The marker utilized was Gene Ruler 100 bp plus. **(B)** 1.2% Agarose gel of the colony PCR from transformed E. coli with the BinHASanyi vector and the insert of MP from sour cherry and apricot CVA strains. The marker utilized was Gene ruler 100 bp plus.

4.1.1. Bioinformatic analysis of MP sequences

The sequences of the MP from CVA from the different host species were compared using multiple sequence alignment showing that there was a big similarity between sour cherry strain and sweet cherry strain, and between plum and apricot strain, forming two groups that were slightly different from each other **(Figure 9)**. The alignment showed that most of the amino acid changes are at the end of the sequences. Some of these amino acids' substitutions were conservative and some were not. The type of non-conservative amino acid substitutions was further analyzed **(Table 11 in Annex 2)**. The most common changes were from Aspartic Acid (D) to Asparagine (N) (acidic to amine amino acid), Serine (S) to Glycine (G) (nucleophilic to small amino acid), and Lysine (K) to Glutamic Acid (E) (basic to acidic amino acid).

Additionally, these sequences were further compared with other CVA MP sequences obtained from the GenBank database and based on this analysis, a phylogenetic tree was constructed **(Figure 10)**. The tree clearly shows two different groups for the MP of CVA. On one side there is sour cherry and sweet cherry together, and on the other apricot and plum together, belonging to two big different clusters.

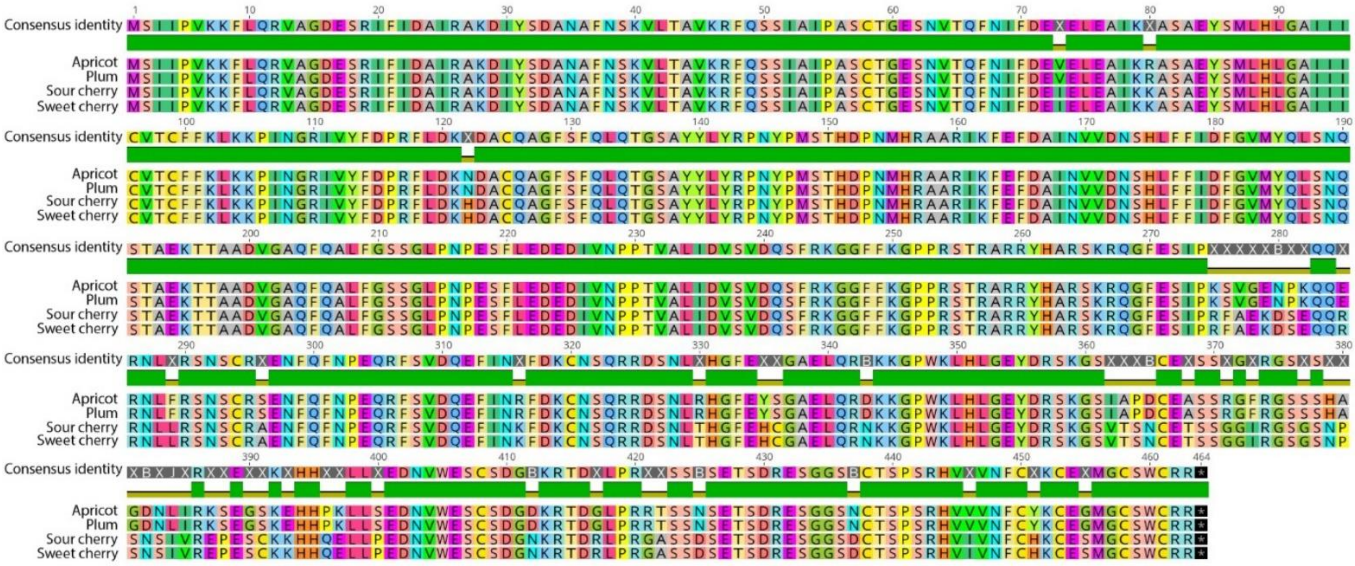


Figure 9: Multiple alignment between sequences of MP of CVA strains from four Hungarian hosts (sweet cherry, sour cherry, plum, and apricot), comparing them with the consensus sequence or reference genome (GenBank: Accession No. NC_003689.1). Sweet cherry and sour cherry shared a big similarity, as well as plum and apricot. Both groups are slightly different from each other. The spaces observed in the green bar across the whole sequence show the amino acid substitutions.

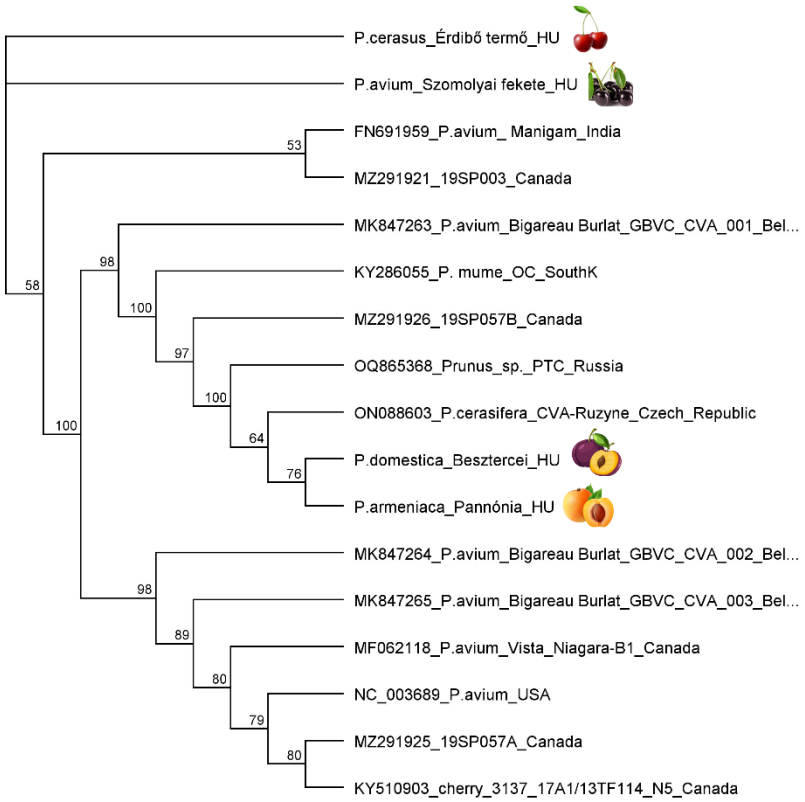


Figure 10: Phylogenetic tree showing the differences between the MP sequences of CVA obtained from the four Hungarian hosts and the MP sequences obtained from the GenBank database.

4.2. Testing whether MP is a suppressor of RNA silencing

4.2.1. *Agrobacterium*-mediated transient assay results

4.2.1.1. Transient protein expression and verification of the correct size of the expressed protein

Before testing the VSR activity of CVA MPs, it was crucial to confirm that the protein was correctly expressed and was of the expected size. To achieve this, *Agrobacterium* containing P14 VSR (a well-known VSR that has a wide range effect on the RNAi to protect the MP protein from degradation), GFP, and CVA coding sequencing were infiltrated into the plants **(Figure 11A)**. Following infiltration, the expression of the HA-tagged protein was tested via Western blot analysis. The results confirmed that the HA-tagged protein was successfully expressed in the plants, with a size of 46.6 kDa for the protein and an additional 1.1 kDa for the HA tag **(Figure 11B)**.

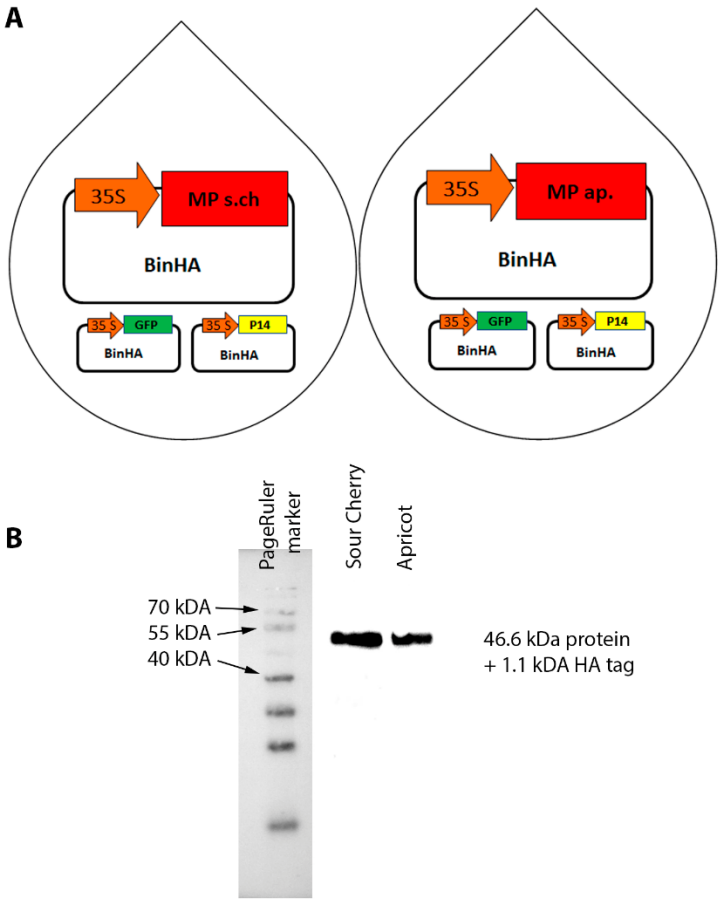


Figure 11: Protein expression and sizing verification. **(A)** Illustration of how the infiltration was performed to verify if the protein was successfully expressed and if it was of the expected size. **(B)** Western blot analysis of MP protein in infiltrated leaf regions using anti-HA tag antibody. The volume was of 25 µL and the marker used was PageRuler Prestained Protein Ladder. The MP is 46.6 kDa + the 1.1 kDA HA tag.

4.2.1.2. Local silencing signal analysis

To evaluate the VSR activity of the MP of sour cherry and apricot CVA strains, it was assessed whether the local silencing was inhibited or not. With this objective, a co-infiltration experiment of GFP-expressing Agrobacteria and the potential VSR coding ORFs in wild-type of *N. benthamiana* was carried out. As a negative control BinHA empty vector, as a positive control P21 with a strong VSR activity were infiltrated into the same leaf where MP of CVA strain from sour cherry and the MP of CVA strain from apricot **(Figure 12A**) was infiltrated.

The plants were examined from the 3.5 days. During these days, the defense silencing mechanism of the plants is activated because of the expression of GFP, which was the case in the negative control that displays a faded fluorescence **(Figure 12B).** If the GFP is co-infiltrated with a protein that has a suppressor activity, the fluorescence signal stays stronger, like in the case of the positive control. If there is no protein to inhibit the RNA silencing response, the GFP will be degraded making the GFP fluorescence signal faded or weaker. In both the MP of CVA strain from sour cherry and apricot, the signal seems stronger than that of the negative control but weaker than that of the positive control.

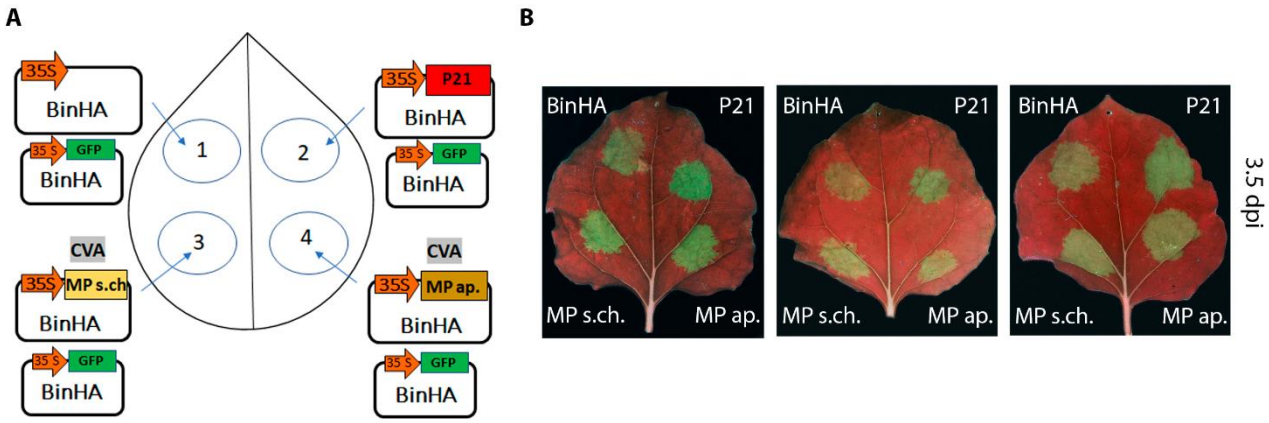


Figure 12: Local silencing signal analysis. **(A)** Illustration of how was done the co-infiltration of GFP-expressing Agrobacteria and the potential VSR coding ORFs (negative control with the BinHA empty vector, the positive control with the strong VSR P21, the MP of CVA strain from sour cherry and the MP of CVA strain from apricot). **(B)** Leaves of co-infiltrated plants showing the green signal of GFP in the infiltrated patches.

To support the differences between the GFP fluorescence observed visually, the intensity of the fluorescence in every patch was measure with the ImageJ software **(Figure 13A)**, and discs of the same infiltrated zone of the leaf were collected for RNA and protein extraction, in order to measure the GFP mRNA and GFP protein expression level by q-PCR and by Western blot, respectively. The fluorescent intensity supported the results observed visually. The GFP mRNA

levels were the lowest in the case of the negative control, followed by the MP of CVA strain from apricot, and lastly with the higher levels the MP of CVA strain from sour cherry **(Figure 13B)**. It was found a 1.38-fold change for the sour cherry strain, and 1.22-fold change for the apricot strain, both compared with the negative control. In the case of the positive control, the levels of p21 GFP mRNA were not as high as expected, being even lower than in the case of the negative control. This might have happened because of a technical difficulty given to the fact that mRNA molecules are sensitive and can sometimes degrade, making it hard to see a correlation between the amount of the mRNA and the proteins extracted that are already expressed and not as sensitive. mRNA levels should not be viewed as the definitive measure of gene expression because mRNA and protein expression levels often do not correlate. Therefore, it is more effective to supplement mRNA data with protein analysis (Buccitelli & Selbach, 2020; Koussounadis *et al.*, 2015).

The results of the Western blot visually show that the levels of the GFP protein expression are higher in the positive control, followed by the MPs of CVA strains from sour cherry and apricot, and in the last place with the lowest level of protein, the negative control **(Figure 13C).** The equal loading was verified by the usage of Ponceau Staining of the membrane. These differences observed visually after the Western blot, were supported later by the measuring of the intensity of the bands with the Image Lab software **(Figure 13D)**.

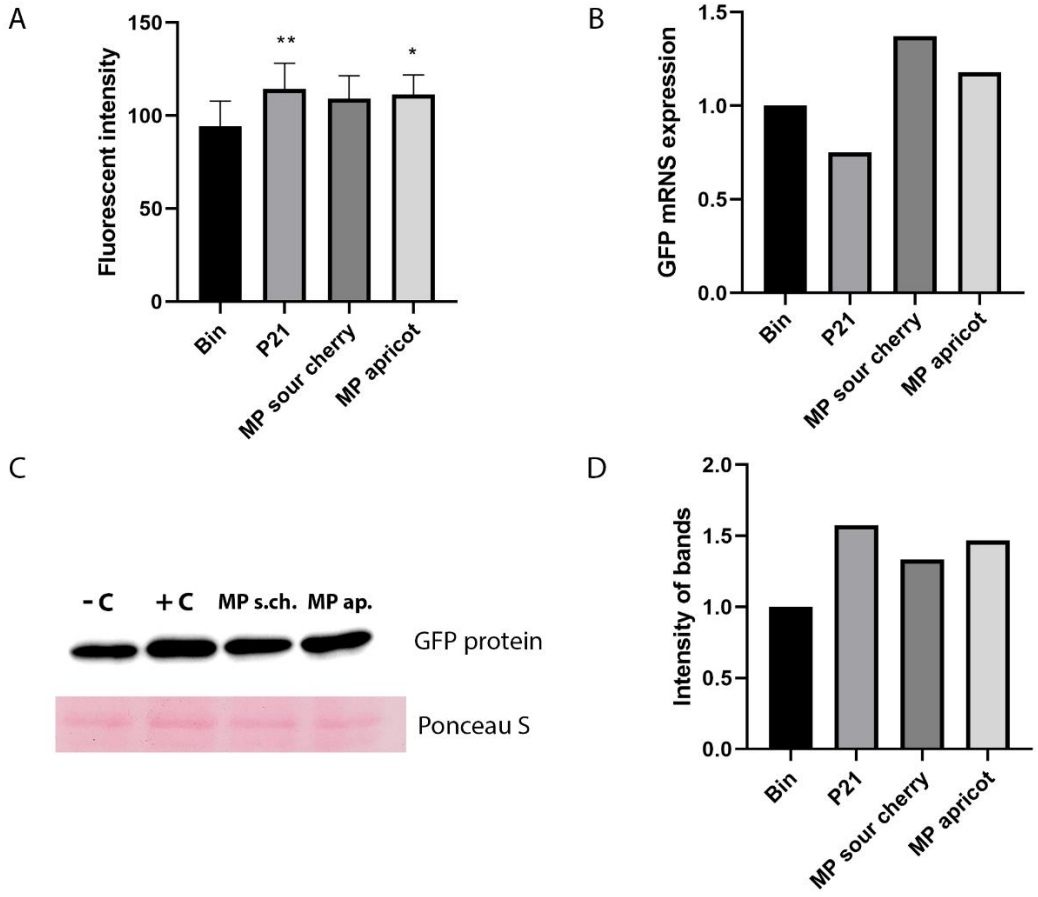


Figure 13: Local silencing signal molecular analysis. **(A)** Intensity of the fluorescence in every patch infiltrated measured with ImageJ software. Asterisks indicate significant differences from the negative control; *p-value* < 0,0001. Error bars indicate the standard error (Bin SD= 13,53; p21 SD= 13,75; MP from sour cherry strain SD= 12,29; MP from apricot strain SD= 10,47) **(B)** Determination of GFP mRNA expression in infiltrated patches by q-PCR (Bin SD= 0,07; p21 SD= 0,24; MP from sour cherry strain SD=0,08; MP from apricot strain SD=0.15) **(C)** Examination of the amount of GFP protein by Western blot. The equal loading was verified by Ponceau S staining of the membrane after Western blotting. **(D)** Intensity of the bands obtained after Western blotting.

4.2.1.3. Testing the systemic VSR activity of the CVA MPs

To evaluate if the MP from sour cherry and apricot CVA strains were inhibiting the systemic silencing, a co-infiltration of GFP transgenic *N. benthamiana* plants (line 16c) with GFP-expressing Agrobacteria and the potential VSR coding ORFs (negative control with the BinHA empty vector, positive control with a strong systemic silencing suppressor p19, MP of CVA strain from sour cherry and MP of CVA strain from apricot) was carried out. GFP transgenic plants allows to see how the silencing signal is spreading in the plant. If the silencing mechanism is induced, the GFP will degrade. This can visually be detected by a red fluorescence signal emitted by the chlorophyll molecules as a red halo (around the infiltrated patches) or red veins (in the new emerging leaves) (Roth, 2004, Yaegashi *et al*., 2007). As presented in **Figure 14**, in the patches infiltrated with the positive control, the GFP fluorescence signal was stronger than in the other conditions, as in the patches infiltrated with the negative control the signal is lower and more faded than in the presence of the other constructs. In the case of the infiltration with MP of CVA strain from sour cherry, and the MP of CVA strain from apricot, it can be seen that the signal is weaker than in the positive control, and its intensity is comparable to that of the negative control.

The RNA silencing can spread between cells through plasmodesmata at short distance, and via phloem at long distances (Yaegashi *et al*., 2008). The short distance silencing can be detected in a 10-20 cell distance from the infiltrated zone, which will be visualized as the red halo around the infiltration patch previously mentioned, as it was the case of the negative control and both MPs from sour cherry and apricot CVA strains at the 7 dpi **(Figure 14)**. In the case of the p19 infiltrated plants (positive control), it was never seen a red halo around the infiltration patch along the experiment. The long-distance RNA silencing can be detected as red veins that are formed in the younger leaves. The negative control plants showed a higher number of leaves showing red veins, sign that the GFP signal is being silenced, while on the case of the potential VSRs of CVA strains from sour cherry and apricot, this was detected but in a smaller amount. In the case of the positive control, it was never detected any leaf showing red veins.

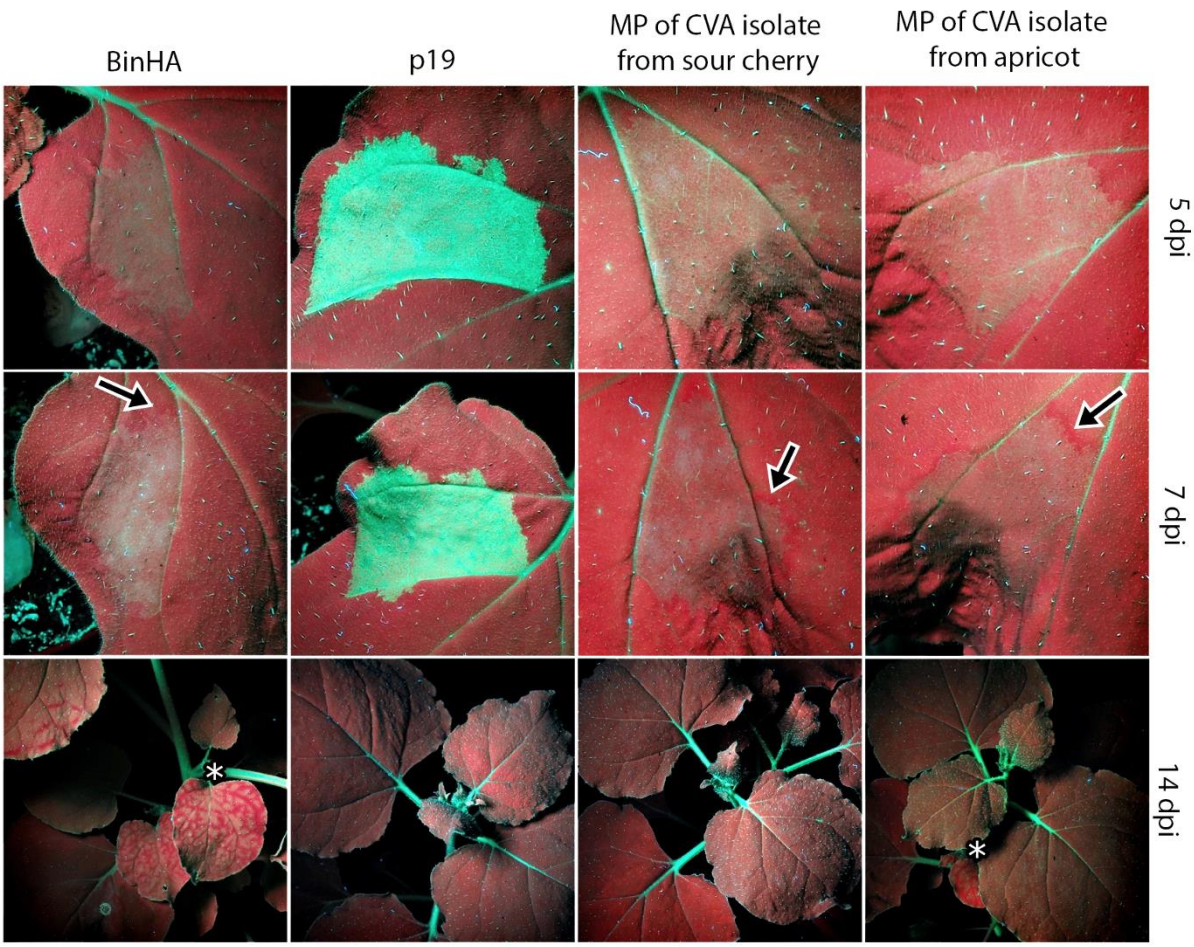


Figure 14: Systemic silencing signal analysis. Transgenic GFP *N. benthamiana* plants co-infiltrated with GFP-expressing Agrobacteria and the potential VSR coding ORFs (negative control with the BinHA empty vector, the positive control with the strong VSR P21, the MP from sour cherry strain and the MP from apricot strain) at 5, 7 and 14 dpi. The arrows show the red halo around the infiltration path, and the asterisks show the new leaves with red veins.

In order to further analyze the systemic silencing response and whether it is being inhibited by the potential VSRs, it was counted the number of leaves **(Figure 15A)** and the quantity of plants and leaves per plant that showed RNA silencing from 7 until 21 dpi. Once finished the experiment, it was calculated the percentage of plants and leaves per plants that showed silencing signals per dpi **(Figure 15A and B)**. The first graph shows that the percentage of leaves showing silencing are highly significative in the case of the negative control comparing to the positive control or with the MP from both strains. On the other hand, when comparing the percentage of plants with systemic silencing, it can be seen that MP from sour cherry strain does not differ from the negative control showing high systemic silencing from the 14 dpi, the positive control shows a null percentage during the whole experiment, and the MP from apricot strain remains as a middle percentage between both controls.

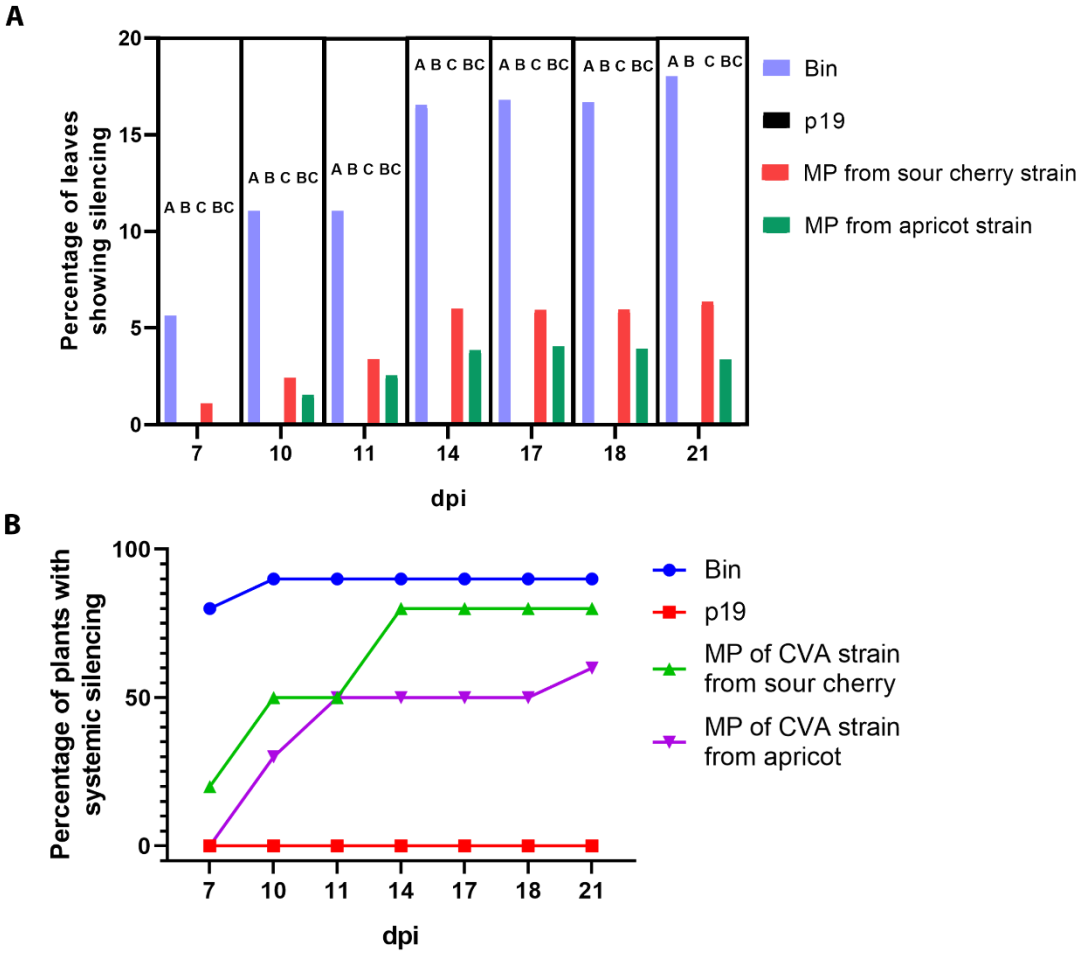


Figure 15: Analysis of plants and systemic silencing signal. **(A)** Percentage of number of leaves that showed silencing per treatment at 7, 10, 11, 14, 17, 18, and 21 dpi. Different letters mean statistically significant differences; *p-value* < 0,0001. **(B)** Percentage of plants that showed systemic silencing per treatment at 7, 10, 11, 14, 17, 18, and 21 dpi.

4.3. Investigation of the CP coding sequences of different CVA strains

Primers were designed to amplify the possibly CVA CP coding sequence. The best annealing temperature for the primers and polymerase were optimized using different annealing temperatures in gradient PCR and showed that the best temperature was 57°C **(Figure 16A)**. After this step, the coding region of the coat protein of the four Hungarian CVA strains originating from different *Prunus* host cultivars (sweet cherry - Szomolyai fekete; plum – Besztercei; sour cherry – Érdi bőtermő; and apricot – Pannónia) were amplified by PCR. The size of the coat protein is of around 600 bp **(Figure 16B)**.

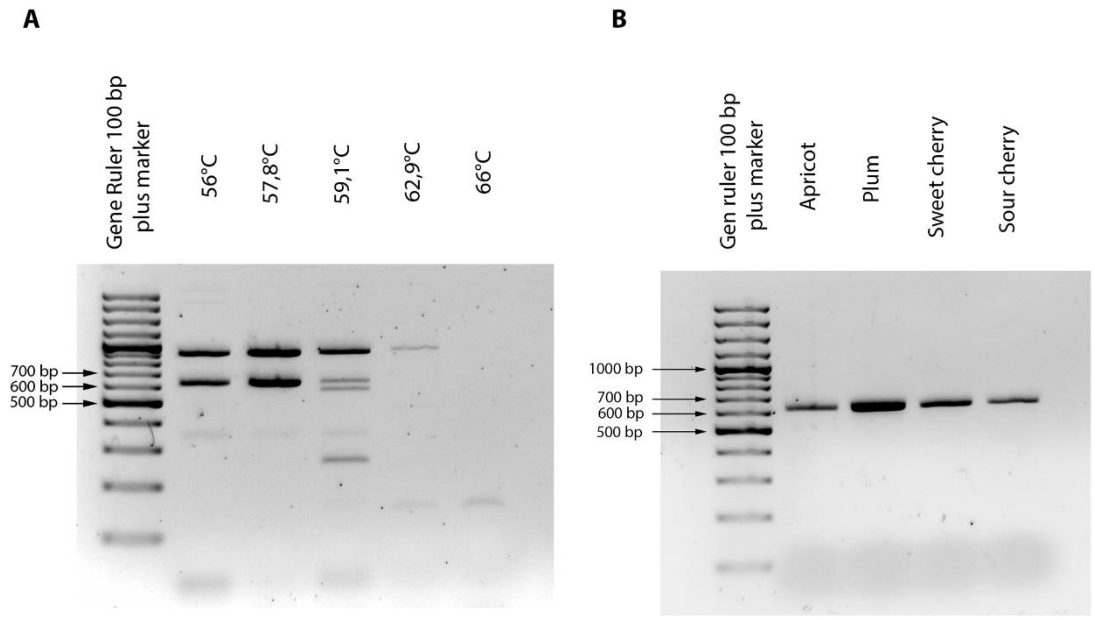


Figure 16: The CP coding region was successfully amplified. **(A)** 1.2% Agarose gel of gradient PCR products performed to test which would be the best annealing temperature for the primers of the coat protein and Q5 polymerase. The temperature chosen was 57°C. The marker used was Gen ruler 100 bp plus. **(B)** 1.2% Agarose gel showing the marker (Gen ruler 100 bp plus), the DNA corresponding to the coat protein of sweet cherry, sour cherry, plum and apricot. The size of the coat protein is around 600 bp.

PCR products from different CVA strains were extracted from the gel, purified and were cloned into pJET1.2/blunt vector. Competent cells of *E. coli* were then transformed, and after liquid culture, three colonies were chosen for plasmid purification. After isolation of the plasmid and digestion with XbaI and Xhol restriction enzymes, it was confirmed that the plasmid contained the inserts and the size for the CP construct and the digested plasmids were of the expected size (2974 bp plasmid + ~600 bp CP) **(Figure 17)**. Samples were then sent for sequencing (sequences can be found in **Annex 1**).

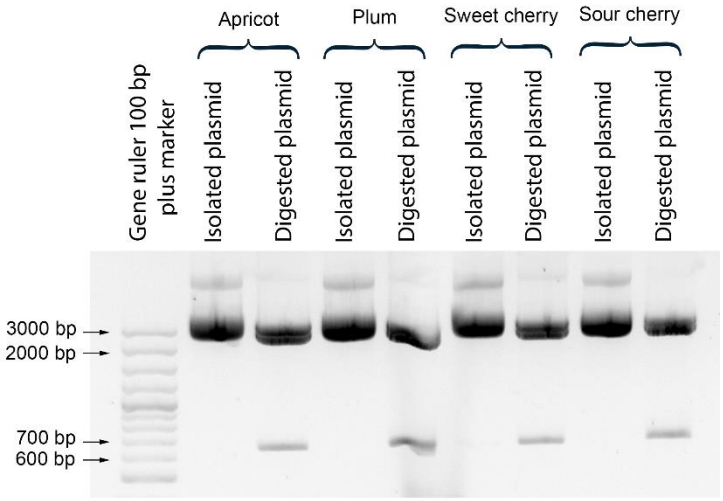


Figure 17: Isolation and digestion of pJET1.2 blunt vector (2974 bp) with the insert of CP (~600 bp) with two restriction enzymes (XbaI and XhoI). The marker utilized was Gene Ruler 100 bp plus.

4.3.1. Bioinformatic analysis of CP sequences

The sequences of CVA CP obtained from Hungarian hosts (apricot, plum, sour cherry, and sweet cherry) were compared by a MUSCLE multiple alignment, showing that they are highly similar and there is only one substitution of amino acids, which is different only in the sweet cherry host in the position 29, where Isoleucine is changed for Valine **(Figure 18)**.

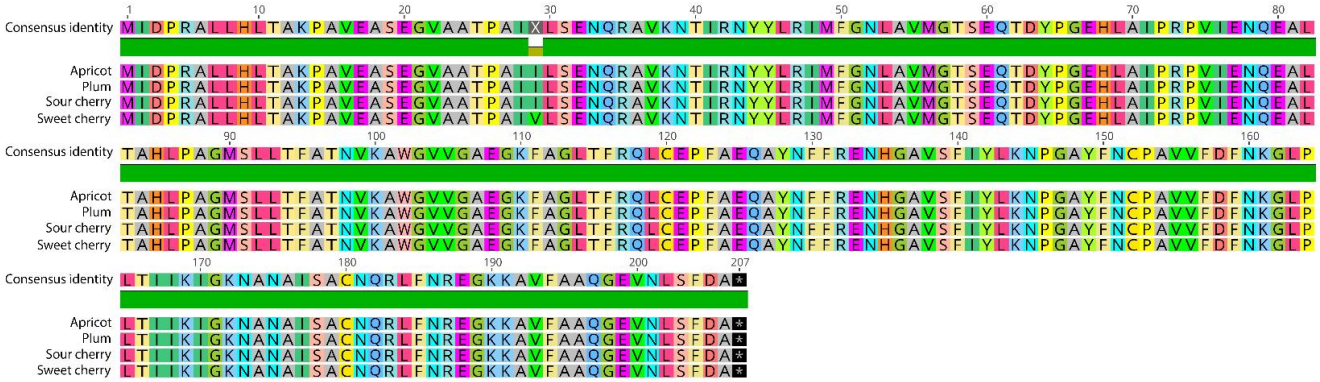
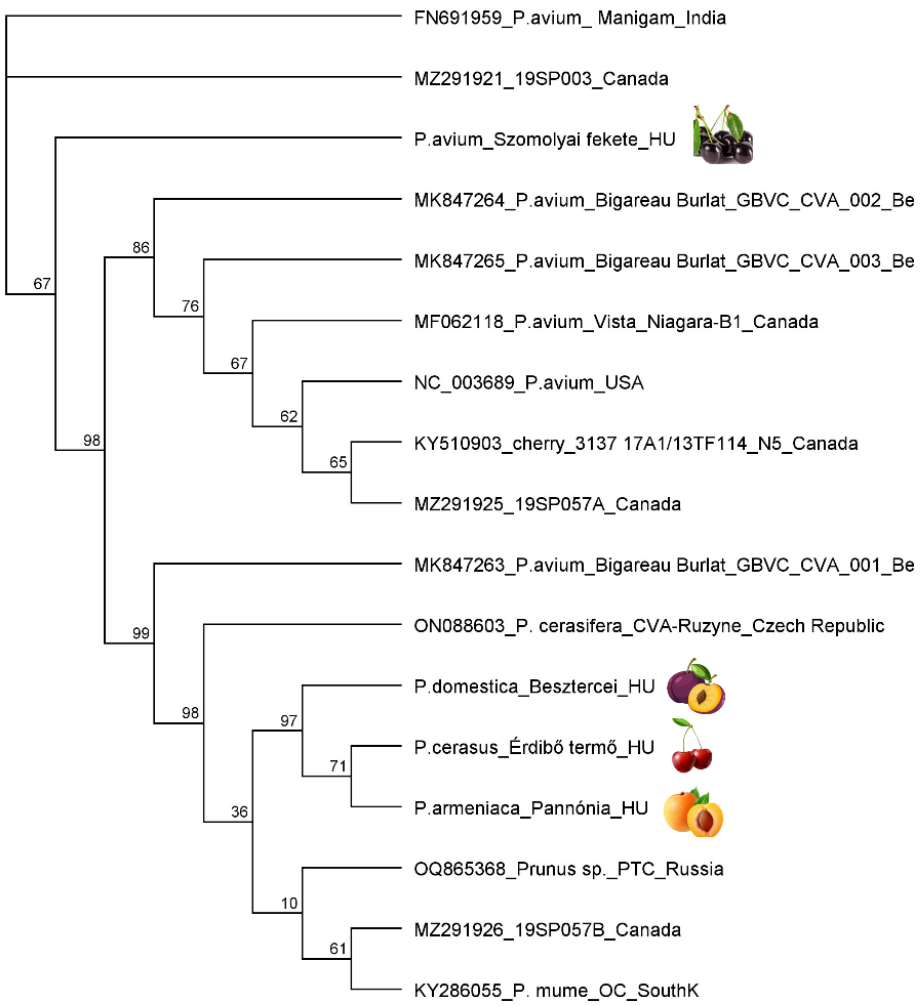


Figure 18: Multiple alignment between sequences of CP of CVA from four Hungarian hosts (sweet cherry, sour cherry, plum, and apricot), comparing them with the consensus sequence or reference genome (GenBank: Accession No. NC_003689.1).

For further analysis, the sequences of these CVA CPs originating from Hungarian hosts were compared with additional sequences retrieved from GenBank. A phylogenetic tree was constructed, revealing that plum, sour cherry, and apricot form a single cluster. In contrast, sweet cherry is located in a distinct and distant cluster **(Figure 19)**. Although there is only one substitution at the amino acid level, there are differences at the nucleotide level.



Distance Matrix

	P.armeniac...	P.avium_Sz...	P.cerasus_É...
P.armeniaca_Pannónia_...		86.312%	100%
P.avium_Szomolyai feket..	86.312%		86.312%
P.cerasus_Érdibő termő...	100%	86.312%	
P.domestica_Besztercei_...	99.678%	86.312%	99.678%

Figure 19: Phylogenetic tree showing the differences between the MP sequences obtained from the four Hungarian hosts and the CP sequences obtained from the GenBank database with its corresponding distance matrix.

5. Discussion

The primary objective of this study was to investigate potential VSR activity of proteins encoded by CVA infecting four different host species. The VSRs can act by inhibiting many possible steps of the pathways of RNA silencing in plants, and the way of inhibition of it can be different. There are two types of suppression of RNA silencing in plants: local and systemic (Roth, 2004). Even though a protein can act as a powerful local suppressor, it may have a lesser or null effect as a systemic suppressor, like in the case of helper component–proteinase (HC-Pro) protein encoded by a potyvirus (Mallory *et al.*, 2001). On the other hand, a protein that mostly affects systemic silencing and has a smaller effect on local silencing can be exemplified by P25 protein of Potato virus X (PVX) (Brosseau & Moffett, 2015). In the case of this work, the inhibition of the local silencing of the MP protein whether it comes from sour cherry or apricot CVA strains during the visual detection, showed a GFP fluorescence signal weaker than the positive control (p21). However, this signal was stronger than the one showed by the negative control. This difference was also supported by the fluorescence intensity analysis; and molecular analysis, in which the GFP mRNA of the MP of CVA strain from sour cherry is 1.38-fold change compared with the negative control, and for the one proceeding from the apricot strain is 1.22. In addition, the levels of GFP protein are higher in the case of the strains than in the negative control. These results suggest that MP from both sour cherry and apricot strains act as weak local VSRs **(Figures 12 & 13)**.

GFP transgenic plants enable visualization of the spread of the silencing signal within the plant. When the silencing mechanism is activated, GFP degrades, which is visually detectable by a red fluorescence emitted by chlorophyll molecules, appearing as a red halo around infiltrated patches or red veins in newly emerging leaves (Roth, 2004; Yaegashi *et al.*, 2007). In the case of the analysis of the suppression of the systemic silencing in this work, it can be seen that the percentage of plants with systemic silencing for the sour cherry strain, was small in the beginning, but later was not different than the negative control. However, the percentage of leaves presenting red veins was lower than in the negative control, meaning that at all, it may be acting as a weak suppressor of the systemic response. Additionally, it could be said that it did not inhibit the mechanism of silencing but it delayed the spreading of the silencing signal. For the MP of the CVA apricot strain, the percentage of plants showing systemic silencing was maintained during the whole experiment as a mean between both controls, showing that its suppressor activity of the systemic signaling is stronger than the one of sour cherry strain, but not as strong as p19 **(Figure 15B)**. Although the red halo was detected in the case of the apricot variant, which means that it did not inhibit the short distance spread of the silencing signal, eventually it delayed the long-

distance spread. This is something that is observed when considering the percentage of leaves that showed red veins, which is a very low number.

There are additional studies in the literature that have identified MP or CP as VSRs. Particularly for MP as a VSR, examples include the citrus leaf blotch virus (CLBV), apple chlorotic leaf spot virus (ACLSV), and potato virus M (PVM), all of which belong to the same family as CVA, *Betaflexiviridae* (Renovell *et al*., 2012; Senshu *et al*., 2011; Yaegashi *et al*., 2008; Yaegashi *et al*., 2007).

CBLV is a virus that belongs to the genus *Citrivirus*, and encodes a 227 kDa replication protein, a 40 kDa MP, and a 41 kDA CP. In *Agrobacterium*-mediated transient assays in *N. benthamiana* line 16c, CLBV MP demonstrated the ability to suppress local GFP silencing but not short or long-distance spread of the silencing signal. This suppression was evidenced by a visible increase in green fluorescence at 5 dpi in areas co-infiltrated with the MP, while a characteristic silenced red ring was observed by 11 dpi. Interestingly, in this study, this red ring phenomenon was observed as early as 7 dpi, indicating a similar but slightly accelerated response of the defense system of the plant.

ACLSV belongs to the *Trichovirus* genus, and its genome encodes a 216 kDa replication protein, a 50 kDa MP called P50, and 21 kDa CP. In *Agrobacterium*-mediated transient assays on *N. benthamiana* 16c, P50 was shown to suppress systemic but not local silencing. Contrasting this with the findings for CVA, the MPs from both the sour cherry and apricot strains exhibited higher levels of GFP mRNA compared to the negative control but lower compared to the positive control p19 in both strains, indicating weak local silencing suppression. However, ACLSV's P50 did not alter GFP mRNA accumulation compared to the negative control, indicating its inability to suppress local silencing. As for the systemic silencing, P50 proved to be more efficient than the MPs in this work since at the 14 dpi P50 displayed only 26.6% of partial disappearance of the GDP fluorescence, while in the case of the CVA it was 80% and 50% for the MP from sour cherry and apricot strain respectively.

The genome of PVM, a member of the genus *Carlavirus,* encodes a replicase protein, a triple gene block proteins 1, 2, and 3 (TGBp1, -2, and -3) that function as MPs, a CP, and nucleic acid-binding protein. In *Agrogacterium*-mediated assays in GFP-expressing *N. benthamiana* line 16c, TGBp1 inhibited the systemic spread of GFP silencing at short and long-distance preventing the appearance of a red ring around the infiltrated area, and with an efficiency of 35.7%. In this case, TGBp1 appears to be a stronger systemic VSR than the MPs from CVA strains comparing with their efficiency previously mentioned.

Taking into consideration the next objectives of this work, both the MP and CP sequences were amplified and compared among the strains, showing that in the case of MP, sour cherry and

sweet cherry seemed to be more related, while apricot and plum seemed to share more similarity **(Figure 10)**. However, in the case of CP, the ones that shared more similarity were plum, sour cherry and apricot **(Figure 19)**, leaving sour cherry in a different distant cluster. Although there was not a high variability between the protein sequences of CP, there was differences between the nucleotide sequences. This could explain this division in different clusters of the strains.

In the case of MP, most of the amino acid substitutions were at the end of the sequences, with non-conservative substitutions from acidic to amine amino acid, nucleophilic to small amino acid, and basic to acidic amino acid. Several studies provide evidence that substitutions in amino acids of viral proteins can significantly impact their function as VSRs in plants. Naturally occurring amino acid substitutions in VSR proteins can enhance viral adaptation and influence suppressor activity, as in the case of alterations in the P0 protein of poleroviruses, that specific changes in amino acids can lead to variability in their ability to suppress RNA silencing across different virus strains (Cai *et al.*, 2023). Another study on the P0 protein from Pea mild chlorosis virus (PMCV) demonstrates that particular amino acid deletions and mutations affect its suppressor function. This indicates that the VSR activity of P0 proteins is highly sensitive to specific amino acid compositions (Sun *et al.*, 2020). This could be the case of the MP protein from CVA as well since the substitutions taken place in this work can alter the structure and function of the protein, potentially altering its ability to bind RNA or interact with other proteins, or alter its stability, which will later impact its VSR activity.

In the future, it will be important to further characterize the VSR activity found in the MP of CVA from apricot and sour cherry strains. Specifically, it will be critical to determine the exact step of the plant silencing mechanism pathway in which they act and to understand how amino acid differences in the MPs between strains influence their VSR capabilities. Additionally, comparing the results of this study with the potential ability of CP to act as a suppressor, which was not explored due to time reasons, would be highly valuable. If CP is found to function as a suppressor, its activity should be further characterized in detail.

6. Conclusions

- MPs from sour cherry and apricot infecting CVA strains act as weak local VSRs.
- The MP of the CVA apricot strain showed stronger suppressor activity of the systemic signaling than the one of sour cherry strain, but not as strong as p19. It did not inhibit the short distance spread of the silencing signal, but it may be a moderate suppressor of the long-distance spread.
- The MP from the sour cherry strain, on the other hand, may act as a weak suppressor of the systemic response. It did not inhibit completely the mechanism of silencing, but it delayed the spreading of the silencing signal.
- When compared MP and CP sequences among the strains: In the case of MP, sour cherry and sweet cherry seemed to be more related, while apricot and plum seemed to share more similarity, and this may have been because of differences at the amino acid level; In the case of CP, the ones that shared more similarity were plum, sour cherry and apricot, leaving sour cherry in a different distant cluster, and this may have been because of differences at the nucleotide level.
- Amino acid substitutions in MP sequences of CVA strains may potentially influence their different VSR activity.
- Further research should focus on characterizing the exact steps of the plant silencing pathway affected by these MPs and exploring the potential suppressor activity of CPs.
- Future studies should also investigate the suppressor activity of CP, which was not examined in this study, to provide a more comprehensive understanding of the VSR capabilities of CVA proteins.
- This study provides valuable insights into the role of MP proteins from CVA strains in the suppression of RNA silencing in plants, and these findings contribute to our understanding of viral adaptation and the mechanisms of RNA silencing in plants.

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Annex 1

>CVA_MP_sweetcherry

ATGTCGATCATACCAGTCAAGAAGTTTCTCCAAAGAGTTGCAGGAGATGAATCAAGAATATTCATTGACG
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>CVA_MP_plum

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>CVA_MP_sourcherry

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Annex 2

Table 11: List of amino acid substitutions along with their position in the sequence of CVA MP. The following color codes are used to categorize the amino acids: light Blue for hydrophobic amino acids, yellow for small amino acids, green for nucleophilic amino acids, purple for aromatic amino acids, red for acidic amino acids, dark Blue for amide amino acids, and grey for basic amino acids.

Sour cherry	Apricot	Position of aminoacid
I (ISOLEUCINE)	V (VALINE)	73
K (LYSINE)	R (ARGININE)	80
H (HISTIDINE)	N (ASPARAGINE)	122
R (ARGININE)	K (LYSINE)	275
F (PHENYLALANINE)	S (SERINE)	276
A (ALANINE)	V (VALINE)	277
E (GLUTAMIC ACID)	G (GLYCINE)	278
K (LYSINE)	E (GLUTAMIC ACID)	279
D (ASPARTIC ACID)	N (ASPARAGINE)	280
S (SERINE)	P (PROLINE)	281
E (GLUTAMIC ACID)	K (LYSINE)	282
R (ARGININE)	E (GLUTAMIC ACID)	285
L (LEUCINE)	F (PHENYLALANINE)	289
A (ALANINE)	S (SERINE)	296
K (LYSINE)	R (ARGININE)	316
T (THREONINE)	R (ARGININE)	330
H (HISTIDINE)	Y (TYROSINE)	335
C (CYSTEINE)	S (SERINE)	336
N (ASPARAGINE)	D (ASPARTIC ACID)	343
V (VALINE)	I (ISOLEUCINE)	362
T (THREONINE)	A (ALANINE)	363
S (SERINE)	P (PROLINE)	364
N (ASPARAGINE)	D (ASPARTIC ACID)	365
T (THREONINE)	A (ALANINE)	368
G (GLYCINE)	R (ARGININE)	371
I (ISOLEUCINE)	F (PHENYLALANINE)	373
G (GLYCINE)	S (SERINE)	377
N (ASPARAGINE)	H (HISTIDINE)	379
P (PROLINE)	A (ALANINE)	380
S (SERINE)	G (GLYCINE)	381
N (ASPARAGINE)	D (ASPARTIC ACID)	382
S (SERINE)	N (ASPARAGINE)	383
I (ISOLEUCINE)	L (LEUCINE)	384
V (VALINE)	I (ISOLEUCINE)	385
E (GLUTAMIC ACID)	K (LYSINE)	387
P (PROLINE)	S (SERINE)	388
S (SERINE)	G (GLYCINE)	390
C (CYSTEINE)	S (SERINE)	391
K (LYSINE)	E (GLUTAMIC ACID)	393
Q (GLUTAMINE)	P (PROLINE)	396
E (GLUTAMIC ACID)	K (LYSINE)	397
P (PROLINE)	S (SERINE)	400

N (ASPARAGINE)	D (ASPARTIC ACID)	412
R (ARGININE)	G (GLYCINE)	417
G (GLYCINE)	R (ARGININE)	421
A (ALANINE)	T (THREONINE)	422
D (ASPARTIC ACID)	N (ASPARAGINE)	425
D (ASPARTIC ACID)	N (ASPARAGINE)	437
I (ISOLEUCINE)	V (VALINE)	446
H (HISTIDINE)	Y (TYROSINE)	451
S (SERINE)	G (GLYCINE)	455