

# Development of a new method for eradication of viruses by induction of posttranscriptional gene silencing in transgenic potato plants

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**Abstract:** A new method of improving potato plants from a viral infection has been proposed. DNA fragments containing the full coding sequence of PVS 25K protein in sense and antisense orientations were cloned from 5' and 3' ends of intron I of *Ricinus communis* catalase gene *cat1 Ricinus communis* into binary agrobacterial vector pCAMBIA2300 under control of 35S CaMV promoter and terminator of nopaline synthase gene. Using the recombinant DNA as a transgene, transformation of six varieties of test-tube potato plants, which were infected with PVM, PVS and/or PVY individually or in complex, was carried out. Further field trials of 2017-2018 allowed us to select four promising transgenic lines that were free from viral infection.

**Key words:** potato virus; PVS; PVM; PVY; RNA-interference; transgene; ELISA.

## 1. Introduction

The main focus of potato protection against viral diseases is on the creation of resistant varieties using traditional breeding methods, the recovery of seed material and the protection of planting against reinfection. The existing technologies for obtaining virus-free seed, both using the method of cultivation of apical meristem and using chemo- and heat therapy, as well as cryotherapy, are still expensive and time-consuming and, as a rule, do not eliminate the possibility of secondary infection of plants in the soil. In this regard, potato varieties with genetically fixed resistance to viral diseases are of particular importance. The creation of such varieties by traditional methods of breeding requires decades; moreover, donors of genes for resistance to viral diseases are mostly wild plant species that cannot always be crossed with cultivated varieties using classical methods of selection.

The resistance of transgenic plants to viral infections is considered as a separate type of specific plant resistance (pathogen-derived resistance, PDR). It is known that this kind of resistance is due to the mechanisms of RNA interference (or post-transcriptional gene silencing, PTGS or RNA silencing) and is characterized by highly specific degradation of viral RNA when interacting with transgenic RNA (Simon-Mateo and Garcia, 2011). For this aim, transgenes having a high degree of homology with the nucleotide sequences of the viral gRNA are used.

It was shown that transforming plants containing sense and antisense orientation of the transgene coding sequence, separated by an intron sequence, the so-called "hairpin" structure, is more resistant to viral infection than plants transformed with a vector that carries only a sense or an antisense sequence (Smith et al., 2000; Wesley et al., 2001). The introduction of such "hairpin" structures into cells leads to the formation of double-stranded RNA (dsRNA), which actually trigger the RNA interference mechanism in order to degrade foreign viral RNA in the event of a pathogen penetration (Moissiard and Voinnet, 2004).

Potato viruses M and S (PVM and PVS) are the most common viruses in Kazakhstan (Loebenstein and Manadilova,

2003; Khassanov, Vologin, 2018; Alexandrova et al., 2018). Until now, no resistance gene donors have been detected for viral diseases caused by these viruses. PVS is a representative of the group *Carlaviruses* of the family *Betaflexiviridae* (King et al. 2011). The virus is carried by aphids in a non-persistent manner and infects plants of the families *Solanaceae* and *Chenopodiaceae*. Genomic RNA (gRNA) of the virus is represented by positively charged single-stranded mRNA of about 8535 nucleotides in length and has a cap structure at the 5' end and a poly (A) tail at the 3' end (Monis et al., 1987; Mackenzie et al., 1989). Six reading frames were found inside the gRNA. ORF1 encodes a polypeptide of 223K in size, inside of which are coding sequences of methyltransferase, peptidase, helicase, and RNA-dependent RNA polymerase. ORF2, ORF3 and ORF4 encode 25K, 12K and 7K proteins that are included in the triple gene block (TGB), characteristic of all Carlaviruses (Morozov et al., 1989; Matoušek et al., 2005). These proteins are responsible for the spread of the virus from one cell to the next and have a region of significant homology with the sequences of the representatives of the potexvirus group. ORF5 encodes a 34K coat protein (CP), and ORF6 encodes a cysteine-rich 11K protein, which has RNA-binding activity.

A method for the eradication of viral material was proposed in the framework of this study, based on the use of source material infected with one or more viruses to create transgenic potato plants capable of expressing a "hairpin" structure with the coding sequence of the PVS 25K protein.

## 2. Materials and methods

The 35S CaMV promoter and terminator of the nopaline synthase gene (nos) were cloned from transient expression vector pBI221 (NCBI GenBank acc. No: AF501128) into binary agrobacterial vector pCAMBIA 2300 (NCBI GenBank acc. No: AF234315) between *HindIII* and *BamHI*, *SacI* and *EcoRI*, respectively.

The coding sequence of the PVS 25K protein in two orientations (sense and antisense) was inserted into secondary plasmid vector pRAJ17 created on the basis of pBluescriptII

KS+ and containing intron 1 of the I catalase *cat1* *Ricinus communis* catalase gene *cat1* between the *Bam*HI / *Nco*I and *Sal*I / *Sac*I restriction sites. The following primers were selected for cloning the coding sequence of 25K protein in sense (# 306 and # 308) or into antisense orientation (# 307 and # 309) (the restriction sites are underlined, restrictase names are shown to the right):

```
#306 5'- cgtaggatcc ttagcggcg tgtaagtgg -3' NcoI
#307 5'- cgtaggatcc atgaggatat ttgatagctt -3' SacI
#308 5'- cgtaggatcc atgaggatat ttgatagctt -3' BamHI
#309 5'- cgtagtcgac ttagcggcg gtgtaagtgg -3' SalI
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The sense or antisense sequences of the 25K protein cloned into pRAJ17 using *Bam*HI and *Nco*I (located on the 5' end of the intron) or *Sal*I и *Sac*I (on 3' end of the intron). Then the DNA fragment from *Bam*HI and *Sac*I was recombined into pCAMBIA 2300 with the 35S CaMV promoter and the nos-terminator. As a result, recombinant DNA with the [35S CaMV – 25K Sense – intron – 25K Asense – nos] cassette was created (Figure 1).

Potato plant transformation was carried out according to a standard method (Raldugina et al., 2011). DNA and RNA from transgenic plant leaves were isolated by a standard method using Trizol according to the manufacturer's recommendations (Sigma). Amplification of DNA fragments was performed using *Pwo* polymerase (Roche) according to the manufacturer's method. The program for amplification: stage 1: 5 min at 94 °C, 1 cycle; stage 2: 30 sec at 94 °C, 30 sec at 54 °C, 1 min 30 seconds at 72 °C, 30 cycles; stage 3: 5 min at 72 °C, 1 cycle. The presence of PVS, PVM and PVY infection was determined by the standard DAS-ELISA method using commercial kits from BIOREBA as recommended by the manufacturer. The measurements were carried out on a Stat Fax 2100 photometer (Awareness) at 405 nm.

### 3. Results and discussion

The presence of the 25K protein is characteristic of all Carlaviruses, its coding sequence is known as part of the TGB. The property of the PVM 25K protein is its ability to suppress RNA silencing (Senshu et al., 2011). In this study, DNA fragments containing the full coding sequence of the PVS 25K protein were cloned from the 5' and 3' ends relative to the intron I of the *Ricinus communis* catalase gene *cat1* in the sense (Sense) and antisense (Asense) orientations into binary agrobacterial vector pCAMBIA 2300 under the control of the 35S CaMV promoter and the nos-terminator (Figure 1).

Potato plants of the varieties 'Dunyasha', 'Zeren', 'Kormilitsa', 'Albinka', 'Fortune', 'Picasso', 'Santa', 'Tien-Shansky' and 'Tokhtar' which were free from viral infection or infected with PVM, PVS and/or PVY alone or in complex were transformed by cocultivation with agrobacteria carrying the created recombinant DNA. The transformation efficiency for each variety was evaluated by the number of the regenerants containing the transgene (Table 1). Normal shoots were obtained only in 'Albinka', 'Dunyasha', 'Zeren' and 'Kormilitsa' after regeneration and transformation.

The plants were analyzed for the presence of recombinant DNA constructs, as well as RNA transcripts corresponding to the transgene. Transgenic plants that showed a positive response during these analyses were grown in soil and fur-

ther tested for the presence of viruses using the DAS-ELISA method. According to our observations, a decrease in ELISA readings from a high value at the moment of planting into the soil to fewer readings during the first six months of observations was caused not only by the successful triggered RNA interference in cells during further plant development after transformation, but also by laboratory manipulations. The viruses did not have time to accumulate in the shoots of tubetest plants during grafting when the continuous intensive apical growth occurred. Therefore, there was a decrease in ELISA values even in untransformed plant regenerants which was observed, for example, for 'Zeren' and 'Dunyasha' infected with PVM. However, samples of these plants taken later again revealed the presence of viral infection after growing for two months in the soil in the greenhouse. That was the reason for the prolonged observation of the lines that had gone through the eradication procedure.

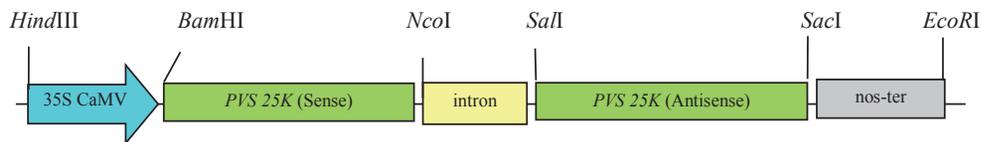
It was also noted that the original 'Zeren' plants which initially carried PVM + PVS + PVY infection had no more PVM during field trials, although this variety gave a positive response to the presence of this virus during the first six months after transformation. As a rule, several viruses in one plant can coexist if they belong to different groups. Moreover, if the titer of a more virulent pathogen remains at the same level, the titer of a less virulent pathogen grows during the growing season of the plant (Nyalugwe et al., 2012; Hameed et al., 2014). The results presented here are not consistent with the previous findings.

The enzyme immunoassay was performed every 30 days for 6 months after the transfer of transgenic plants to the soil in the greenhouse. Transgenic lines TL-59, TL-44, TL-62, TL-65 and all 'Albinka' lines had no changes and were infected by PVM and PVY as before transformation. As for the other varieties, there were lines that showed a decrease in ELISA value up to the complete absence of a positive response. As a result, the 4 transgenic lines were selected as being free of virus. TL-61 and TL-67 ('Dunyasha'), as well as TL-103 ('Kormilitsa') infected with PVM before transformation had a negative response. Moreover, complex infection with PVM + PVS + PVY was eliminated in TL-119 ('Zeren').

In 2017 and 2018, microtubers of 14 transgenic lines TL-39, TL-43, TL-46, TL-48, TL-74 and TL-159 ('Albinka'), TL-59 and TL-119 ('Zeren'), TL-44, TL-61, TL-62, TL-65, TL-67 ('Dunyasha') and TL-103 ('Kormilitsa') were planted in the field. Besides transgenic plants, the plants infected by different viruses (Table 1) and control virus-free plants of the same varieties were also grown to create a viral background and to check the rates of virus contamination. The presence of PVM, PVS and/or PVY had already been seen in virus-free control plants after the first ELISA in 2017.

During each season, leaf samples were collected from each plant three times for ELISA 1, 2 and 3 months after planting. Lines TL-103, TL-44, TL-61 and TL-67 remained free of PVM. PVM, PVS and PVY were eliminated in TL-119 plants.

Virus-free tubers were used for planting in 2018. After ELISA analysis, PVM and PVS were not detected in TL-103 and TL-119. PVM was not found in TL-61 and TL-67. TL-119 showed eradication of three viruses after the first six months



**Figure 1.** Scheme of the recombinant DNA cassette created as part of agrobacterial plasmid vector pCAMBIA2300 and used as a transgene for potato plant transformation.

**Table 1**  
Transformation efficiency of various potato varieties

Name of varieties	Virus infection	Number of regenerants (units)	Number of transgenic shoots (units)	Transformation efficiency, %
'Albinka'	PVM, PVY	18	8	44.4
'Dunyasha'	PVM	13	5	38.5
'Zeren'	PVM, PVS, PVY	15	4	26.7
'Kormilitsa'	PVM	5	1	20.0
'Picasso'	PVM, PVY	0	0	0
'Santa'	PVM, PVY	0	0	0
'Tokhtar'	PVM	0	0	0
'Tien-Shansky'	PVM	1	0	0
'Fortune'	PVM, PVS	0	0	0

in the greenhouse. However, during field trials, a positive response to PVY was shown in 2017 by one out of five plants planted and in 2018 by seven out of 9 plants planted. PVY belongs to *Potyvirus*, PVS and PVM are Carlaviruses. The recombinant DNA construct developed was directed against PVS, but in the case of PVM it could be a trigger of RNA interference due to homology between genomic sequences. Although the amino acid sequences of the 25K proteins of PVS, PVM and PVX have more differences than capsid proteins, conservative domains about 20 amino acids in size were found at the N-terminus of these proteins.

#### 4. Conclusion

The problem of multiple infections in potato fields in Kazakhstan still persists. A new methods of treatment of plants infected with related and unrelated viruses is proposed with the example of a transgene carrying the complete coding sequence of the PVS 25K protein in two orientations and able to induce RNA interference in plant cells. As a result, four virus-free lines were obtained. Thus, it has been proven that the coding sequence of the PVS 25K protein is a good target for the action of RNA interference.

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