

BLOCKING THE SYSTEMIC SPREAD OF POTATO VIRUS Y IN THE TISSUES OF POTATOES BY POSTTRANSCRIPTIONAL GENE SILENCING

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Abstract

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Plant virus diseases are the second biggest group after the fungal diseases in agricultural production. Enormous economic loss in agriculture is caused by the virus diseases. The traditional prevention and control of the virus diseases are mainly agricultural control measures. Potato virus Y (PVY) is the type species of genus *Potyvirus* from family *Potyviridae*. It is transmitted by aphids in non-persistent manner. Using insecticides is useless against spreading the infection because the virus is transmitted in non-persistent manner. Using resistant plant cultivars is not effective because of the fast evolution of the virus mainly through mutations and recombination between its strains. In the current paper we propose a new way of controlling the virus by blocking the replication and the transmission through the plant by RNA based vaccination of plants with specific to viral HC-Pro gene dsRNAs and siRNAs. This technology will give a chance against PVY invasion without changing the valuable qualities of the sensitive to the virus cultivars of potatoes.

Key words: potatoes, PVY^N, RNAi

Introduction

Some of the most important and popular crops from the botanical family *Solanaceae*, are potato, tomato, eggplant, pepper and tobacco. The potato and the tobacco are with significant economic importance.

In the terms of human consumption potato (*Solanum tuberosum*) is the third important crop in the world after rice and wheat. It was stated that in the first decade of the 21st century the annual menu of an average global citizen includes around 33 kg of potatoes (United Nations Food and Agricultural Organization, 2009). According to FAOSTAT data (2013) the global production of potato is 333 955 646 tones per 2009, 334 262 522 tones per 2010 and 374 382 247

tones per 2011. It is grown in over 100 countries around the world (source: FAOSTAT). The production and the importance of the potato vary between the different countries but the common is that it is very important for the economics of the developing countries. Nowadays the biggest producers of potato are China and India, where 1/3 of the world's production is harvested. It is a fundamental element in the food security for millions of people across South America, Africa, and Asia, including Central Asia. The potato is essential crop in Central and Eastern Europe as well (Hijmans and Robert, 2001). In Bulgaria, according to the report of the Ministry of Agriculture and Food the total production of potatoes is 251 205 tones for 2010 and 232 314 tones for 2011. The total land used for potato production during 2011 is 16 218.7

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ha (Annual report on the condition and the development of agriculture, 2012).

Potatoes can be infected by many different viruses that can affect negatively the harvest and the tuber quality. Virus diseases can often be diagnosed by mosaic forms on leaves, stunting of the plant, leaf malformations, and tuber malformations. Some of the most common viruses that attack the potato are: Potato virus Y (PVY) (Petrov et al., 2008; Petrov and Lyubenova, 2011; Petrov, 2012), Potato leaf roll virus (PLRV), Potato virus S (PVS), Potato virus X (PVX), Potato mop top virus (PMTV), Tobacco rattle virus (TRV) (Dikova, 2005, 2006a, 2006b), Alfalfa mosaic virus (AMV) and more (Ward and Shukla, 1991; Agindotan et al., 2007).

The virus causes wide range of symptoms which depend on the virus strain, the cultivar of the host plant, climate conditions, and the region. The most common symptoms of the disease on potatoes are: mosaic, leaf wrinkling, mild to

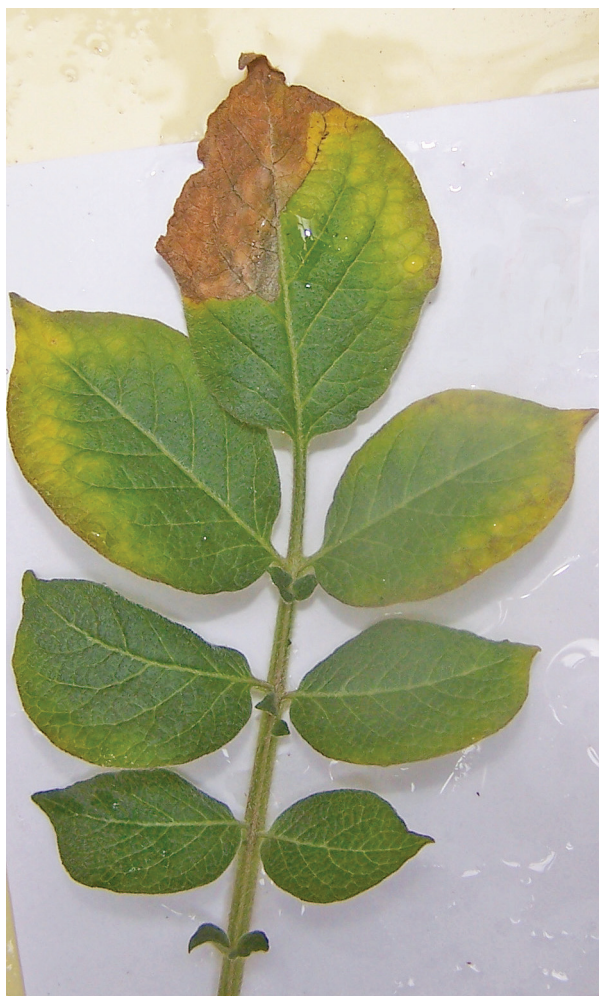


Fig. 1. Symptoms of PVY^N on potato leaves cv. Arinda

strong mottling, often leading to leaf deformation, chlorosis, and necrosis, necrotic spots and rings on the potato tubers, drying and defoliation. (Buchen-Osmond, 1987). (Figure 1)

From the beginning of its identification, PVY is considered as a complex of virus isolates (Smith, 1931). Potato virus C (PVC) was recognized as PVY (Bawden, 1943). When it was first reported in 1930 (Salaman, 1930; Bawden, 1936; Dykstra, 1936), it was the first one from a strain group, named later PVY^C. Other strain group PVY^N (De Bokx, 1961), was reported for the first time in 1935 in a tobacco field near experimental potato plants (Smith and Dennis, 1940), after that in Peruvian and Bolivian potato cultivars in 1941-1942 (Nobrega, 1944; Silberschmidt, 1960). This strain group caused severe epidemics on the potato and tobacco plants in Europe in 1950 (Klinkowski, 1960; Horvath, 1967; Weidemann, 1988). In 1950 it was proven that PVY damaged the tomato plants in Australia (Sturgess, 1956), in South and North America (Silberschmidt, 1956; Silberschmidt, 1957; Simons, 1959), and the pepper plants in Florida (Simons, 1959) and Israel (Nit-zany, 1962). PVY^O group, also called Ordinary strains, are wide distributed and cause severe symptoms as mottling and leaf curling, necrosis on the *Physalis floridana* and leaf spots on the tobacco (Van der Vlugt, 1993). PVY^N strains induce vein necrosis on tobacco, leaf spots on potatoes and necrosis on *P. floridana*, (Van der Vlugt, 1993). PVY^{NTN} was first reported in Hungary in 1978 (Beczner, 1984; Le Romancer, 1994), and since then a lot of reports about it were received in Europe (Weidemann, 1996). PVY^{NTN} strain resembles PVY^N, but induces necrotic ring spots on potato tubers. Nowadays this strain is considered as a subgroup of PVY^N group. PVY^{N-Wilga} was first reported in Poland in 1984 as similar to PVY^N (Chrzanowska, 1991), and later was concluded that it's serologically related to PVY^O strains (Chrzanowska, 1994). It caused vein necrosis on tobacco, which was typical for PVY^N strain group, but its RNA sequence was closer to PVY^O and PVY^{NTN} (Shukla, 1994).

First report for the spread of Potato virus Y (PVY) in Bulgaria was made from Kovachevski (1942), who had found single cases of symptoms on pepper, caused by this virus. Later, the author proved that the virus caused widespread disease of tobacco and crispness of potato. He first reported that PVY induced necrotic symptoms on tobacco and experimentally transmitted this virus with aphids from the genus *Myzus persicae* (Kovachevski, 1951).

RNA gene silencing, part of which is posttranscriptional gene silencing (PTGS), is a general term describing related gene regulatory mechanisms, led by RNA in plants (Vance et al., 2001). The first cases of gene silencing concerned transgenic plants bearing artificially introduced genes. The process is initiated by double stranded RNAs (dsRNAs) - mole-

cules that are produced during viral replication. The dsRNAs are recognized by the plant as a “non-own” and subsequently cut by Dicer-like cellular enzymes to form small interfering RNAs (siRNAs) with a length of 21–25 bp (Hammond et al., 2000). These siRNAs are the main component of the RNA gene silencing (Denli et al., 2003). They initiate complementary-specific RNA degradation by forming a multi-component cell complex (RISC), inducing RNA gene silencing that destroys cognate mRNAs (Martinez et al., 2002). Remarkable feature of the RNA gene silencing is its ability to spread both from cell to cell and over long distances causing systemic RNA silencing throughout the whole organism by complementary-specific signal silencing, obtained after induction of RNA gene silencing in single cells (Mlotshwa et al., 2002).

In response, plant viruses encode proteins capable of suppressing RNA gene silencing (Voinnet and Baulcombe, 1997; Mlotshwa et al., 2002). The first reported viral suppressor of gene silencing were HC-Pro and 2b proteins encoded respectively from Potyviruses and Cucumoviruses (Kasschau and Carrington, 1998; Li et al., 1999). Potyviral HC-Pro is a multifunctional protein that participates in the transport of virions with aphids as well as in the movement of the virus in the plant and suppression of RNA-dependent gene silencing, establishing as a defense mechanism against viruses (Kasschau and Carrington, 1998; Revers et al., 1999). HC-Pro is a potential suppressor of intracellular gene silencing, but the signal is continuously expressed and exported outside the HC-Pro expressing cells, with the result that can reduce the level of viral spread in the healthy tissues of infected plants (Mallory et al., 2001).

There are still not known effective substances for reduction the viral infection, which is imperative for developing of new approaches to block the replication of PVY.

Materials and Methods

The material consists of

- plants – 18 plant pots with Potatoes cv. Arinda;
- virus: PVY strain N obtained from potato cv. Marabel from the virus collection of Institute of soil sciences, agro technologies and plant protection (ISSAPP)
- referent compounds: dsRNA for the S segment of Phi6, siRNAs for the S segment of Phi6

Mechanical inoculation of plants with PVY

The plants are inoculated by Noordam (1973). Prior to inoculation, the plants were placed in a room with low light (shading), sprinkled with water and the leaves were dusted with carborundum 400–600 meshes.

One gram of the symptomatic plant foliage was homogenized in 1 ml of cooled to 4°C 0.1 M potassium sodium phosphate buffer, pH 8.0 containing 0.2% Na₂SO₃ and 0.2% ascorbic acid. Inoculations were performed by gently rubbing the leaves with this homogenate. After 3–5 minutes, the plants were washed with water.

Serological diagnostic test

The serological diagnostic test is DAS-ELISA (Double Antibody Sandwich Enzyme Linked Immunosorbent Assay):

The analysis was conducted by the method of Clark and Adams (1977). We have used a commercial kit of LOEWE Biochemica GmbH, Sauerlach, Germany. ELISA plates are loaded with antiserum (IgG) for PVY, with dilutions (according to the instructions of the manufacturer) in 0.05 M carbonate buffer. The samples were incubated for 4 hours at 37° C, and the unbound components were washed out with PBS-T buffer for 5 min. All samples were grounded in extraction buffer containing 1% PVP (polyvinyl pyrrolidone) in a ratio of 1:10. The plates were incubated at 4°C for 16 h. Following the third wash step alkaline-phosphatase conjugate for PVY was added and the plates were incubated for 4 h at 37°C. The used substrate is p-nitrophenyl phosphate (p-nitrophenyl phosphate, Sigma) in diethanolamine buffer (pH 9.8) at a ratio of 1mg/ml. The reaction proceeded in the light at room temperature and was stopped with 3N NaOH. The adsorption of the color reaction is measured at multifunctional detector (DTX 880) at a wavelength of 405 nm.

The positive samples had optical density (OD) over the threshold (Cut-off) which is two times the value of the negative control.

Molecular methods for virus diagnostics: RNA extraction from potatoes infected with PVY

Extraction of total RNA was performed with RNEasy Plant Mini Kit (Qiagen, Germany). Extraction was carried out according to the instructions of the manufacturer.

In vitro system for the production of dsRNA

dsRNA was synthesized by a combination of *in vitro* transcription and replication of DNA template (according to Replicator RNAi Kit, Finnzymes, Finland). DNA template for synthesis of dsRNA was obtained by PCR using Phusion High-Fidelity DNA polymerase. Primers for the PCR are designed so that the resulting PCR fragment contained a target sequence (HC-Pro of PVY), flanked by T7 promoter sequences in the 5' end and phi6 qRdRP promoter sequences in the 3' end. PCR DNA product was purified and transcribed by T7 viral RNA polymerase to ssRNA. This ssRNA was replicated to dsRNA by virus phi6 qRdRP. The sequences of our designed primers are: HC-Pro dsRNA 1 (5'-TAA TAC

GAC TCA CTA TAG GG TAG GAT TCT GTC GAA TGC CGA CAA TTT T -3'), HC-Pro dsRNA 2 (5'-GGA AAA AAA TAC TGC AGA CCA ACT CTA TAA TGT TT -3').

***In vivo* system for the production of dsRNA**

The system was constructed in the cells of the bacterium *Pseudomonas syringae*, which continuously expressed T7 RNA polymerase (from plasmid pLM1086). pLM991 brought a cDNA phi6 Lkan – segment of bacteriophage, which contained all the necessary components to form a polymerase complex and the gene for resistance to the antibiotic kanamycin. The other plasmid contained phi6 S-segment, 5' packaging and 3'-replication signals, including our target gene (HC-Pro). T7 RNA polymerase synthesized ssRNA temporarily from cDNA plasmids. ssRNA of Lkan – segment managed translation of viral proteins, leading to the formation of empty polymerase complexes. Packaging began with specific ssRNA segments of S-specific ssRNA followed by the L-segment. During packaging, exactly complementary copy was synthesized in virions by viral RNA-dependent RNA polymerase (RdRP) (Aalto et al., 2007).

Production of siRNAs

The PowerCut Dicer is a recombinant endoribonuclease from *Giardia intestinalis*. It cleaves dsRNA efficiently, producing fragments with a length of 25-27 nucleotides, yielding a pool of small interfering RNAs (siRNAs). Since every siRNA has its own off-targets, pooling is thought to reduce the individual nonspecific contributions of each sequence revealing the “true” RNAi-phenotypes.

Results and Discussion

In vitro system for generating dsRNA combined T7 RNA polymerase to synthesize ssRNA templates from PVY cDNA template of the selected fragment of the viral genome and viral Phy6 RdRP polymerase formed de novo initiation and synthesis of dsRNA from the used template ssRNA.

Through the *in vivo* system were received phi6-specific dsRNAs by transfected bacteria *P. syringae* with ColEI-based plasmids containing cDNA copies of viral genomic segments under the control of the T7 promoter. The system was stable on RNA level. Selection of stable cell, carried bacteriophage polymerase complexes was achieved by introducing a gene for kanamycin resistance in the region of the 3' UTR of the L segment of phi6 viral genome. Plasmids containing the T7 promoter sequence and heterologous cDNA sequence, surrounded by the viral packaging and replication signals, were transcribed and packaged into viral polymerase complexes, followed by synthesis of a fully complementary RNA chains.

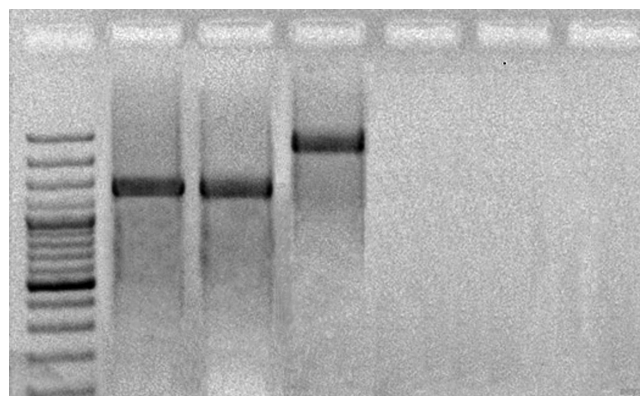


Fig. 2. dsRNAs of HC-Pro gene region of PVY and S segment of Phi6

Legend: 1 – 100 bp DNA ladder; 2 – dsRNAs of HC-Pro gene region of PVY 1386 bp, produced by *in vitro* system; 3 – dsRNAs of HC-Pro gene region of PVY 1386 bp, produced by *in vivo* system; 4 – dsRNAs of S segment of Phi6 2948 bp

In vitro and *in vivo* recombinant viral Phi6 RdRP-based systems allowed the generation of medium-sized or large quantities of dsRNAs to attack our target genetic sequence. *In vitro* system was efficient, high quality (almost error-free amplification of dsRNAs) and convenient method of obtaining high quality dsRNAs from ssRNAs of practical infinite length sequences chosen up to 10,000 bp, such as Potato virus Y HC-Pro gene, 1386 bp (Figure 2). From these specific to Hc-Pro gene dsRNAs we produce siRNAs with Power Cut Dicer (Figure 3)

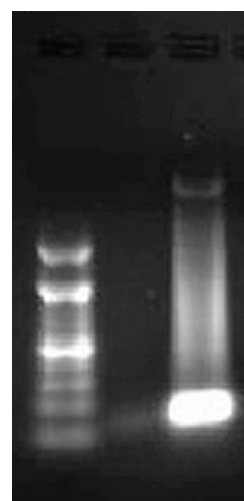


Fig. 3. siRNAs of HC-Pro gene region of PVY

Legend: 1 – 10 bp DNA ladder; 2 – siRNAs of HC-Pro gene region of PVY 25 bp

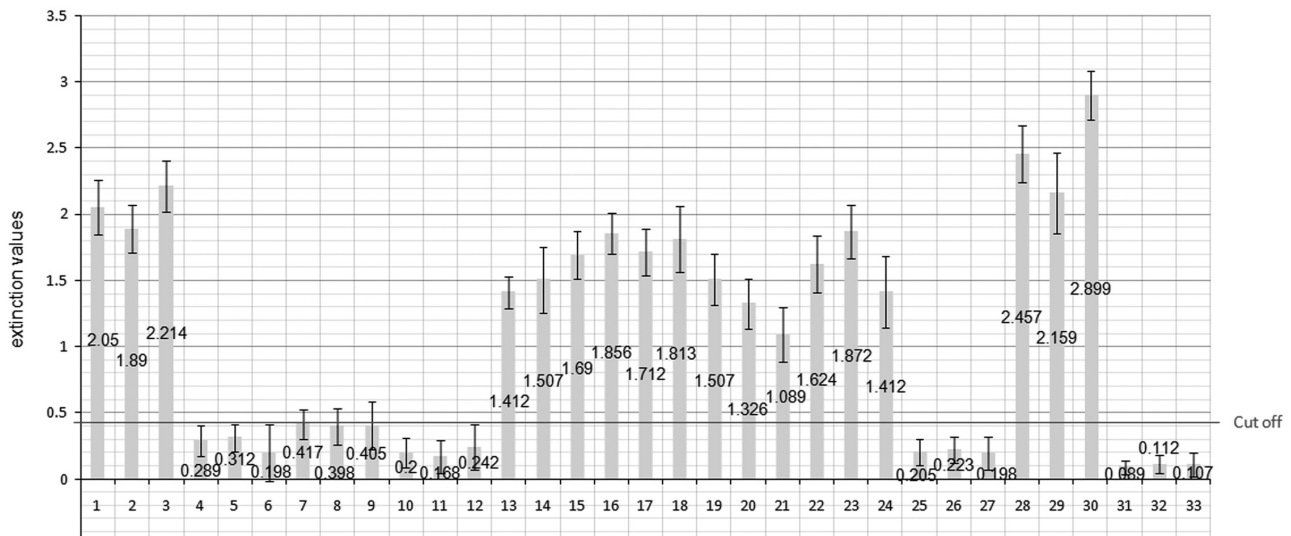


Fig. 4. DAS-ELISA of potato plants after treated with dsRNA and si RNAs

Legend: 1÷3 – Optical Density (OD) values of samples from old leaves (leaves of the plants in the moment of treatment and virus inoculation) of potato plants treated with HC-Pro dsRNAs and inoculated with PVY; 4÷6 – OD values of samples from new leaves (leaves grown after treatment) of potato plants treated with HC-Pro dsRNAs and inoculated with PVY; 7÷9 – OD values of samples from old leaves of potato plants treated with HC-Pro siRNAs and inoculated with PVY; 10÷12 – OD values of samples from new leaves of potato plants treated with HC-Pro siRNAs and inoculated with PVY; 13÷15 – OD values of samples from old leaves of potato plants treated with Control dsRNAs from the S segment of Phi 6 and inoculated with PVY; 16÷18 – OD values of samples from new leaves of potato plants treated with Control dsRNAs from the S segment of Phi 6 and inoculated with PVY; 19÷21 – OD values of samples from old leaves of potato plants treated with Control siRNAs from the S segment of Phi 6 and inoculated with PVY; 22÷24 – OD values of samples from new leaves of potato plants treated with Control siRNAs from the S segment of Phi 6 and inoculated with PVY; 25÷27 – OD values of Control leaf samples of healthy potato plants not treated and not inoculated with virus; 28÷30 – OD values of Control leaf samples of potato plants inoculated with PVY and not treated; 31÷33 – OD values of Control ELISA wells only with buffer

Fourteen days after being inoculated with dsRNAs and siRNAs and infected with PVY^N a day after inoculation plants were tested by DAS-ELISA with polyclonal serum IgG (LOEWE) for presence or absence of PVY viral infection in different plant parts (old leaves and newly grown leaves). We received high OD values (2 to 0.4; Figure 4, for 1÷3 and 7÷9 respectively) of samples from old leaves (leaves of the plants which was treated and PVY inoculated) of potato plants treated with HC-Pro dsRNAs and siRNAs and a day after inoculated with PVY which was a confirmation that PVY stayed in old parts of the plants although treatment. For the newly grown leaves of the same potato plants, similar to OD values under the Cut off results were received (Figure 4; 4÷6 for treatment with HC-Pro dsRNAs and 10÷12 for treatment with HC-Pro siRNAs). These small OD values confirm absence of PVY infection in the newly grown parts of potato plants due to the blocking of essential for virus replication HC-Pro gene region

of PVY. As a control in the experiment were used dsRNAs and siRNAs of S segment of bacteriophage Phi6. All treated plants with these unspecific for PVY dsRNAs and siRNAs remain infected with PVY (Figure 4; 13 to 24.)

Conclusion

Posttranscriptional gene silencing was induced in potato plants cv. Arinda by specific dsRNAs and siRNAs for HC-Pro region of PVY^N, which effectively blocked systemic spread of the virus. Blocking the HC-Pro gene of PVY^N in newly grown leaves of potato plants and hence the viral replication in all inoculated plants with the virus was established. The old treated and PVY inoculated leaves of the potato plants remain infected and later defoliate. All new leaves of potato plants cv. Arinda (not treated) grown after treatment with dsRNAs and siRNAs and PVY^N inoculation remain virus free.

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