ACTA MICROBIOLOGICA BULGARICA Volume 34 / 3 (2018)

Resistance to Potato Virus Y in Potatoes Induced at Epigenetic Level

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Abstract

Potyviruses are the largest group of plant virus pathogens, which are constantly expanding their territories of emergence, threatening their hosts worldwide. Potato virus Y(PVY) epidemics have a serious negative impact on agricultural productivity, resulting in significant losses to farmers. The need of efficient technique to control PVY is essential for decreasing yield loss in agriculture.

Plants employ a defense mechanism called RNA silencing, which we used to direct against the Potyviruses. We induced posttranscriptional gene silencing (PTGS) of essential multifunctional virus gene, such as HC-Pro by specific siRNAs which promote sequence-specific viral RNA degradation by forming a multicomponent RNA-induced silencing complex (RISC). By using PTGS against HC-Pro, we achieved potato resistance to PVY.

Keywords: PVY, potato, RNAi, PTGS, resistance, control

Резюме

Потивирусите са най-голямата група от растителни вирусни патогени, които непрекъснато разширяват своите територии и причиняват щети по културните си гостоприемници по целия свят. Епидемиите от картофения вирус Y (PVY)имат сериозно отрицателно въздействие върху селскостопанската продукция, което води до значителни загуби. Необходимостта от ефективна техника за контрол на PVY е от съществено значение за намаляване на загубата на добив в селското стопанство.

Растенията използват защитен механизъм, наречен РНК заглушаване, който може да се използва като се насочи срещу потивирусите. Ние индуцирахме посттранскрипционно генно заглушаване (PTGS) на основен мултифункционален вирусен генкато HC-Proчрез специфични миРНКи, които индуцират специфична деградация на вирусната РНК чрез формиране на многокомпонентен РНК-индуциран заглушаващ комплекс (RISC). Чрез използването на PTGS постигнахме устойчивост на картофи спрямо PVY.

Introduction

The potato is an essential part of the food for millions of people across the continents - South America, Africa, and Asia, and an economically important crop in Central and Eastern Europe (Hijmans and Spooner, 2001). India is the third largest producer of potatos in the world (MAFW, 2017-18), and in Bulgaria potatoes had the largest share invegetable production in 2015, and the second largest in 2016 (MAF, 2016; MAFF, 2017).

Potato virus Y (PVY) and Potato leaf roll virus (PLRV) are amongst the most common viruses attacking the potatoes in Bulgaria. PVY causes a wide range of symptoms depending on the virus strain, the host plant cultivar, the climate conditions, and the region. The most common symptoms of the disease in potatoes are mosaic, leaf wrinkling, mild to strong mottling, often leading to leaf deformation, chlorosis and necrosis, necrotic spots and rings on the potato tubers, drying and defoliation (Buchen-Osmond, 1987).

PVY was first isolated in 1930 (Smith, 1931). Strain PVY^N was reported for the first time in 1935 in tobacco grown near a potato field (Smith and Dennis, 1940), and after that in potato cultivars in

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Peru and Bolivia in 1941-1942 (Nobrega and Silberschmidt, 1944; Silberschmidt, 1960). This strain group caused severe epidemics in both crops in Europe in 1950 (Klinkowski and Schmelzer, 1960; Weidemann, 1988) and also caused damage to tomato plants in Australia (Sturgess, 1956), South and North America (Silberschmidt, 1956, 1957; Simons, 1959), and to pepper plants in Florida (Simons, 1959) and Israel (Nitzany and Tanne, 1962). The *P*VY^{O} group is widely distributed and causes leaf spots in tobacco and severe symptoms such as mottling and leaf curling (Vander Vlugt *et al*., 1993). PVY^{N} strains induce vein necrosis in tobacco, and leaf spots in potatoes (Van der Vlugt *et al*., 1993). The PVY^{NTN} strain resembles *P*VY^{N} but induces necrotic ring spots on potato tubers. PVY$^{N\text{-}Wilga}$ was first reported to be similar to PVY^{N}, because it caused vein necrosis in tobacco, but was found to be serologically related to PVY strains and genetically closer to PVY and PVY^{NTN} (Chrzanowska, 1991; Shukla, 1994).

In Bulgaria, PVY was reported for the first time in pepper in the 1940s by Kovachevski (1942). Further investigations revealed thata widespread disease of tobacco and potato plants was caused by the same virus(Kovachevski, 1951). Only recently, it was found that necrotic ring spots on potato tubers were induced by an aggressive strain of PVY close to PVY^{NTN}, but not identical with it (Petrov et al, 2008). The strain was characterized as PVY$^{N/NTN}$ (Petrov, 2012a).

Different methods for control have been exploited with variable success. Electrotherapy and hot-water thermotherapy of potato tubers compared to conventional virus elimination techniques, such as meristem culture and hot-air treatment for long periods of time, are technically simple, faster and economically less costly. The combination of electrotherapy and thermotherapy resulted in high (>90%) elimination of PVY but reduced the germination rate (Petrov and Lyubenova, 2011a). Substances based on salicylic acid were used in various schemes of treatment in order to achieve systemic acquired resistance (SAR) against plant pathogenic viruses. Treatment with BION^{TM} and EXIN^{TM} resulted in 46-92% protection against PVY^{Wilga} in tomato plants (Petrov and Andonova, 2012).

In recent years, researchers have tried to control the viruses at epigenetic level. Viruses have a stage of double stranded RNAs (dsRNA) production during their replication in the cell, which are recognized by the plant as a „non-own“ and cut by cellular Dicer-like enzymes to form small interfering RNAs (siRNAs) (Denli and Hannon, 2003). The siRNAs initiate the formation of a multicomponent cell complex (RISC), which destroys complementary-specific viral mRNAs (Martinez *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 suppressors of gene silencing were HC-Pro and 2b proteins encoded in Potyviruses and Cucumoviruses, respectively (Kasschau and Carrington, 1998; Li *et al*., 1999). Potyviral HC-Pro is a multifunctional protein that also participates in the transport of virions with aphids and within the plant (Kasschau and Carrington, 1998; Revers *et al*., 1999).

Attacking the formation of viral suppressor proteins holds the key to unlocking the host plant self-defense mechanisms by posttranscriptional gene silencing (PTGS). PTGS spreads from cell to cell and thus, induction of RNA gene silencing in a single cell causes systemic signal in the whole plant (Mlotshwa *et al*., 2002).

The aim of this study was to achieve control of virus infection with one of the most damaging strains, PVY$^{N/NTN}$, by PTGS, targeting a specific viral gene region for an essential suppressor protein HC-Pro.

Material and Methods

Virus strain PVY$^{N/NTN}$, obtained from potato cv. Marabel from the virus collection of the Institute of Soil Science, Agrotechnologies and Plant Protection “N. Pushkarov” (ISSAPP) was used for this study. Fifteen plant pots with potatoes cv. Marabel served for the experiment.

Inoculation of plants

Twelve plant pots were inoculated as described 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% Na_2SO_3 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.

Three plant pots were used as positive controls. Nine plant pots were inoculated with siRNAs. Inoculation of the potato plants with siRNAs was similar to mechanical inoculation with PVY$^{N/NTN}$. We used carborundum and siRNAs.

Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA)

The analysis was conducted by the method of Clark and Adams (1977) with a commercial kit of LOEWE Biochemica GmbH, Sauerlach, Germany, 14 days after treatment of the plants with siRNAs. ELISA plates were loaded with polyclonal antiserum (IgG) for PVY, with dilutions in 0.05 M carbonate buffer according to the instructions of the manufacturer. The samples were incubated at 37°C for 4 hours 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) at a ratio of 1:10. The plates were incubated at 4℃ for 16 hours. Following the third wash, step alkaline-phosphatase conjugate for PVY was added and the plates were incubated at 37°*C* for 4 hours. The substrate used was p-nitrophenyl phosphate (Sigma) in diethanolamine buffer (pH 9.8) at a ratio of 1 mg/ml. The reaction proceeded in the light at room temperature and was stopped with 3N NaOH. The adsorption of the color reaction was measured in a multifunctional detector (DTX 880) at a wavelength of 405 nm.

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

RNA extraction from potatoes infected with PVY

Extraction of total RNA was performed with RNEasy Plant Mini Kit (Qiagen, Germany) 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 a DNA template by 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 were 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') (Petrov, 2012a; 2012b), designed so that the resulting PCR fragment contained the target sequence (*HC-Pro* of $PVY^{N/NTN}$), which is 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. ssRNA was replicated to dsRNA by virus phi6 qRdRP.

Production of siRNAs

We used a recombinant endoribonuclease from *Giardia intestinalis* - PowerCut Dicer, to cleave dsRNA to fragments with a length of 25-27 nucleotides and yield a pool of small interfering RNAs (siRNAs) (Petrov, 2012a; 2012b).

Results

HC-Pro is a multifunctional protein needed for virus spread and transfer, which also increases viral pathogenicity through suppression of PTGS. In the absence of functional HC-Pro, the viral RNA is targeted by gene silencing of the host plant (Kasschau and Carrington, 1998). The protein contains highly conservative domains, which can be used as targets for gene silencing (Petrov *et al*., 2018). Because of its features, HC-Pro was used as a target for RNAi in this study.

The polymerase system of bacteriophage phi6 used in this study generated specific dsRNAs of the selected region PVY *HC-Pro*. We received PVY HC-Pro dsRNA with length 1412 bp (Fig. 1). From this *HC-Pro* gene-specific dsRNAs, we produced siRNA pools with Power Cut Dicer.

The leaves of potatoes cv. Marabel were inoculated with a siRNA pool, complementary to the *HC-Pro* gene region of PVY. Seven days after inoculation, the plants were infected with $PVY^{N/NTN}$ and after further seven days they were observed for symptoms and tested by DAS-ELISA for presence or absence of PVY viral infection.

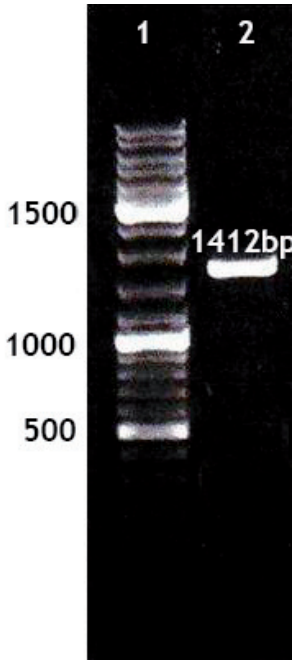

Fig. 1. PVY HC-Pro dsRNA with length1412 bp

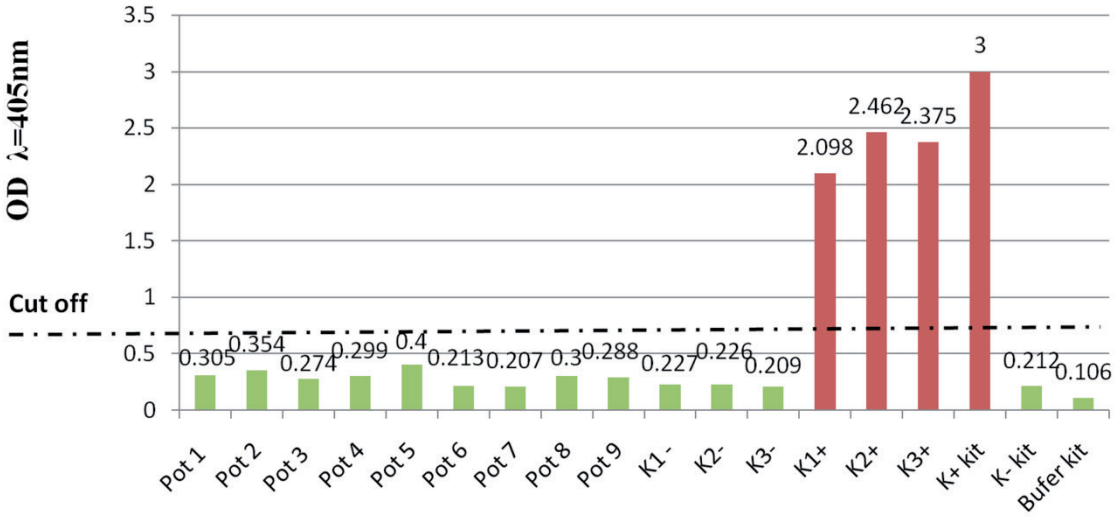

Fig. 2. DAS-ELISA of potato plants after treatment with siRNAs for *HC-Pro* of PVY$^{N/NTN}$ and after infection with PVY$^{N/NTN}$

None of the plants treated with HC-Pro-siRNAs and infected with PVY$^{N/NTN}$ developed visual symptoms of disease. The optical density (OD) values of the plant samples of these pots remained under the cut-off value and close to the healthy control plants. The DAS-ELISA value (0.4) of one of the treated plant pots was near the Cut-off but still close in value. Symptoms of infection in this pot were also not visible. High OD values were received for samples from leaves of potato plant pots inoculated with PVY$^{N/NTN}$ only (K+), indicating successful viral infection with PVY$^{N/NTN}$ (Fig. 2).

(Pot 1-9: potato plant pots treated with *HC-Pro*-siRNAs and inoculated with PVY$^{N/NTN}$; K1-, K2-, K3-: healthy potato plants not treated and not inoculated with virus; K1+, K2+, K3+: potato plants inoculated with PVY$^{N/NTN}$ and not treated with *HC-Pro*-siRNAs;K+kit: positive control from ELISA kit; K-kit: negative control from ELISA kit; Buffer kit: buffer from the kit)

Treatments with siRNAs specific to the *HC-Pro* gene region of PVY$^{N/NTN}$ resulted in growth of healthy plants, not prone to PVY$^{N/NTN}$ disease development (Fig. 2, pot 1-9). The strategy applied in this study for control of the viral infection was based on the knowledge of the mechanisms of PVY replication and plant defense responses against pathogens, assuming that blocking a key protein for the viral replication and the suppression of PTGS will lead to blocking of the development of the infection process itself.

Discussion

Treatments with siRNAs specific to the *HC-Pro* gene region of PVY$^{N/NTN}$ resulted in development of healthy plants "immune" to PVY$^{N/NTN}$ infection, which can be assigned to activation of PTGS, targeting the *HC-Pro* transcript of PVY$^{N/NTN}$ in the plant. In all of the tested plants, the DAS-ELISA values corresponded to lack of viral replication.

The results refer to full control of the viral replication by blocking the expression of the *HC-Pro* gene. The selected target is a multifunctional protein important also for the spread of the virus in the host plant tissues and among plants by aphid vectors. It can be stated that by blocking this specific target, the spread of the virus is also blocked in short distance into the surrounding plant cells and tissues, and in long distance via vectors that fail to assimilate it without normal functioning of the HC-Pro protein. One of the most important features of HC-Pro is that this protein also functions as a viral suppressor of PTGS. By blocking the gene coding for this protein, we attack the viral mechanism which overcomes the plant defense responses, allowing the effective induction of PTGS of plant cells against the viral invasion.

PTGS mechanisms have been largely studied since the 1990s. However, knowledge of PTGS was used mainly for construction of transgenic plants (Eamens *et al.*, 2008). The use of epigenetic control of viruses via PTGS by introducing artificial RNAs in plants is a novel strategy. It has been applied in tobacco plants against PVY^{O}, where the

viral replication was blocked in 82% of the tested plants (Petrov, 2012b). Full control of the virus was achieved with potatoes cv. Agria, inoculated with *HC-Pro*-specific siRNAs and subsequently infected with PVY^{NTN} virus strain. PTGS was induced by these siRNAs, which effectively blocked the viral replication and the systemic spread of the virus (Petrov *et al*., 2015a). Blocking the *HC-Pro* gene of PVY^{N} strain with complementary dsRNAs and siRNAs was established in newly grown leaves of potato plants cv. Arinda. The old leaves treated and PVY-inoculated remained infected and later defoliated while all new (not treated) leaves grown after the dsRNAs/siRNAs treatment and PVY^{N} inoculation remained virus-free (Petrov *et al*., 2015b). In this study, we successfully achieved epigenetic control of one of the most damaging strains - $PVY^{N/NTN}$, of a food crop.

Epigenetic control of viral infection via PTGS features two key advances. The crop losses due to viral diseases can be greatly reduced, which places the strategy used in this study in the unoccupied place for direct control of viral infection in plant protection. At the same time, the resistance is not inherited because of the lack of interfering with the plant genome. The sensitive crop varieties with valuable food and taste properties can be retained unaltered. Production remains ecologically clean, free of chemical compounds, transgenic proteins, allergens, mutagens, etc., which pose threats to human health. The strategy can be defined as vaccinating of crop seedlings against diseases, and provides perspectives on its use against other plant pathogens.

Conclusion

Epigenetic control of viral infection was achieved via induction of posttranscriptional gene silencing in potato plants cv. Marabel by specific siRNAs targeting the *HC-Pro* gene region of $PVY^{N/NTN}$. This strategy effectively blocked systemic spread of the virus and inhibited viral replication in host plants.

Acknowledgments:

We wish to thank the National Science Fund of Bulgaria for the financial support for contract ДНТС India 01/1.

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