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PRIMING OF *SOLANUM TUBEROSUM IN VITRO* PLANTS WITH PVY-SPECIFIC INTERFERING RNAS ACTIVATES ANTI-VIRAL RESISTANCE

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ABSTRACT

Helper component-proteinase (HC-Pro) is a multifunctional suppressor protein synthesized by potato virus Y (PVY), which is able to neutralize the protective mechanisms of RNA interference (RNA-i) in potato plants, thereby causing systemic infection of the host plant and significant damage to the tuber material. This article demonstrates how one of the main functions of HC-Pro, the capture and retention of short interfering RNAs, 21-23 nt in size (siRNA) on the surface of protein subunits, can be used to create virus-resistant potato plants in vitro. The proposed method is based on the selective dissociation of the HC-Pro/siRNA complex from PVY-infected plants and siRNA-priming of healthy plants. Purified preparations of PVY-specific siRNAs were obtained using column gel filtration followed by immunoprecipitation and phenol-chloroform extraction. Injections of siRNA into the leaf plate of virus-free potato microplants, cultivars bred at Amanzholov University, led to a decrease in the accumulation of viral particles in the cytoplasm of plants inoculated with the wild PVY strain and the formation of resistance to PVY for the entire growing season.

Key words: *Solanum tuberosum*, HC-Pro, PVY, RISC, siRNA, RNA interference.

INTRODUCTION

Phytopathogenic viruses cause enormous damage to agriculture and the economy. The existing methods of fighting viruses are reduced to obtaining virus-free seed material, but this method is not always effective, since a new strain of viral infection appears every season, and obtaining virus-free planting material is a very expensive, laborious and lengthy process. In this regard, the work on the timely diagnosis of infectious plant diseases and the development of new strategies to combat viruses are especially relevant.

One of the potential tools for increasing plant resistance to viruses is RNA interference (RNA-i) [1]. An essential feature of the protective mechanism of RNA interference is the presence of short double-stranded interfering RNA (siRNA, *short interfering RNA*) with a length of 21-23 nucleotides, which are powerful modulators of RNA interference [2, 3, 4]. When the RNA-i process is triggered, the viral double-stranded RNA (dsRNA) is fragmented by the DISER enzyme (ribonuclease from the RNase III family) into complementary short interfering RNAs (siRNA) of the viral RNA, which are further involved in the endoribonuclease effector complex RISC (*RNA-induced silencing complex*) and are directly involved in the recognition and subsequent degradation of viral RNA targets [5, 6, 7]. Researchers have demonstrated how, using the phenomenon of protective RNA interference, significant resistance to viruses can be conferred by creating genetically modified plants [8]. Our work clearly demonstrates a fundamentally new methodological approach to using PVY-infected plants as a donor of PVY-specific 21-23 nt siRNA, followed by priming of healthy potato plants to activate antiviral resistance without resorting to modification of the plant genome. Our proposed method is based on preliminary activation of RISC by exogenous PVY-specific siRNA for recognition and enzymatic cleavage of viral RNA [9].

Viruses from the genus Potyviridae encode the HC-Pro suppressor protein, which is a multifunctional viral protein and is involved in such important processes as virus replication, systemic and intercellular distribution of viral particles [10]. However, the most important biological function of HC-Pro is its participation in the suppression of RNA-i defense mechanisms, in particular, in the capture and retention of short 21-23 nt siRNA molecules, which are necessary for the successful activation of RNA-i defense mechanisms [5]. Detailed biochemical studies of HC-Pro have shown that its ability to form dimers and multimers is critical to its function as an RNA-i suppressor. The researchers determined the functional role of the FRNK site in the structure of the HC-Pro protein and showed that this function directly affects the selective binding of siRNA and the intensity of symptoms of a viral disease [12]. In addition, the suppressor function of HC-Pro may also be associated with a decrease in siRNA stability, since transgenic expression of the protein leads to a significant decrease in the 5'-terminal modification of viral 21-nucleotide siRNAs. Also, HC-Pro was found to inhibit functional siRNA methylation and siRNA binding to the RISC effector complex [13].

Since the structural and biochemical characteristics of the HC-Pro suppressor protein play a crucial role in the detection and uptake of siRNA, in particular, in the stabilization of siRNA on the surface of HC-Pro protein subunits and the formation of the HC-Pro/siRNA complex, we formulated a working hypothesis that PVY- infected plants containing the HC-Pro/siRNA complex can serve as a source of virus-specific short interfering RNAs that can be used for exogenous introduction into potato plants under sterile conditions. Virus-specific siRNAs, when they enter the cytoplasm of plant cells, can directly participate in the activation of the effector endoribonuclease complex RISC and initiate the plant's natural immune response against the PVY virus.

The main goal of this work is to prime potato plants of Amanzholov University breeding varieties with PVY-specific short interfering RNAs to activate the protective mechanisms of RNA interference against viral invasion. In order to achieve the goal, following key objectives were set: 1) to develop a preparative amount of PVY-specific siRNA from PVY-infected plants; 2) treat healthy plants of Tavria, Vostochny-1 and Isolda varieties with preparations containing PVY-specific siRNA; 3) test treated plants for resistance to PVY virus in laboratory and field conditions.

The studied data allow us to conclude that a strategy based on the use of short interfering RNAs allows us to develop a fundamentally new effective method for imparting antiviral resistance to potato plants.

MATERIALS AND METHODS

Information about the object of the study and the structure of the experiment.

Potato plants (*lat. Solánum tuberósum*), genus Solanum, Solanaceae family, classification by author Kaspar Baugin, 1596, were used as the object of the study.

Plants were grown on a Murashige-Skoog liquid nutrient medium [14] in a special growing chamber at an air humidity of 85%, a temperature of 19–20°C, and a 16-hour light period. In each experiment, 60 plants were used and 3 biological replications were carried out under the same conditions.

The research was carried out using immunological, spectrophotometric methods, ELISA, PAGE, HPLC, as well as methods of statistical data processing

Plants were inoculated with Potato virus Y (PVY), Potyvirus family, genus Potyviridae, and its isolates. The experiments were carried out according to the following scheme:

1. Growing potato plants *in vitro*.
2. Inoculation of plants with RNA-containing PVY virus.
3. Isolation of HC-Pro/siRNA complexes from tissues of infected plants using column gel filtration followed by purification by immunoprecipitation.
4. Dissociation of the HC-Pro/siRNA complex to obtain purified siRNA.
5. Preparative production of PVY-specific siRNAs.
6. Priming of healthy plants with preparations containing purified siRNA.
7. Laboratory and field testing of siRNA-treated plants for resistance to PVY.
8. ELISA diagnostics of experimental plants with the phenotype of recovery and analysis of the accumulation of viral particles.

Determination of viral infections in plants was carried out using enzyme-linked immunosorbent assay (ELISA) and real-time polymerase chain reaction (*real time RT-PCR*).

Fractionation of the HC-Pro/siRNA Complex by Column Gel Filtration.

Fresh leaf material of PVY-infected plants Solánum tuberósum in the amount of 6 g was subjected to freezing with liquid nitrogen (-196°C) and ground with a pestle in a porcelain mortar to a homogeneous mass. Extraction buffer was added to the resulting homogenate in a volume of 1 ml. (200 mM Tris-HCl, pH 7.4). The extracts were filtered and centrifuged for 15 min at 12000 g and 4°C. The supernatant was loaded onto a column (volume 150 ml, length 0.2 m) and fractionated on an AKTA pure 25 HPLC unit (GE Healthcare, USA) through a Sephacryl S-200 column (Sigma, USA) at a rate of 50 ml/ h. 100 mM Tris-HCl buffer (pH 7.5) was used as the mobile phase. Analysis of protein concentration in the eluate was carried out at a wavelength of A280 nm. Using dextran-1000 (Sigma, United States), the free column volume V0 = 14 ml was determined. Calibration was performed using marker proteins: alcohol dehydrogenase - 150 kDa, bovine serum albumin - 68 kDa, carbonic anhydrase - 40 kDa and cytochrome - 12 kDa. (Sigma, USA). Fractions with a volume of 1.5 ml were collected using an automatic collector, followed by transfer to ice [15].

Affinity purification of the HC-Pro/siRNA complex.

The HC-Pro\siRNA complex from the fractions was purified using anti-rabbit Protein-G agarose according to the method supplied with the commercial kit (Thermo Scientific, USA). Fractions in a volume of 1.5 ml were mixed with antibodies (anti-rabbit) and incubated for 12 hours in a refrigerator at 4°C. Then, 30 µl of Protein-G was poured into each tube and incubated for 2 hours at room temperature. After incubation, 500 µl of immunoprecipitation buffer (25 mM Tris, 150 mM NaCl, pH 7.2) was added to the mixture, centrifuged for 3 min, and the supernatant was removed. The precipitation step was repeated three times. The immune complex was isolated by adding 50 µl of eluting buffer (0.2 M glycine-HCl, pH 2.5), incubating for 5 minutes and centrifuging at 2500 g for 3 minutes.

Dissociation of the HC-Pro/siRNA complex and purification of siRNA from chromatographic fractions.

Nucleic acids were isolated from chromatographic fractions by a modified method using a stabilized phenol solution and a TRI reagent (Sigma, USA) [16]. The samples were sequentially mixed with the TRI reagent, chloroform, isopropanol (99.9%), ethanol (70%) and incubated step by step for 5 min. at room temperature followed by centrifugation for 10 min. at 12000 g and 4°C. At the last stage, the precipitate was dried on ice and diluted with enzyme-free distilled water.

Identification of 21-23 nt siRNA.

Electrophoretic separation of proteins and nucleic acids was carried out according to the standard method of Laemmli [17]. Identification of 21-23 nt siRNA in chromatographic fractions was performed using electrophoresis in 3% agarose gel in standard TAE buffer (0.4 M Tris acetate, 0.01 M EDTA, pH 8.3) with the addition of ethidium bromide. The siRNA bands were detected using a GelDoc gel-documenting system (Bio-Rad, USA). Gene Ruler DNA Ladder, 100 bp (Thermo Scientific, USA) was used as a marker for the number of nucleotides.

Spectral analysis of RNA concentration.

The RNA concentration was determined spectrophotometrically on a NanoDrop 2000c spectral analyzer (Thermo Scientific, USA) at a wavelength of A260 nm. The quality of the preparation was analyzed in the ratio of optical density at wavelengths of A260/280 nm. To do this, RNA samples isolated from chromatographic fractions were loaded into the reading module of the device, followed by measurement of

light absorption at a wavelength of A260 nm.

ELISA diagnostics of plant material for the presence of potato viruses.

Diagnosis of infectious diseases of potatoes (PVY, PVS, PVM, PVX, PLRV) was carried out according to the protocol of the commercial test kit of the ELISA sandwich version of the Federal State Budgetary Scientific Institution "Federal Potato Research Center named after A.G. Lorch", Russian Federation. According to the instructions for the test system, the analysis was carried out according to the generally accepted method [18]. Rabbit antibodies specific to the studied viruses (working dilution 1:500) were sensitized on the bottom of the wells of polystyrene plates in a cover buffer (0,02 M Na_2CO_3, 0,07 M $NaHCO_3$, pH 9,6) and incubated in a refrigerator for 12 hours at +4°C. After sensitization, the plates were washed with wash buffer (phosphate-buffered saline with the addition of 0.05% Tween-20, pH 7.4) to remove unbound reaction components. At the next stage, extracts of plant material and controls were added to the wells. As a negative control, lyophilized samples of the cell extract of a healthy plant were used, after which they were incubated overnight in a refrigerator at +4°C. The plates were then washed 3 times with wash buffer. A specific antiviral conjugate (working dilution 1:500) was added to the wells of the plate and incubated for 1 hour at 37°C in a thermostat. At the final stage, the wells were washed and the substrate was added to show the reaction for 10 minutes. The results of the analysis were taken into account on a Multiskan FC spectrophotometer (Thermo Scientific, USA) at a wavelength of 492 nm.

Real-time RT-PCR testing of plant material for the presence of potato viruses.

Nucleic acids were extracted from plant material using a commercial M-SORB kit (Synthol, Russia) with magnetic particles. One-step reverse transcription and real-time RT-PCR was performed using a commercial kit of reagents (Synthol, Russia) with ANK-32 PCR analyzer (Synthol, Russia). Samples were tested for PVY, PVS, PVM, PVX, PLRV viruses.

RESULTS AND DISCUSSION

PVY-infected plants usage as a source of virus-specific short interfering RNAs.

One of the functions of the suppressor protein PVY-HC-Pro is the size-specific capture and fixation on the surface of protein subunits of double-stranded short interfering RNAs specific to PVY [11, 12, 13]. In this regard, in the cell sap of plants infected with potato virus Y, there are virus-specific short interfering RNAs bound on the surface of HC-Pro subunits. Accordingly, virus-specific siRNAs can be obtained by purification and dissociation of the HC-Pro/siRNA complex from the cell sap of plants infected with PVY mono-infection.

Experimental samples of potato plants of varieties Magistr, Izolda and Tavria, planted in the garden of the East Kazakhstan Agricultural Experimental Station (EKAES), were tested for the presence of viral infections of potatoes (PVY, PVX, PVM, PVS, PVL) with commercial enzyme immunoassay diagnostic test systems, production of the Federal State Budgetary Scientific Institution "Federal Research Center for Potato named after A.G. Lorch" and using a one-step reverse transcription reaction and real-time RT-PCR using a commercial kit of reagents (Synthol, RF). According to the results of combined visual diagnostics, ELISA (Table 1) and RT-PCR (Table 2), plant samples with confirmed PVY monoinfection were selected. The resulting PVY-infected plants were used to

Table 1 - Results of ELISA diagnostics of samples for the presence of viruses, units of optical density

Variety/ sample	Name of the pathogen				
	PLRV	PVM	PVS	PVX	PVY
Magister 1	Negative	Negative	Negative	Negative	Positive 1,846±0,031
Magister 2	Negative	Negative	Negative	Negative	Positive 1,688±0,027
Magister 3	Negative	Negative	Negative	Negative	Positive 1,372±0,031
Magister 4	Positive 1,294±0,039	Positive 1,448±0,025	Negative	Negative	Positive 1,865±0,028
Magister 5	Negative	Negative	Negative	Negative	Positive 1,372±0,037
Isolda 1	Negative	Positive 1,841±0,035	Positive 1,480±0,033	Negative	Positive 1,480±0,033
Isolda 2	Negative	Negative	Negative	Negative	Positive 1,042±0,030
Isolda 3	Positive 1,557±0,033	Positive 0,945±0,021	Positive 0,945±0,018	Negative	Positive 1,076±0,023
Isolda 4	Positive 1,335±0,021	Positive 0,935±0,022	Positive 0,935±0,017	Positive 1,059±0,025	Positive 1,312±0,018
Isolda 5	Negative	Negative	Negative	Negative	Positive 1,663±0,027

Tavria 1	Negative	Negative	Negative	Negative	Positive 1,315±0,018
Tavria 2	Negative	Positive 1,717±0,032	Positive 1,150±0,020	Positive 1,014±0,034	Positive 1,310±0,025
Tavria 3	Negative	Positive 1,058±0,031	Negative	Positive 1,379±0,037	Positive 0,900±0,028
Tavria 4	Negative	Negative	Negative	Negative	Positive 1,539±0,036
Tavria 5	Negative	Positive 1,379±0,029	Negative	Positive 0,994±0,019	Positive 1,197±0,025
Positive control	1,195±0,039	0,981**±0,025**	1,289±0,029	1,118**±0,034**	1,247**±0,031**
Negative control	0,067±0,018	0,091**±0,021**	0,068±0,025	0,052**±0,020**	0,089**±0,017**

Note: 3 independent repetitions of the experiment were carried out, the figure shows one of them. The value is the average ± standard error of the average.

Table 2 - Results of PCR diagnostics of samples for the presence of viral infections

Variety/ sample	**Name of the pathogen**				
	PLRV	**PVM**	**PVS**	**PVX**	**PVY**
Magister 1	N/a	N/a	N/a	N/a	Positive 25,26±0,3
Magister 2	N/a	N/a	N/a	N/a	Positive 21,70±0,1
Magister 3	N/a	N/a	N/a	N/a	Positive 19,62±0,1
Magister 4	Positive 19,68±0,3	Positive 19,67±0,2	N/a	Positive 28,85±0,1	N/a
Magister 5	N/a	N/a	N/a	N/a	Positive 19,67±0,3
Isolda 1	N/a	Positive 28,91±0,1	Positive 22,54±0,1	Positive 20,83±0,2	N/a
Isolda 2	N/a	N/a	N/a	N/a	Positive 27,24±0,1
Isolda 3	Positive 19,26±0,3	Positive 27,82±0,2	Positive 22,54±0,1	N/a	Positive 22,54±0,1
Isolda 4	Positive 24,84±0,1	Positive 21,74±0,1	Positive 27,59±0,3	Positive 27,59±0,1	Positive 22,68±0,1
Isolda 5	N/a	N/a	N/a	N/a	Positive 25,80±0,2
Tavria 1	N/a	N/a	N/a	N/a	Positive 23,23±0,2
Tavria 2	N/a	Positive 28,82±0,1	Positive 19,95±0,2	Positive 20,41±0,3	Positive 26,21±0,2
Tavria 3	Positive 24,09±0,1	Positive 25,59±0,2	Positive 26,13±0,1	Positive 21,60±0,3	N/a
Tavria 4	N/a	N/a	N/a	N/a	Positive 26,09±0,1
Tavria 5	N/a	Positive 23,10±0,1	N/a	Positive 27,68±0,3	N/a

Note: N/a – no amplification, the result is negative. Three independent repetitions of the experiment were carried out. The value is the average ± standard error of the average.

propagate the PVY viral material and further study the complex of the HC-Pro suppressor protein with short interfering RNAs.

Purification of the ribonucleic complex of the HC-Pro suppressor protein with PVY-specific short interfering RNAs.

Identification and purification of PVY-specific short interfering RNAs was carried out in 3 stages. At the first stage, extracts of plant material from PVY-infected potato samples were fractionated by size exclusion chromatography on a Sephacryl S-200 column. This matrix was chosen because the resolution of Sephacryl S-200 allows efficient separation of proteins with a molecular weight of up to 200 kDa. Fractions eluted from the column after chromatographic separation were analyzed for the presence of proteins and nucleic acids (Fig. 1A). Using anti-HC-Pro polyclonal rabbit serum, fractions containing the siRNA-associated HC-Pro suppressor protein were identified (Figure 1B). After chromatography, the HC-Pro/siRNA complex from the fractions was precipitated with Protein-G agarose according to the standard method supplied with the commercial kit (data not shown).

At the second stage of purification, the selective separation of the HC-Pro/siRNA complex and the production of a preparative amount of short interfering RNAs specific to PVY were performed. For this, chromatographic fractions containing the HC-Pro/siRNA complex obtained in the previous experiment were treated after immunoprecipitation with trizol, phenol-chloroform, and methanol according to the standard method for RNA purification [16]. As a result, siRNAs separated from the HC-Pro suppressor protein without any structural damage or loss of specificity. Moreover, this method makes it possible to exclude foreign protein and nucleic impurities, as well as the entry of whole genome virus RNA into the preparation.

Purified siRNA samples were checked spectrometrically for total RNA content on a NanoDrop-2000 photometer (Thermo Scientific, USA). The results of spectrophotometry indicate the presence of nucleic acids in fractions 2-5, which corresponds to a molecular weight yield of HC-Pro 60±5 kDa (Table 3). The concentration of RNA in the purified preparations was determined in the range of 120-210 ng/µl. The device did not detect the presence of DNA in the samples, which indicates the purity of RNA in the obtained chromatographic fractions.

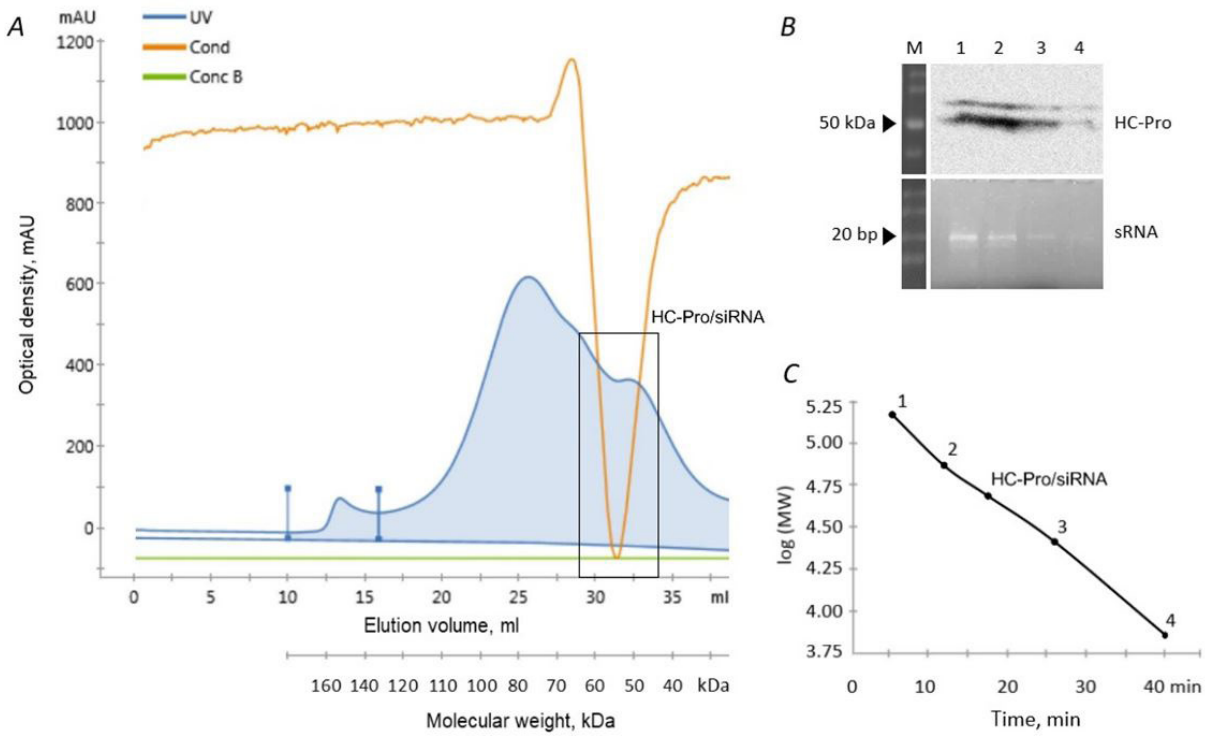

Figure 1 – Purification and identification of components of the HC-Pro/siRNA nucleoprotein complex. A) Chromatogram of the elution profile of proteins isolated from infected plant samples. The black frame shows the elution of the HC-Pro/siRNA complex (60±5 kDa). B) Western blot (top) with anti-HC-Pro polyclonal rabbit serum for identification of the HC-Pro/siRNA complex; agarose gel electrophoresis in the presence of ethidium bromide (bottom) for identification of 21-23 nt siRNA. C) Visualization of the schematic location of the yield of HC-Pro fractions on the calibration curve. The total Molecular weight of the HC-Pro dimer with the associated 21-23 nt siRNA duplex is 60±5 kDa. Note: 3 independent repetitions of the experiment were carried out, the figure shows one of them.

Table 3 - Spectral analysis of RNA concentration in chromatographic fractions containing the HC-Pro/siRNA complex

No.	RNA concentration	Units	A260 (Abs)	A280 (Abs)	260/280	Sample type
1	19,2±1,1	ng/µl	0,479±0,019	0,361±0,017	1,33±0,03	RNA
2	40,2±2,5	ng/µl	1,004±0,028	0,688±0,028	1,46±0,05	RNA

3	137,1±7,4	ng/µl	3,427±0,121	2,226±0,92	1,54±0,1	RNA
4	270,5±12,8	ng/µl	6,761±0,250	4,273±0,165	1,58±0,12	RNA
5	147,3±8,2	ng/µl	3,683±0,138	2,289±0,094	1,61±0,1	RNA
6	97±4,4	ng/µl	2,426±0,104	1,695±0,047	1,43±0,09	RNA
7	38±2,3	ng/µl	0,949±0,086	0,816±0,061	1,16±0,06	RNA
8	34,9±2,0	ng/µl	0,872±0,069	0,685±0,052	1,27±0,02	RNA
9	18,7±0,9	ng/µl	0,468±0,018	0,425±0,018	1,10±0,01	RNA

Note: There were 3 independent repetitions of the experiment. The value is the average ± standard error of the average.

At the final stage, it was necessary to determine the size of RNA purified from chromatographic fractions. For this purpose, electrophoretic analysis of nucleic acids was carried out in agarose gel electrophoresis in the presence of ethidium bromide to visualize RNA. Based on the results of the analysis, it was possible to identify short RNA molecules with a length of 21-23 nucleotides in chromatographic fractions containing the viral suppressor HC-Pro (Figure 1B). The data can be interpreted by the fact that the molecular weight of one pair of RNA nucleotides corresponds to 640 Da, and the mass of HC-Pro is 50 kDa [20]. Based on this information, it can be argued that the total mass of the HC-Pro dimer with the associated 21-23 nt siRNA duplex is 60 ± 5 kDa (Figure 1C), which corresponds to the previously obtained data of other authors [10, 20].

Thus, the presence of the 60 kDa HC-Pro/siRNA complex in PVY-infected Solánum tuberósum plants was shown, which correlates with the data of other authors [10, 11, 12, 20, 21]. A new complex method for the purification of PVY-specific short interfering RNAs was also demonstrated, which can be used in the preparative production of the required amount of siRNA for priming potato plants in order to confer antiviral resistance to PVY.

siRNA priming of healthy in vitro potato planting material allows plants to effectively neutralize the symptoms of a viral infection in the field.

Testing the effectiveness of acquired resistance to the PVY virus was carried out on test-tube microplants in vitro of potato varieties bred at Amanzholov University. A total of 180 plants of the varieties Tavria, Vostochny-1 and Izolda were involved in the experiment. These varieties are included in the State Register of varieties recommended for cultivation in the Republic of Kazakhstan. A necessary condition was laboratory testing of plants for the degree of resistance to PVY viral infection after inoculation with virions and analysis for the presence of viral particles in the cell sap of immunized plants.

All plants were divided into 4 experimental groups: 1 control group of healthy plants; 2 control group infected with wild strain PVY plants; group 3 - PVY-infected plants not subject to siRNA pretreatment; Group 4 - PVY-infected plants pre-treated with siRNA. Experimental plants were continuously monitored with photofixation of symptoms and morphological changes.

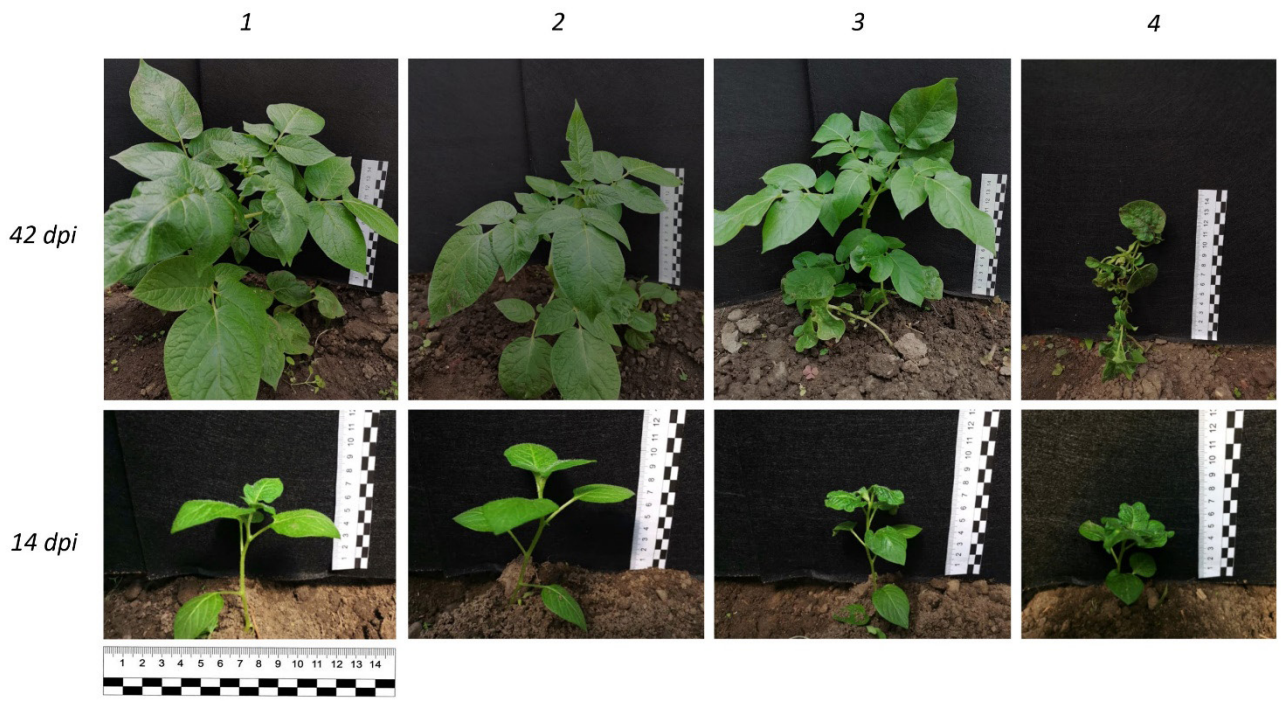

Figure 2 – Healing effect after siRNA-priming of potato plants in vitro in the field. 1 - control group of healthy plants; 2 – control group of siRNA-treated plants without subsequent PVY inoculation; 3 – experimental group of plants treated with 120 ng/µl of PVY-specific siRNA and inoculated with PVY; 4 - control group of PVY-infected plants. Note: 3 independent repetitions of the experiment were carried out, the figure shows one of them.

As a method for delivering siRNA into plant tissues, the method of injection with a microsyringe into a leaf plate under sterile conditions was used. After the introduction of exogenous PVY-specific siRNAs (5 days after priming), the plants were planted indoors. The period of adaptation to natural conditions was 5 days, then the plants in the field were inoculated with wild type PVY. After 7-9 days after infection, plants that were not pre-treated with viral siRNAs showed symptoms characteristic of PVY: development of a wrinkled mosaic of leaves, damage to the veins on the leaf blade, necrotic spots, death of the leaf blade, a significant slowdown in the growth and development of the terrestrial part of plants [10].

The group of plants immunized with siRNA preparations did not lag behind in growth and development from the control group of healthy plants, however, they showed minor symptoms on the first and second leaf tiers, which disappeared on days 21-27 after inoculation with the virus (Figure 2).

At the end of the growing season, the minitubers of the experimental plants were also checked for symptoms of PVY. The symptoms of the disease on the tuber material of the experimental plants did not appear, the disease proceeded stably, without causing any damage to the minitubers. The control group of PVY-infected plants showed significant developmental deficiencies and the presence of viral minituber lesions, compared to the experimental groups of siRNA-treated plants (Figure 3).

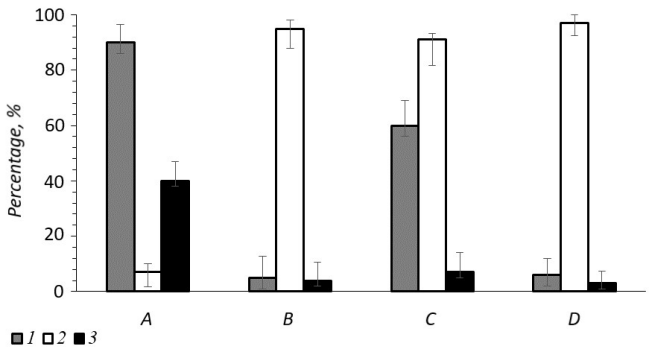

Figure 4 – Dynamics of the pathogenesis of PVY infection on experimental plants during the growing season. A) control group of PVY-infected plants; B) control group of siRNA-treated plants without subsequent PVY inoculation; C) experimental group of plants treated with 120 ng/µl of PVY-specific siRNA and inoculated with PVY; D) control group of healthy plants. 1 - plants with symptoms of PVY; 2 - healthy plants or plants with a phenotype of recovery after a PVY infection; 3 - collapsed plants. Note: 3 independent repetitions of the experiment were carried out, the figure shows one of them. The value is the average ± standard error of the average.

son at an early stage of PVY infection in siRNA-treated plants showed minor symptoms of the disease, which almost completely disappeared on days 21–27 after PVY inoculation. The development of the ground part of plants and tuber material proceeded within the normal range until the end of the growing season. This section shows the health-improving effect on test-tube plants in vitro and minitubers of potato varieties

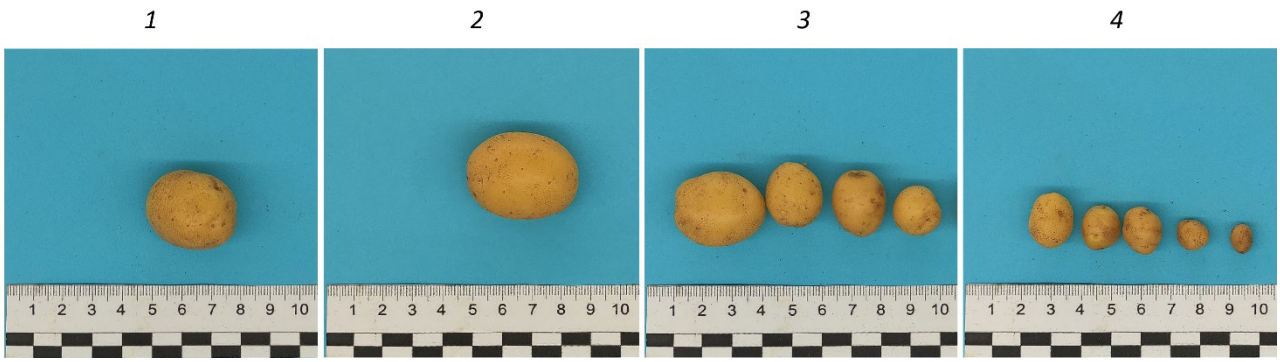

Figure 3 – Preventive effect on tuber material after siRNA-priming of potato plants in vitro. 1 – control group, minitubers of healthy plants; 2 – control group, minitubers of siRNA-treated plants without subsequent PVY inoculation; 3 – experimental group, minitubers of plants treated with 120 ng/µl of PVY-specific siRNA and inoculated with PVY; 4 - control group, minitubers of PVY-infected plants. Note: 3 independent repetitions of the experiment were carried out, the figure shows one of them.

The presence of symptoms at an early stage of development in siRNA-immunized plants after PVY inoculation was recorded in 60±4.1% of cases. Symptoms in the control group of plants treated with siRNA without further inoculation with the virus were recorded only in 5±1.25% of plants, which indicates a high degree of purification of siRNA preparations and the safety of their use on healthy plants. Plant collapse was statistically observed in less than 3±0.92% of virus-negative control groups. Plants from the positive control group for the PVY virus showed the presence of disease symptoms in 96±2.37% and plant death in 40±8.2% of cases. According to the results of laboratory tests, the viability of siRNA-immunized plants after PVY infection is 92±3.12%, the presence of leaf wrinkling symptoms was detected in 60±4.1% of immunized plants. No viral symptoms or lesions were identified on minitubers of siRNA-treated plants (Figure 4).

Thus, dynamic visual diagnostics during the growing season bred at Amanzholov University, planted in closed ground for the full growing season from June to September 2021. Next, the effect of plant siRNA priming on the accumulation of viral particles in the cytoplasm of the host plant will be shown.

Treatment of plants with preparations containing PVY-specific siRNAs significantly reduces the concentration of viral particles in plant cells and tissues.

Experimental in vitro siRNA-immunized potato plants of Izolda, Tavriya and Vostochny-1 varieties, planted in the field, were tested for the presence of potato viral infections (PVY, PVX, PVM, PVS, PVL) using commercial enzyme immunoassay diagnostic test systems produced by the Federal State Budget Scientific institutions «Federal Research Center for Potato named after A.G. Lorch» According to the test results, it was found that the concentration of surface antigens of the PVY virus in the cell sap of virus-susceptible plants

subjected to pretreatment with virus-specific siRNAs is reduced by 80±7.1% relative to the control group of PVY-positive plants (Table 4). The results can be interpreted by the molecular properties of the RISC effector complex, which was programmed upon exogenous introduction of PVY virus-specific siRNAs into the cytoplasm for recognition and enzymatic cleavage of viral RNA [8].

Table 4 - Dynamics of the decrease in the accumulation of viral particles in siRNA-treated plants

Potato variety	Groups of experimental plants tested for PVY		
	1 (PVY+)	*2* (siRNA+)	*3* (siRNA+PVY)
Vostochny-1	Positive 1,965±0,031	Negative 0,081±0,038	Positive 0,577±0,033
Vostochny-1	Positive 1,738±0,032	Negative 0,031±0,033	Positive 0,624±0,030
Vostochny-1	Positive 2,001±0,025	Negative 0,028±0,038	Positive 0,538±0,025
Isolda	Positive 1,709±0,034	Negative 0,045±0,037	Positive 0,517±0,019
Isolda	Positive 1,957±0,025	Negative 0,092±0,028	Positive 0,588±0,022
Isolda	Positive 1,763±0,033	Negative 0,090±0,034	Positive 0,625±0,024
Tavria	Positive 1,676±0,026	Negative 0,039±0,036	Positive 0,648±0,038
Tavria	Positive 1,743±0,038	Negative 0,022±0,025	Positive 0,506±0,034
Tavria	Positive 1,988±0,036	Negative 0,044±0,018	Positive 0,555±0,019
Positive control	1,847±0,025	1,752**±0,031**	1,898±0,036
Negative control	0,056±0,016	0,049±0,017	0,072±0,019

Note: 1 - Control group of PVY-infected plants; 2 – control group of siRNA-treated plants without subsequent PVY inoculation; 3 – experimental group of plants treated with 120 ng/µl of PVY-specific siRNA and inoculated with PVY. Three independent repetitions of the experiment were carried out. The value is the average ± standard error of the average.

Thus, upon infection with a wild strain of PVY, RISC endoribonuclease activity significantly reduces the concentration of viral particles in the cytoplasm, inhibiting further reproduction and spread of the virus between host plant cells. The results obtained allow us to state that pretreatment of in vitro potato microplants with preparations containing 120 ± 20 ng/µl of PVY-specific siRNA immediately before planting in open ground and infection with a wild strain of the PVY virus prevents systemic death of the plant, damage to tuber material and gives immunized plants stable resistance to the course of the disease.

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Compliance with ethical standards

This article does not contain any studies involving humans as research objects.

Conflict of interests

The authors declare no conflict of interest. The sponsors were not involved in the design of the study; when collecting, analyzing or interpreting data; in writing the manuscript or in the decision to publish the results.

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ПРАЙМИНГ *IN VITRO* РАСТЕНИЙ *SOLANUM TUBEROSUM* PVY-СПЕЦИФИЧНЫМИ КОРОТКИМИ ИНТЕРФЕРИРУЮЩИМИ РНК АКТИВИРУЕТ АНТИВИРУСНУЮ РЕЗИСТЕНТНОСТЬ

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АБСТРАКТ

Helper component-proteinase (HC-Pro) – мультифункциональный супрессорный белок, синтезируемый вирусом картофеля Y (PVY), способен нейтрализовать защитные механизмы РНК-интерференции (RNA-i) у растений картофеля, тем самым вызывая системное заражение растения-хозяина и значительное поражение клубневого материала. В данной статье наглядно показано, каким образом можно использовать одну из основных функций HC-Pro – захват и удержание коротких интерферирующих РНК, размером 21-23 nt, (siRNA) на поверхности субъединиц белка – для создания вирус-резистентных растений картофеля in vitro. В основе предлагаемого способа лежит селективная диссоциация комплекса HC-Pro/siRNA из PVY-инфицированных растений и siRNA-прайминг здоровых растений. С помощью колоночной гель-фильтрации с последующей иммунопреципитацией и фенол-хлороформной экстракцией были получены очищенные препараты PVY-специфичных siRNA. Инъекции siRNA в листовую пластину безвирусных микрорастений картофеля, сортов селекции Университета Аманжолова, приводили к снижению накопления вирусных частиц в цитоплазме инокулированных диким штаммом PVY растений и формированию устойчивости к PVY на весь период вегетации.

Ключевые слова: *Solanum tuberosum*, HC-Pro, PVY, RISC, siRNA, РНК-интерференция.

ӘОК: 581.1; 578.232; 632.08; 632.938

IN VITRO SOLANUM TUBEROSUM ӨСІМДІКТЕРІН PVY - СПЕЦИФИКАЛЫҚ ҚЫСҚА ИНТЕРФЕРЕНТТІ РНҚ-МЕН ӨҢДЕУ ВИРУСҚА ҚАРСЫ ТӨЗІМДІЛІКТІ БЕЛСЕНДІРЕДІ

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АҢДАТПА

Helper component-proteinase (HC-Pro) – картоп өсімдіктеріндегі РНҚ интерференциясының (RNA-i) қорғаныс механизмдерін бейтараптандыру арқылы өсімдіктің жүйелі зақымдануы мен түйнек материалына айтарлықтай зиян келтіретін у (PVY) картоп вирусымен синтезделетін көп қызметті супрессорлы ақуыз. Бұл мақалада вирусқа төзімді in vitro картоп өсімдіктерін алу үшін HC-Pro – ның ақуыз суббірліктерінің бетінде 21-23 nt (siRNA) өлшемді, қысқа интерферентті РНҚ-ны ұстау және ұстап тұру қызметін қалай қолдануға болатындығы нақты көрсетілген. Ұсынылған әдіс PVY жұқтырған өсімдіктерде HC-Pro/siRNA кешенінің селективті диссоциациялануна және сау өсімдіктердің siRNA-мен өңделуіне негізделген. Колонкалық гелдік-фильтрлеу, иммунопреципитация және фенол-хлороформды экстракциялау көмегімен PVY - спецификалық siRNA тазартылған препараттары алынды.

Аманжолов университетінің селекциялық сорттарына жататын картоптың вируссыз микроөсімдіктерінің жапырақ тақталарына siRNA енгізу жабайы PVY штамы егілген өсімдіктердің цитоплазмасында вирустық бөлшектердің жинақталуының төмендеуіне және бүкіл вегетация кезеңінде PVY-ке төзімділіктің қалыптасуына әкелді.

Түйінді сөздер: *Solanum tuberosum*, HC-Pro, PVY, RISC, siRNA, РНҚ-интерференция.