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Genetically Transforming Russian Potato Cultivars for Resistance to Colorado Beetle

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Abstract

Using *Agrobacterium*-mediated transformation procedure, a total of 204 primary transformants of the Elizaveta, Lugovskoi and Nevski potato varieties carrying the *cry* IIIa gene were obtained. A number of biomolecular tests, including Southern hybridization, insert copy number screening, immunofluorescence analysis for assessment of the target gene expression levels, and PCR control for testing of the insert integrity, was carried out for these transformants, as well as the biosafety field trials for the transgenic lines selected during the biomolecular analysis. As a result, 3 transgenic lines (E2, N1, and L5) carrying one insert copy per genome with target gene expression level over 10 ppm were selected.

Keywords: Biosafety; *CryIIIA*, field tests; δ-endotoxins; Genetic transformation; Resistance against Colorado beetle; *Solanum tuberosum*

Abbreviations: NAA: 1-naphtalene acetic acid; Zea: zeatin 6-(4-hydroxy-3-methyl-but-2-enylamino] purine; BAP: 6-BAP-N^6-benzyladenine; GLY: glyphosate; Cb: Carbenicillin disodium salt; GA_3: Gibberellic acid A_3

Introduction

The estimated losses that the Colorado beetle (*Leptinotarsa decemlineata* Say) brings to potato crops vary from 18% of the potential yield for large state potato producers to 40–90% for private producers. According to the expert estimates of the Russian Federation Ministry of Agriculture, the latter are responsible for 90% of the gross potato yield. While various methods (mechanical, chemical, and biological) are used to control this pest, but most of them are either inefficient (mechanical) or harmful to the environment (chemical), because, as a rule, pesticides possess no selective effect and attack harmful and useful entomofauna equally. Moreover, persisting in the environment and foodstuffs, they become a potential source of carcinogens and toxins.

The common strategy to create genetically modified plants resistant to insect pests is the usage of natural proteinaceous insecticides (δ-endotoxins); the genes encoding these proteins were extracted from various strains of the bacterium *Bacillus thuringiensis* [1-5]. Such transgenic plants were named Bt-protected plants. In 1993, transgenic potato plants with amazingly high resistance to Colorado beetle were obtained [6]. Bt-protected corn, cotton, and potato were originally implemented into agricultural practice in the United States in 1995-1996. Large-scale tests and long-term agricultural application in various countries confirmed the safety of the products obtained from Bt-crops for humans and environment [7-9].

Toxicological researches on mammals and studies of Bt-toxin digestion in the gastrointestinal tract confirmed that these proteins were nontoxic and did not cause any problems in respect to their allergenic potency. It was found that foodstuffs and their components derived from Bt-plants were identical to the same products obtained from regular plants in virtually all respects [10,11].

All these findings were the inspiration for the onset and successful realization of the program on creating genetically modified varieties of Russian selection resistant to Colorado beetle.

This work is dedicated to creation of new biotechnological potato varieties by means of genetic engineering via *Agrobacteria*-mediated transformation by inserting a genetic vector carrying the *cryIIIa* gene into the genome of Elizaveta, Lugovskoi and Nevski potato plant varieties.

Materials and Methods

Plant material and potato transformation

Source plants of the Elizaveta, Lugovskoi, and Nevski varieties, which were free from virus and viroid infections, were cultured *in vitro* under aseptic conditions. For this purpose, source plant stems were cut into cuttings with one axillary bud and incubated in Petri dishes on agar nutrient medium containing mineral salts and vitamins (MSbase medium, supplemental material S1) at the temperature of 18-21°C and photoperiod of 16 hours day/8 hours night (light intensity 120 μE) for 3 – 5 weeks.

The *Agrobacterium tumefaciens* GV 3101 strain [12,13] carrying the pMON38943 plasmid kindly provided by Monsanto Co (supplemental material S2) was used for plant transformation.

Potato explant transformation was performed according to the technique suggested by Block [14] according to the protocols optimized to comply with the reaction of the variety genotype [15-17].

A. tumefaciens GV 3101 strain was cultured in Luria-Bertani (LB) nutrient medium with antibiotics (streptomycin, spectinomycin, and kanamycin, 50 mg/l each and chloramphenicol, 25 mg/l) for 48 hours in the dark, at 28°C with shaking (100-140 rpm).

The internodes were cut into segments without axillary buds, 5-10 mm long, and placed onto the surface of sterile paper filter on the Callus Inducing Medium (supplemental material S1) 24 hours before transformation.

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Overnight culture of *A. tumefaciens* was diluted up to OD_{650}=0.12 with liquid CIM medium. Diluted strain suspension (0.2-0.5 ml) was applied uniformly over the surface of solid nutrient medium, for which purpose the filters with explants were removed carefully and then put back over the applied suspension. The explants were arranged on the wet filter for co-cultivation for 48 hours at 18-20°C.

To initiate callus formation, the explants were transferred onto CIM nutrient medium with plant growth regulators in appropriate concentrations (supplemental material S1). Regeneration and selection were carried out starting from the 8th day in Regeneration Medium supplemented with 4.23 mg/l of glyphosate (N-phosphonomethyl glycine) as a selective agent. The emerging green embryos were cultured in the Root Inducing nutrient medium. As a control, the same quantity of liquid CM nutrient medium was used instead of *A. tumefaciens* CBE121 strain suspension.

To test the efficiency of the regeneration process, regeneration medium in the control versions was used without selective agent (Regeneration medium, supplemental material S1).

Transgenic detection using Polymerase Chain Reaction (PCR)

Regenerated shoots selected on selective medium with the herbicide were tested for the presence of heterologous gene by PCR with the primer set specific for the NOS-T/*CryIIIA* junction of the transgenic insert.

For further work, only the shoots were selected for which a 540-bp PCR product was revealed. For more detailed PCR analysis, additional reactions followed by digestion of resulted PCR products with the *PvuI* restriction enzyme were carried out as shown in supplemental material S3. After digestion, the presence of the expected restriction fragments was sufficient to confirm the existence of a transgenic insert in the investigated plants. The results of detailed PCR/restriction analysis of transgenic potato lines E2, L1 and N5 are presented on Figure 1.

ELISA assay

ELISA assay were carried out using the kit by Monsanto Co. (USA) according to the manufacturer's instruction. ELISA results were read using a UNIPLAN plate reader (Russia). Mathematic treatment of the results was carried out using the standard Microsoft Excel 2003 package.

Southern blotting analysis

Genomic DNA was extracted from the control and experimental lines according to the standard technique (CTAB) [18].

Radioactively labeled probes for Southern hybridization were prepared on the basis of the PCR fragments received on the vector DNA used for potato transformation. The diagram of the PCR fragment arrangement within the expression cassette is shown on Figure 1. Herewith, the overlap area of each of the probes with the *HindIII*-marker fragment (1800 base pairs long) was at least 300 base pairs.

The label was inserted into the probe by multiple linear polymerase reaction using one of the primers of each PCR-fragment. Therefore, obtained probes were single-stranded, which prevents self-hybridization of the probe and increases the hybridization efficiency.

For Southern blot analysis, the obtained genome DNA samples of transgenic potato plants were digested with restriction endonucleases *HindIII* and *EcoRV* (Fermentas), the restriction products were decomposed by electrophoresis in 0.7% agarose gel. Standard quantities of DNA (1 ng, 0.1 ng and 0.01 ng) of the pMON3843 vector treated with *HindIII* restriction endonuclease were used as the control. Restriction endonuclease treatment was performed under the conditions recommended by the manufacturers, with the 1 μg DNA3/10 U of the enzyme.

Upon the end of electrophoresis, DNA was transferred to Hybond XL (Amersham) membranes using the capillary transfer method [19]. Hybridization with the labeled probe and further washing off non-hybridized probe were performed according to the standard protocol recommended for Hybond XL. The membranes were exposed with a screen film for up to 170 hours. The results of the Southern blotting are shown on Figure 2.

Checking the amino acid equivalence of the expressed protein of transgenic potato plants and *CryIIIA*

For transgenic potato lines with a single *CryIIIA* gene insertion, we obtained PCR fragments of the coding area of the *CryIIIA* gene and analyzed their base sequence. Received sequences were translated into amino acids and compared to each other using the BioEdit software package [20].

Field trials

In 2000, limited field trials (registered site no. 09-P/1999) were held to cultivate and obtain tuberous material of transgenic plants.

During the period from 2001 to 2003, on the plots registered by the Inter-Agency Committee on Genetic Engineering at the Russian Ministry for Industry and Science (ICGE) no. 09-P/1999 (Scientific Research Institute of Phytopathology, Russian Academy of Agricultural Sciences, B. Vyazemy village, Moscow region) and no. 07-P/2002 ("Rogachevo" agrocompany, Dmitrovsky district, Moscow region, data not shown), limited field trials were carried out according to the international UPOV system and assessment of agrotechnical characteristics [21].

Colorado potato beetle resistance field trials: During the period from 2003 to 2004, at the site registered by ICGE no. 24/P-99 (Scientific Research Institute of Biological Protection of Plants, Krasnodar krai,

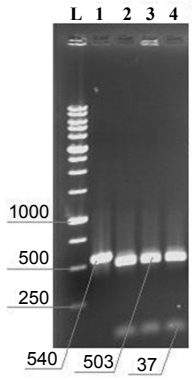

Figure 1: An example of restriction analysis of the genomic DNA extracted from potato transformed lines.
L – Gene Ruler 1 kb DNA Ladder (Lithuania, Fermentas #SM0311), 1 – position of PCR fragment obtained with primer pair Int1-Int2; 2, 3, 4 – Pvu I restricts for Int1-Int2 PCR fragments obtained on DNA of E2, L5 and N1 lines, correspondingly.
The lengths of GeneRuler fragments are given at the left, the lengths of PCR and restriction fragments are given at bottom.

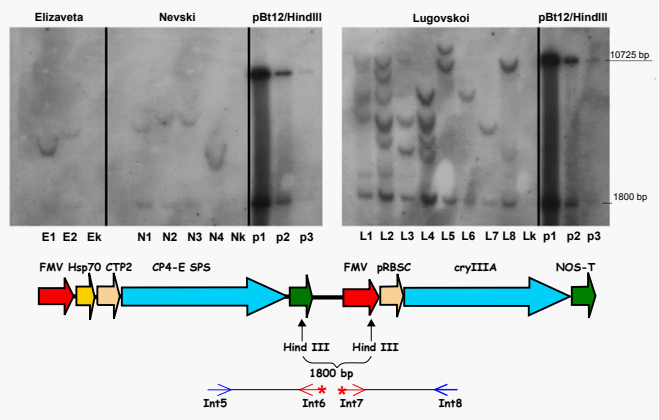

Figure 2: Southern blotting of HindIII/EcoRV digested potato genomic DNA. Designing the probes for Southern blotting.
E1-E2 – various Elizaveta transgenic lines, 10 mkg per line; N1-N4 – various Nevski transgenic lines, 10 mkg per line; L1-L8 – various Lugovskoi transgenic lines, 10 mkg per line; p1, p2, p3 – 1000 pg, 100 pg and 10 pg of HindIII digested pBt12 vector DNA, correspondingly.

Russia), limited field trials of transgenic potato lines were carried out in order to assess the expression of the introduced feature (resistance to Colorado beetle) in the field.

In 2003, resistance was monitored for three months starting from the beginning of Colorado beetle attack at plants to natural dying off of the aerial parts potato plants. The material under study was planted in three repeats of 30 plants for each line. The average number of different stages of pest development at one plant, the number of plants attacked by the pest, and the percentage of leaf surface damage by the pest were determined. The controls were plants of the original Nevski, Lugovskoi and Elizaveta varieties. Potato plants of the Superior Newleaf (*Monsanto*, US) variety were used as the standard.

In 2004, comparative monitoring of the biological efficiency of Bt-potato and chemical defense with insecticides offered by Avgust company, Russia against the Colorado beetle was carried out (Table 2). At each of the sites, 200 potato tubers of transgenic lines and source varieties, respectively, were planted. The area of each site was 200 m^2.

Results and Discussion

Transformation efficiency

First studies on potato transformation have been published in the 1980s. The possibility to obtain transgenic potato via bacterial transformation using *A. tumefaciens* was originally demonstrated in 1986 [22] and confirmed in our laboratory in 1990 – 1992 [23,24]. Since the transformation process is genotype-dependent, transformation was conducted according to the protocols priliminarily optimized for a specific variety. However, the transformation efficiency (percentage ratio of primary transformants to the total number of regenerated shoots), calculated by the results of checking regenerant DNAs in PCR reaction for the *CryIIIA* gene content, varied significantly depending on the genotype (Table 1). Out of 1363 checked transformants, in 204 the presence of the *CryIIIA* gene sequence was confirmed; thus, the transformation efficiency for different potato varieties differed more than two fold [25,26].

Immunoenzyme screening of selected transformants

For 204 transformants selected according to PCR analysis, immunofluorescence testing was performed to select the lines with the Bt protein expression exceeding 10 ppm in the leaves (Table 2). Such level of Bt-toxin is considered sufficient to prevent development of resistance to it in Colorado beetles [27,28]. Transgenic lines with the *CryIIIA* protein expression level over 10 ppm/g tissue were cultivated *in vitro* for further research.

Following the results of this research, 3 selected transgenic lines of the Elizaveta variety, 8 lines of the Nevski variety, and 13 lines of the Lugovskoi variety were selected, for which transgenic insertion abundance in genomic DNA was later defined.

Southern analysis of insertion sequence abundance into the genomic DNA of potato transgenic lines

Insertion abundance is one of the most important characteristics of a transgenic plant, because it defines both the plant biosafety level and the methods for the quantitative analysis of the foodstuffs produced from it. New approaches based on real-time PCR and microchip analysis were recently proposed to define the abundance [29]. To obtain reliable data by these methods, however, a standard reference sequence for potato genomic DNA is needed, which should meet several requirements – species-specifity, uniqueness or a known number of copies in genomic DNA, and equal number of the copies among different varieties. By now, reference targets complying with these requirements have not been suggested for potato genomic DNA. In this work we therefore used the generally acknowledged method for determination of gene abundance in genomic DNA, Southern hybridization [19]. The choice of restriction endonuclease for Southern blotting was made basing on the analysis of the general restriction map of the DNA fragment to be inserted. Therewith, it was considered that the transmittable genetic maker contained two recognition sites for the *HindIII* ferment, which should have led to emergence of a marker fragment indicating the presence of transgenic insertion in the plant genomic DNA. The *EcoRV* enzyme recognition site was absent from the transmittable maker, so the genomic DNA of the studied transgenic lines was additionally treated with *EcoRV* restriction endonuclease to shorten the restriction fragments containing a transgenic insertion. Figure 2 shows the arrangement of PCR fragments used for creating the probes for Southern hybridization. Int6 and Int7 primers were selected so as to be within the *HindIII*-fragment of transgenic insertion at the distance of approximately 300 bases from the *HindIII* sites. Thus, each probe hybridized with two different restriction fragments.

It can be seen from the data of radioautogram (Figure 2) that all examined transgenic lines of the Elizaveta and Nevski varieties had one copy of the transgenic insertion, while only half of the Lugovskoi

Potato variety	*Number of regenerated plants tested*	*Number of verified transformants*	*Transformation efficiency* (%)*
Elizaveta	472	44	9.3%
Nevski	415	61	14.7%
Lugovskoi	476	99	20.8%

*- calculated according to *cry*IIIa PCR detection.

Table 1: Transformation efficiency of different potato varieties.

Potato variety	*Number of analyzed transformants*	*No. of transformants with Bt level ≤10 ppm*	*No. of transformants with Bt level >10 ppm*
Elizaveta	44	37	7
Nevski	61	38	23
Lugovskoi	99	56	43

Table 2: Bt-toxin expression levels in studied leaves.

variety lines (L5-L8) carried one copy each, but in L2 line's DNA as many as three different insertions were observed.

Based on these results, one line of each variety was selected for further molecular genetic analysis: E2, N1 and L5. Later these lines were patented as Elizaveta plus 2904 kgs/1, Nevski plus 0311 mbc and Lugovskoi plus 1610 amk varieties, correspondingly.

Determination of the equivalence of expressed Bt-toxin

It was shown earlier that the Bt-toxin synthesized in potato transgenic lines of the Russet Burbank NewLeaf™ variety was nontoxic for mice up to a dose of 5220 mg/kg [30]. The genetic maker we used for potato transformation encoded the *CryIIIA* protein identical in amino acid content to the one expressed in the Russet Burbank NewLeaf™ variety lines. Consequently, the task for the next research stage was checking the identity of the *CryIIIA* proteins expressed in the lines we obtained and the *CryIIIA* protein from Russet Burbank NewLeaf™ GM potato. For all selected transformed lines we obtained PCR fragments corresponding to the *CryIIIA*-coding area of transgenic insertion and defined their base sequence. After that, DNA sequences of the PCR fragments were translated to amino acid ones and matching sequences were aligned with the *CryIIIA* protein sequence encoded by the corresponding gene of the expression cassette of pBt12 transforming vector. Analysis of the data obtained suggested that all the proteins examined were completely identical to each other (S. 4). Thus, the Bt-toxin expressed in potato transgenic lines we obtained should possess the same levels of toxicity and allergenic capacity as Bt-toxin of transgenic potato of the Russet Burbank NewLeaf™ variety has.

Transgenic line tests on distinguishability, uniformity, and stability according to the UPOV System (field plot no. 09-P/1999)

Since phenotypic characteristics of transgenic and source (control) potato lines could differ between each other, the task for the next research stage was detailed definition of the agronomical characteristics of the obtained lines according to the UPOV testing procedure acknowledged both in the EU and in Russia as a standard method for description of a new plant variety. As obtained Bt-lines are planned to be implemented in industry, it is required to undergo the state registration rules, with the new lines included into the State Register of Varieties and Selection Achievements Accepted for Cultivation in the Russian Federation.

For this purpose, assessment of Bt-lines 0311 mbc (N1), 1210 amk (L5), 2904/1 kgs (E2), and the source Nevski, Lugovskoi and Elizaveta varieties as standard and control for 42 variety characteristics and resistance to Colorado beetle was conducted in the field (on registered plots) for three years.

Basing on the obtained results, it can be concluded that there are no sufficient differences in variety characteristics between transgenic potato Bt-lines and source varieties. Thus, the only sufficient feature distinguishing the transgenic lines from the source non-transgenic varieties is resistance to Colorado beetle.

Field trials of transgenic lines on resistance to colorado beetle (field plot no. 24/P-99)

The main criterion of the efficiency of our work was the level of the effect of Colorado beetle on the obtained transgenic potato lines. As

Line number	Plant occupation by CB, %	Leaf damage, %	Plant occupation by CB, %	Leaf damage, %	Plant occupation by CB, %	Leaf damage, %	Plant occupation by CB, %	Leaf damage, %
Register date	27.05.2003		17.06.2003		15.07.2003		06.08.2003	
N1	7,3	0	50,2	0	8,0	0	8,0	0
Control Nevski	100	75,0	100	100	–	100	–	100
L5	24,0	0	58,0	0	12,0	0	12,0	0
Control Lugovskoi	100	40,0	100	100	–	100	–	100
E2	15,0	0	35,0	0	16,0	0	0	0
Control Elizaveta	100	30,0	100	100	–	100	–	100
Superior Newleaf variety (standard)	10,0	0	10,0	0	5,0	0	0	0

Table 3: Trial results on Colorado beetle resistance (first year of trial, 2003).

Line number	Plant occupation by CB, %	Leaf damage, %	Plant occupation by CB, %	Leaf damage, %	Plant occupation by CB, %	Leaf damage, %	Plant occupation by CB, %	Leaf damage, %
	10.06		30.06		9.07		22.07	
N1	0	0	0,1	0	0,1	0	8	0
Control Nevski	10	10	60	50	80	80	100	100
Chemical protection Control Nevski	10	10	60	40	30	30	40	40
L5	0,01	0	0,1	0	0	0	1,0	0
Control Lugovskoi	10	10	50	20	80	50	90	85
Chemical protection Control Lugovskoi	8	10	50	20	20	30	30	40
E2	0	0	0,1	0	1	0	1	0
Control Elizaveta	10	10	50	40	70	60	95	95
Chemical protection Control Elizaveta	10	10	60	30	20	40	30	50
Superior Newleaf	0,01	0	0	0	0,9	0	5	0
Control Superior	8	10	45	30	60	60	95	80
Chemical protection Control Superior	8	10	50	35	30	30	40	40

Table 4: Trial results on Colorado beetle resistance (second year of trial, 2004).

the research results shown in Table 3 demonstrated, all tested Bt-lines exhibited complete resistance to Colorado beetle; the corresponding indices were at the level of the Superior Newleaf standard variety. During the first year of trial, in spite of the pest occupation of transgenic plants during the vegetation period from 7.3 to 58.0 % (with average pest number from 0.1 to 3.1 imago specimen and from 0.1 to 0.3 larval specimen per 1 plant and no egg-laying), leaf surface damage on the GM potato lines was virtually 0%, while 100% of the source non-transgenic potato varieties (with high level of occupation – from 2.2 to 3.3 egg-layings and from 15 to 25 larvae per plant) were destroyed by the pest (Table 3).

During the second year of field trial, no larvae or egg-layings of the pest were detected on transgenic potato plants, only imagoes were observed in a number not exceeding 0.1 to 1.0 specimen per one plant with total occupation not exceeding 8.0% of the total number of plants (Table 4). By the last register date, the control plants were destroyed by the pest completely. Comparative monitoring of the biological efficiency and chemical protection of Bt-potato showed that transgenic potato had advantages over chemical treatment, because leaf damage in the chemically protected versions varied from 40 to 50%, while all Bt-lines demonstrated complete (100%) resistance to Colorado beetle.

In 2009 all the three transgenic varieties Lugovskoi plus (1210 amk, L5), Nevski plus (0311 mbc, N1), and Elizaveta plus (2904 kgs, E2) received selection achievement patents and got included into the State Register of Selection Achievements.

All tests conclusions

Therefore, based on the conducted research, it can be concluding that:

- Three potato varieties of Russian selection were successfully transformed with pBt12 vector that had the genes in its expression cassette content coding resistance to the herbicide glyphosate and Bt-toxin *CryIIIA* providing resistance to Colorado beetle;
- Biomolecular analysis of the primary transformants made it possible to select 3 transgenic lines (Elizaveta, Lugovskoi, and Nevski varieties) carrying one copy of the *cryIIIA* gene, with a high expression level and without phenotypic distinctions from the source varieties;
- Multiyear field trials of the obtained transgenic lines on Colorado beetle resistance and the persistence of the inserted genetic maker revealed high (100%) resistance of the obtained lines to Colorado beetle and advantages of the Bt-potato usage over chemical protection;
- Assessment of distinguishability, uniformity, and stability performed according to the UPOV international system showed conformity of the characteristics of the obtained line variety to those of the source potato varieties, excluding the feature of resistance to Colorado beetle;
- Patents on selection achievements were received and three transgenic varieties, Lugovskoi plus (1210 amk, L5), Nevski plus (0311 mbc, N1) and Elizaveta plus (2904 kgs, E2), got listed in the State Register of Varieties and Selection Achievements of the Russian Federation.

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