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OBTAINING TRANSGENIC POTATO PLANTS FOR COUNTERING *PHYTOPHTHORA INFESTANS* VIA "HIGS" METHOD IMPLEMENTATION

Abstract

Oomycetes are a group of organisms that include some of the most dangerous plant pathogens capable of causing epiphytoties that lead to reduced crop yields and famine in some countries. The only available method of combating them – repeated application of fungicides in fields and careful selection of seed material – is ineffective for most of these pathogens. This paper proposes to use the HIGS (host-induced gene silencing) approach so that genetically modified plants, when infected with late blight, would cause the pathogen to silence its effector protein genes, which would prevent the pathogen from growing and reproducing in plant.

To implement the HIGS method against the pathogen *Phytophthora infestans*, we developed two genetically engineered constructs, one of which uses the *P. infestans* RXLR-effector gene AVR3a-b as the RNA interference target, and the other uses a region of the PITG_03155 effector gene, which is conserved for both *P. infestans* and *P. cactorum*. After Agrobacterium-mediated genetic transformation of potato plants, we obtained four transgenic plants carrying a construct based on the AVR3a-b gene (two each of the "Milena" and "Gala" varieties) and one of "Milena" variety carrying a construct based on the PITG_03155 gene. These plants will be micro clonally propagated and tested for the presence of interfering RNA to the effector genes of *P. infestans*, after which the plant lines are planned to be planted under natural pathogenic background conditions and a comparative analysis with control plants for disease incidence and resistance will be conducted.

Key words: *Phytophthora infestans*, RNA interference, Host Induced Gene Silencing, transgenic plants, siRNA, *Avr3a*, PITG_03155.

Introduction

The oomycete group includes a large number of economically important pathogenic species [1]. According to a number of physiological characteristics and genetic analysis of conservative regions, oomycetes belong to the kingdom *Chromista*, as well as brown and diatom algae [2]. The greatest economic damage is caused by species belonging to the genera: *Phytophthora*, *Plasmopara*, *Pythium*, *Albugo*, *Aphanomyces*, *Bremia*, *Pseudoperonospora*, *Peronospora*.

In particular, *Phytophthora infestans* affects tomatoes and potatoes. Numerous studies [3,4] have shown that losses directly from *Phytophthora infestans* damage to potatoes average 10-30%. In years with favorable climatic conditions for the development of late blight, crop losses can reach 50-70%, as was shown in 2007 in Pakistan [5]. Most economic analyses suggest that global losses from *P. infestans* in potato crops amount to $3-5 billion per year [6].

The main problem in controlling late blight is its low susceptibility to standard fungicides, which leads to the search for new biotechnological methods of combating these infectious agents. One of the promising methods is obtaining resistant plants using RNA interference against the pathogen. This is so-called HIGS (Host Induced Gene Silencing) concept, which is based on that RNA interference of a pathogen necessary gene is caused in the cells of the host plant, and from it, small interfering RNAs enter pathogen cells during the infection, where they carry out silencing of its genes [7]. The general mechanism of RNA interference is known as a highly specific process of mRNA degradation due to the presence of complementary double-stranded RNA (dsRNA) [8]. Long dsRNA are cut by Dicer group enzymes into small segments of 21-24 nt – small interfering RNA

(siRNA). These siRNAs bind to the RISC complex (RNA-inducing silencing complex), which leaves in its composition the antisense strand (AS-RNA) of siRNA, which determines the specificity of mRNA destruction during silencing. It is known that both plants and animals are capable of exchanging siRNA with closely related pathogenic organisms [9], which is the basis of the HIGS concept.

RNA interference is used to combat phytopathogens, including viruses, insect pests, bacteria, fungi, oomycetes [7,10]. Thus, using HIGS, barley was obtained that was resistant to powdery mildew caused by *Blumeria graminis* [11]. *Jahan et.al.* [12] obtained transgenic potato plants that expressed siRNA to three *P. infestans* genes – *PiGPB1, PiCESA2,* and *PiPEC*; plants showed a reliable slowdown in plant disease.

In this work, we developed two genetically engineered constructs, one of which uses the *P. infestans* RXLR-effector gene AVR3a-b as the RNA interference target, and the other uses a region of PITG_03155 effector gene, which is conserved for both *P. infestans* and *P. cactorum*. We obtained four transgenic plants carrying the construct based on AVR3a-b gene (two of "Milena" and two of "Gala" variety) and one "Milena" plant carrying the construct based on PITG_03155 gene. These plants will be tested for the presence of specific siRNA, after which the plant lines will be planted under natural pathogenic background conditions and a comparative analysis with control plants for disease incidence and resistance will be conducted.

Methods and materials

Identification of target genes. At the first stage, a database of *Phytophthora* effector sequences registered in the NCBI (national center of biotechnological information) was collected. In addition to *P. infestans P. cactorum, P. citrophthora, P. megakarya, P. nicotianae, P. palmivora, P. ramorum* were analyzed; as a result, a database was obtained that included 451 sequences ranging in length from 2889 nt to 153 nt. The blastN network analyzer was used to analyze conserved regions. Of these sequences, 23 had conserved regions of sufficient length that were found in two or more oomycetes at once. Each of the 23 sequences was analyzed by blastN both against the entire oomycete group and against higher plants, using the "somehow similar sequences" module. Each of the sequences was then analyzed in the commercial search engines BLOCK-iT RNAi Designer ("ThermoFisher"), siRNA Target Finder ("GenScript"), siRNA Wizard 3.1 ("InvivoGen") to determine the possibility of generating siRNA from them. Two selected *P. infestans* sequences overlapping with *P. cactorum* sequences were used to construct genetic engineered cassettes.

Construction of genetic engineered cassettes. The 'PITG_03155+UBQ10-intr1' cassette was constructed around the sequence of the first intron of the ubiquitin 10 gene. Its 304 nt long sequence has donor and acceptor splicing sites. A 122 nt long fragment of the *P. cactorum* PITG_03155 gene (Genebank MT897014.1 or XM_002906196.1 for *P. infestans*) was located on both sides of this sequence in a complementary direction. The cassette was flanked on both sides by *Pst*I and *Bam*HI restriction endonuclease sites for subsequent cloning into the agrobacterial pCambia2000 vector (with a promoter and transcription terminator of the cauliflower mosaic virus CaMV).

The 'Avr3a-b+CAT2-intr3' cassette was constructed around the sequence of the third intron (1830–1931 nt) of castor bean catalase-2 (CAT-2). It was taken from the UniProt database (ID D21162), to which donor and acceptor sites were added. On both sides of this sequence, in a complementary direction to each other, a 200 nt long region of *the P. infestans* PITG_14371 gene, also known as *Avr3a* (Genebank XM_002898796.1), supplemented by a 92 nt long region of the *P. infestans Avr3b* gene (Genebank XM_002897915). The cassette was also flanked on both sides by *Pst*I and *Bam*HI restriction endonuclease sites. Synthesis of both cassettes was ordered from GenScript in pUC57 plasmids.

Cloning of constructs and genetic transformation of plants. Standard methods of genetic engineering were used for cloning the synthesized constructs into the agrobacterial vector pCambia2000. The EHA-105 *A. tumefaciense* strain was used to transform potato plants according to the protocol [13]. Kanamycin at a concentration of 30 mg/l was used as a selective agent, starting from the second week after transformation. Total DNA was isolated from the leaf blades of

regenerated plants using the “GenUP Plant DNA Kit” (“Biotechrabbit”) according to the manufacturer's instructions.

PCR analysis of regenerated plants. The presence of transgene inserts in plants was tested by PCR analysis using hot-start Taq-DNA polymerase from “Biotechrabbit” with a proprietary buffer and additional reagents with the following final concentrations: 0.2 mM dNTP, 2 mM $MgCl_2$ and 30 pM primers. 3 µl of total plant DNA sample were added to 15 µl of the reaction mixture. PCR conditions were as follows: 1. 95°C 3 min – 1 cycle; 2. 95°C 20 sec, 58°C 20 sec, 72°C 40 sec – 30 cycles; 3. 72°C 5 min – 1 cycle. Primary screening for the presence of the sequence flanked by the transcriptional promoter and terminator of cauliflower mosaic virus was performed with the following oligonucleotide primers: 35S_3end (TGATATCTCCACTGACGTAAGGGAT) – forward primer to the 3’-terminal sequence of the 35S promoter and 35Ster (CGCTCATGTGTTGAGCATATAAG) – reverse primer to the 3’-terminal sequence of the CaMV terminator. Positive samples were tested by PCR analysis for the presence of a specific insert with the following primers: Fw_infPITG (ACCATATCCTTGCACACATTGGG) and Rv_infPITG (AACGACGGAGTTCAGCAACGAG) – for the cassette ‘PITG_03155+UBQ10-intr1’; Fw_infAvr3a (CGGCAGAAGACTTCTTCGCAAGA) and Rv_infAvr3a (TCTCCGTCAACGCAGCCACATT) – for the cassette ‘Avr3a-b+CAT2-intr3’. Agrobacterium contamination was tested by PCR analysis with the following primers: FwVirD (GAAGAAAGCCGAAATAAAGAG) and RevVirD (TTGAACGTATAGTCGCCGATA) – for the sequence of the *virD* gene of *Agrobacterium tumefaciense*.

Results and discussion

Determination of target genes and construction of genetic engineered cassettes.

One of the technologies for inducing RNA interference in transgenic plants is the expression of transgenic hpRNA (hairpin RNA) [14], the essence of which is that the transgenic insert contains a section of the target gene sequence in both sense and antisense orientations, separated by either an intron or a spacer that forms a loop in the mRNA. This technology was used in this work; two different plant introns were chosen as a separator, functionally suitable for the sequences of the selected target genes.

The *Avr3a* target gene was preliminarily analyzed in a number of different *P. infestans* strains for the presence of highly conserved regions; we selected the region of the first 200 nt of the sequence as the most conserved for the majority of *P. infestans* strains as the target. It was also decided to investigate the possibility of generating siRNA to different target genes from one genetic engineered cassette, therefore the 200 nt region was supplemented with two regions of the *P. infestans Avr3b* sequence (45 nt and 47 nt). The *Avr3b* sequence is highly conserved not only among *P. infestans* strains, but also among other species of the genus *Phytophthora* (Figure 1), such as *P. ramorum, P. parasitica, P. plurivora, P. capsici, P. sojae, P. agathidicida, P. cinnamomi*. Thus, the total length of the “Avr3a-b” fragment is 292 nt, which is a sufficient size for successful siRNA generation according to literature data [15]. The silencing cassette based on the “Avr3a-b” fragment was provided with intron 3 from the castor oil catalase 2 gene. Intron 3 of the CAT2 gene retains its functionality in most dicotyledonous plants, including *Solanaceae* [16].

The *P. infestans* target gene PITG_03155 was also preliminarily analyzed for the presence of highly conserved regions; blast-analysis results revealed the presence of a 122 nt sequence that is completely conserved among *P. infestans* strains and has single substitutions for *P. cactorum* strains. The 122 nt fragment length is not the most optimal for use in hpRNA technology; however, there are a number of examples of the successful use of small sequences for target gene silencing [17]. Also, to increase the likelihood of successful induction of RNA interference from the PITG_03155 region, we constructed a cassette with intron 1 of the ubiquitin 10 gene. It is known that the first intron of the ubiquitin 10 gene (UBQ10) promotes significant accumulation of those mRNAs in which it is present [18], which increases the likelihood of siRNA generation. Thus, the small size (122 nt) of the PITG_03155 region will be compensated by the large amount of transcribed dsRNA.

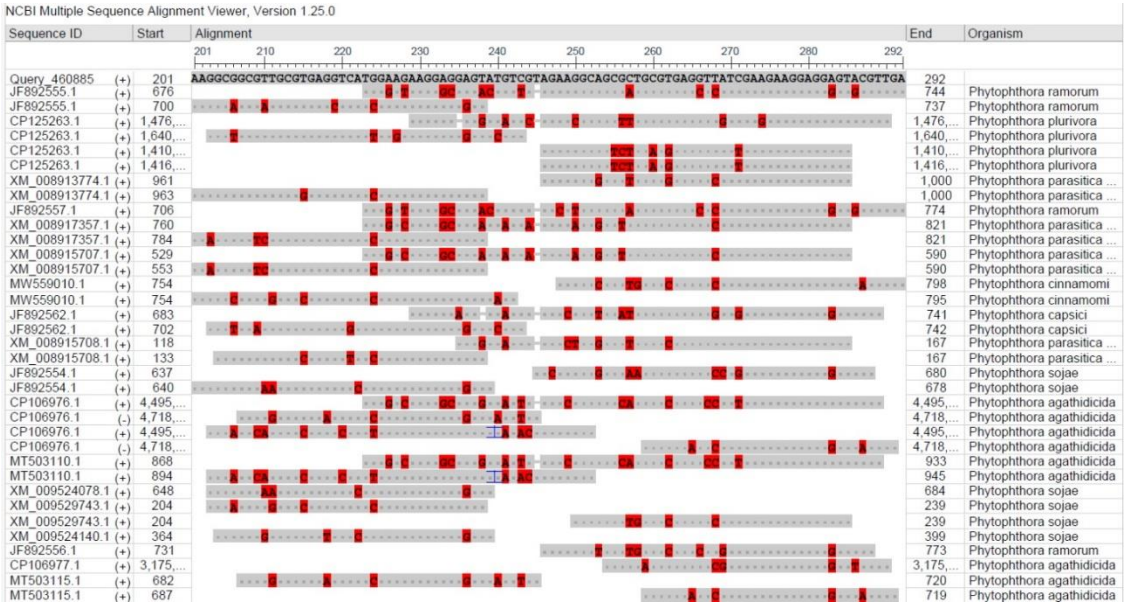


Figure 1 – Results of BLAST-analysis of the *Avr3b* gene region in a number of species of the oomycete group

Figure 2 shows both constructed sequences (the diagrams were generated in VectorNTI Suit 11). The fragments ‘Avr3a-b’ and ‘PITG_03155’ were also used for blastN-analysis in the polyphyletic group of green plants; no homologous sequences with any species were found, which suggests that the interfering RNAs are safe for the host plants.

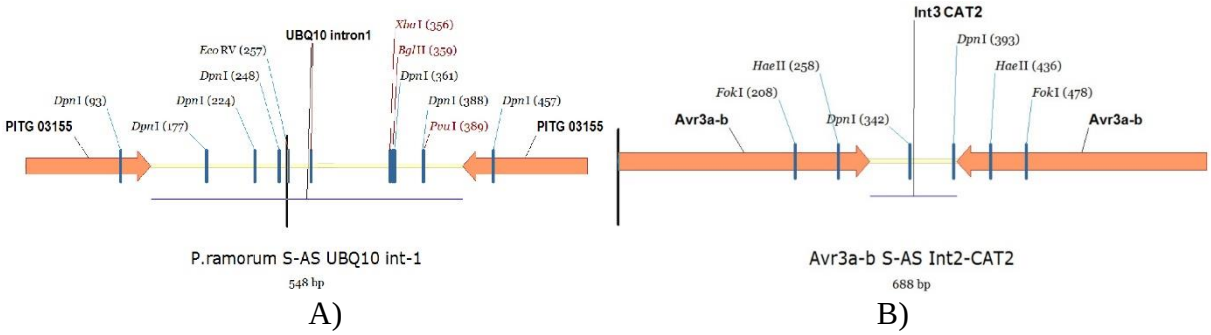


Figure 2 – Scheme of silencing inserts: A) carrying regions of the PITG_03155 gene; B) carrying regions of the *Avr3a* and *Avr3b* genes in sense (S) and antisense (AS) orientations.

Next, we analyzed the possibility of generating siRNA from the selected fragments ‘Avr3a-b’ and ‘PITG_03155’. The analysis was performed in three commercial programs: BLOCK-iT RNAi Designer (“ThermoFisher”), siRNA Target Finder (“GenScript”), siRNA Wizard 3.1 (“InvivoGen”). The number of possible siRNAs for the fragment ‘PITG_03155’ varied from 10 (“ThermoFisher”) to 2 (“InvivoGen”), and for the fragment ‘Avr3a-b’ – from 10 (“ThermoFisher” and “GenScript”) to 5 (“InvivoGen”). Moreover, the number of possible siRNAs from the *Avr3a* and *Avr3b* regions was different in different programs: BLOCK-iT RNAi Designer – 9 (*Avr3a*) and 1 (*Avr3b*), siRNA Target Finder – 8 (*Avr3a*) and 2 (*Avr3b*), siRNA Wizard 3.1 – 1 (*Avr3a*) and 4 (*Avr3b*). Nevertheless, all three programs predicted the formation of siRNA from both parts of the composite fragment “Avr3a-b”. Figure 3 shows the analysis of siRNA generation in the BLOCK-iT RNAi Designer program for both fragments.

A)

10 Stealth RNAi™ siRNA target sequences (Up to 10 top scoring Stealth RNAi™ siRNA sequences are reported, sorted by the Start position and ranked as ★★★★★ to ★★★☆☆ to indicate knockdown probability). Select the sequence to order and click "Continue".

Select	No.	Start	Sequence(DNA)	Region	GC%	Rank[1]
☐	1	2	ACCATATCCTTGCACACATTGGGTT		44.0	★★★★☆
☐	2	8	TCCTTGCACACATTGGGTTTGCGTT		48.0	★★★★☆
☐	3	13	GCACACATTGGGTTTGCGTTGTTCA		48.0	★★★★★
☐	4	16	CACATTGGGTTTGCGTTGTTCAGTG		48.0	★★★★½
☐	5	21	TGGGTTTGCGTTGTTCAGTGGCGTT		52.0	★★★★☆
☐	6	55	CGAGTGACGAGACCGAATATCCGAA		52.0	★★★½☆
☐	7	91	GATCTCGTTGCTGAACTCCGTCGTT		52.0	★★★★☆
☐	8	93	TCTCGTTGCTGAACTCCGTCGTTTA		48.0	★★★★½
☐	9	95	TCGTTGCTGAACTCCGTCGTTTATT		44.0	★★★½☆
☐	10	96	CGTTGCTGAACTCCGTCGTTTATTG		48.0	★★★½☆

B)

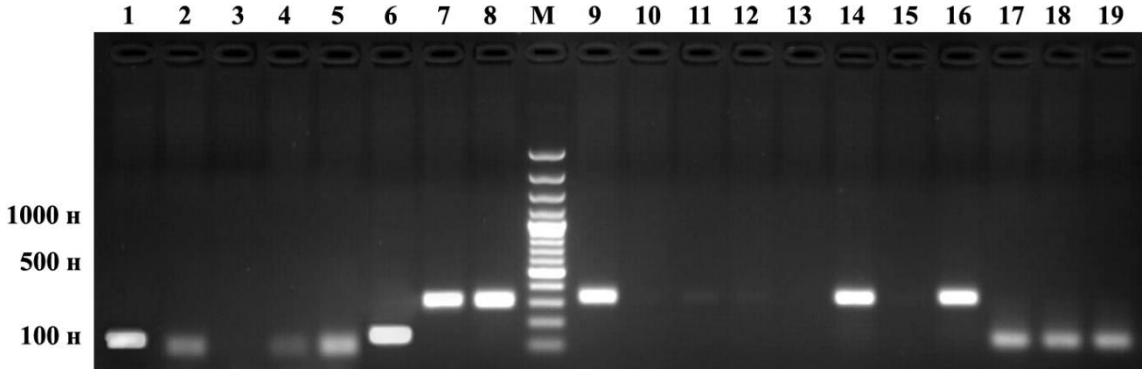
10 Stealth RNAi™ siRNA target sequences (Up to 10 top scoring Stealth RNAi™ siRNA sequences are reported, sorted by the Start position and ranked as ★★★★★ to ★★★☆☆ to indicate knockdown probability). Select the sequence to order and click "Continue".

Select	No.	Start	Sequence(DNA)	Region	GC%	Rank[1]
☐	1	44	GAACGAAGAGAATGAAGAAACGTCT		40.0	★★★★½
☐	2	80	CCCAAATTTCAATTTGGCGAATCTA		36.0	★★★★★
☐	3	94	TGGCGAATCTAAATGAGGAGATGTT		40.0	★★★★½
☐	4	96	GCGAATCTAAATGAGGAGATGTTTA		36.0	★★★★★
☐	5	109	AGGAGATGTTTAATGTGGCTGCGTT		44.0	★★★★★
☐	6	161	AGCGAAACAGCTTATGGGTAATGAT		40.0	★★★★½
☐	7	162	GCGAAACAGCTTATGGGTAATGATA		40.0	★★★★★
☐	8	163	CGAAACAGCTTATGGGTAATGATAA		36.0	★★★★★
☐	9	176	GGGTAATGATAAGCTGGCGGATGCT		52.0	★★★★½
☐	10	225	GAAGAAGGAGGAGTATGTCGTAGAA		44.0	★★★★½

Figure 3 – Variants of siRNA generation analysis from the PITG_03155 gene region (A) and the ‘Avr3a + Avr3b’ fragment (B) in the BLOCK-iT RNAi Designer program (“ThermoFisher”)

Plants regeneration and PCR analysis.

Regeneration of plants from stem cuttings began 3-4 weeks after transformation. Plants of the “Milena” variety showed a high level of regeneration, while the “Gala” variety showed a below average level. A total of 34 regenerates were obtained – 23 “Milena” and 11 “Gala”. After PCR screening of regenerates with primers for the promoter and terminator of the cauliflower mosaic virus, 17 plants were selected, with DNA samples of which PCR analysis was carried out for the presence of target fragments with specific primers. Figure 4 shows the obtained result.



The samples are designated by numbers: 1-6 – analyzed for the presence of the insert ‘PITG_03155’ (1 – positive control plasmid pSS-PITG); 7-19 – analyzed for the presence of the insert ‘Avr3a-b’ (16 – positive control plasmid pSS-Avr); M – GeneRuler 100 bp (“Thermo”).

Figure 4 – PCR analysis of regenerated plants, PCR-products in 1% agarose gel

Positive plasmid controls showed PCR fragments of the expected size (292 nt ‘Avr3a-b’ and 122 nt ‘PITG_03155’). Of the analyzed regenerates, 4 showed the presence of ‘Avr3a-b’ insert – lines Avr_M11, Avr_M15 (variety "Milena") and Avr_Г7, Avr_Г4 (variety "Gala"). One regenerate showed the presence of ‘PITG_03155’ insert – line PITG_M3 (variety "Milena"). Plants of all the obtained transgenic lines do not show any deviations from the norm – morphological features are the

same as in the original variety, growth rate and the ability to regenerate during cuttings also did not change.

Conclusions

HIGS technology is widely used abroad to combat various types of plant pathogens. A number of studies have shown the effectiveness of this technology in controlling the spread of infections caused by oomycetes, including *P. infestans*. In this paper, a HIGS approach was proposed for inducing interference in potato plants against the effector genes of *P. infestans* PITG_03155 and *Avr3a* and *Avr3b*. For the first time, we propose the induction of siRNA to two genes (*Avr3a* and *Avr3b*) from one genetic engineered construct. Also, for the first time, a fragment of the target gene (PITG_03155) is selected in such a way that siRNA induction occurs in two different pathogen species – *P. infestans* and *P. cactorum*.

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PHYTOPHTHORA INFESTANS КЕҢЕСІНДЕГІ «HIGS» ӘДІСІН ЖҮЗЕГЕ АСЫРУ ҮШІН ТРАНСГЕНДІК КАРТОП ӨСІМДЕРІН АЛУ

Аңдатпа

Оомицеттер - белгілі бір елдерде шығымдылықтың төмендеуіне және аштыққа әкелетін эпифитотияларды тудыруы мүмкін ең қауіпті өсімдік патогендерін қамтитын организмдер тобы. Олармен күресудің жалғыз қолжетімді жолы – егістіктерде фунгицидтерді қайталап қолдану және тұқымдық материалды мұқият таңдау – бұл қоздырғыштардың көпшілігі үшін тиімсіз. Бұл жұмыста HIGS (host-induced gene silencing) әдісін қолдану генетикалық түрлендірілген өсімдіктер кеш қоздырғышпен жұқтырған кезде қоздырғыштың патогенді организмнің өнуіне және оның көбеюіне жол бермейтін эффекторлық белоктардың үнсіздігін тудырады.

Phytophthora infestans қоздырғышына қарсы HIGS әдісін енгізу үшін біз екі гендік инженерия құрылымын жасадық, олардың бірі РНҚ кедергі нысанасы ретінде RXLR-эффекторлық ген *P. infestans* AVR3a-b, ал екіншісі PITG_03155 эффекторлық генінің бөлімін пайдаланады – ол *P. infestans* және *P. cactorum* үшін консервіленген. Картоп өсімдіктерінің агробактериалды-генетикалық трансформациясынан кейін AVR3a-b геніне негізделген құрылымды алып жүретін төрт трансгенді өсімдік (әрқайсысы «Милена» және «Гала» екі сорты) және PITG_03155 геніне негізделген құрылымды алып жүретін бір «Милена» сорты

алынды. Бұл өсімдіктер микроклоналды түрде көбейтіледі және *P. infestans* эффекторлық гендеріне кедергі жасайтын РНҚ бар-жоғына зерттеледі, содан кейін өсімдік линияларын табиғи патогендік фон жағдайында отырғызу жоспарлануда және аурулардың жиілігі мен төзімділігі бойынша бақылау өсімдіктерімен салыстырмалы талдау жасалады; жүзеге асырылатын болады.

***Кілт сөздер**: Phytophthora infestans*, РНК-интерференция, Host Induced Gene Silencing, трансгенді өсімдіктер, siRNA, *Avr3a*, PITG_03155.

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ПОЛУЧЕНИЕ ТРАНСГЕННЫХ РАСТЕНИЙ КАРТОФЕЛЯ ДЛЯ РЕАЛИЗАЦИИ МЕТОДА “HIGS” В РАМКАХ БОРЬБЫ С *PHYTOPHTHORA INFESTANS*

Аннотация

Оомицеты являются группой организмов, к которым относятся одни из наиболее опасных патогенов растений, способных вызывать эпифитотии, приводящие к сокращению урожайности и голоду в отдельных странах. Единственный доступный способ борьбы с ними – многократное применение фунгицидов на полях и тщательный отбор посевного материала – малоэффективен для большинства из этих патогенов. В данной работе предлагается использовать подход HIGS (host-induced gene silencing) для того, чтобы генетически модифицированные растения при их заражении фитофторой вызывали бы у патогена сайленсинг генов его эффекторных белков, что препятствовало бы прорастанию патогенного организма в растении и его размножению.

Для реализации метода HIGS против патогена *Phytophthora infestans* нами разработано две генно-инженерных конструкции, в одной из которых в качестве мишени РНК-интерференции используется ген RXLR-эффектора *P. infestans* AVR3a-b, а в другой – участок гена эффектора PITG_03155, консервативного и для *P. infestans*, и для *P. cactorum*. После агробактериальной генетической трансформации растений картофеля получили четыре трансгенных растения, несущих конструкцию на основе гена AVR3a-b (по два сортов «Милена» и «Гала») и одно сорта «Милена», несущее конструкцию на основе гена PITG_03155. Эти растения будут микроклонально размножены и исследованы на наличие интерферирующих РНК к генам эффекторов *P. infestans*, после чего линии растений планируется высадить в условиях естественного патогенного фона и провести сравнительный анализ с контрольными растениями на заболеваемость и устойчивость.

***Ключевые слова:** Phytophthora infestans*, РНК-интерференция, Host Induced Gene Silencing, трансгенные растения, siRNA, *Avr3a*, PITG_03155.