

## Strategy for modification of the bovine beta-lactoglobulin gene using components of the CRISPR/Cas9 system in plasmid form

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Using on-line programs, sites were selected for obtaining double-stranded breaks in the BLG gene of cattle. The strategy for making double-stranded cuts in the BLG gene was developed taking into account the polymorphic variant of the gene (A-allele): DNA was isolated from bovine sperm used for fertilization of cow eggs in vitro. Four pX330 plasmids encoding Cas9 endonuclease and gRNAs specific to the selected BLG gene sequences were obtained. A strategy was developed for analyzing possible genetic modifications resulting from the operation of the CRISPR/Cas9 system components and the genetic construct microinjected into zygotes (NHEJ, HDR). The pBLGcmvEGFP plasmid containing the green fluorescent protein gene under the cytomegalovirus promoter was proposed as a model genetic construct for replacing the BLG gene. The use of a plasmid containing the reporter protein gene under its own regulatory sequences, flanked by homology arms to the beta-lactoglobulin gene, can be useful for evaluating the effectiveness of site-specific activity of the CRISPR/Cas9 system components in vitro.

**Key words:** genetic constructs,  $\beta$ -lactoglobulin, BLG gene, cattle, CRISPR/Cas9, microinjection, non-homologous end joining (NHEJ), homology directed repair (HDR), PCR.

### Introduction

In the last decade, significant advances have been made in the development of genome editing tools: ZFN - zinc-finger nucleases, TALEN - transcription activator-like effector nucleases, and CRISPR/Cas9 - clustered regularly interspaced short palindromic repeats. These tools are based on generating a double-stranded break (DSB) of DNA, followed by one of two repair pathways - non-homologous end joining (NHEJ) or homology directed repair (HDR). Compared to traditional approaches, these tools significantly reduce the time and effort required to create a genetically modified (GM) animal. Another advantage of using new genome editing tools is the use of direct injection (microinjection, MI) into developing embryos to induce target mutations, which avoids the disadvantages of the somatic cell nuclear transfer (SCNT) method. New genome editing technologies have significantly improved the efficiency of creating large, low-offspring, genetically modified animals for both biomedical and agricultural purposes (Larkina et al., 2018). Genomic editing technologies, especially variants of CRISPR technology, can make a real breakthrough to increase the production of livestock products, in terms of improving the efficiency of feed assimilation, reducing the impact of agriculture on the environment, strengthening pest control, and preserving animal health. The era of genome editing has already arrived, which requires a serious assessment of potential opportunities and threats, benefits and risks, ethical and scientific views, and legal regulation (Menchaca et al., 2020). Cattle breeding is carried out in three directions - meat, dairy-meat and dairy - in order to obtain products of the desired category. Over the past 30 years, transgenic cows have been created for such purposes as: expression of pharmacological proteins; increasing the proportion of beta- and kappa-casein in milk; increasing disease resistance; increasing meat productivity.

The whey protein  $\beta$ -lactoglobulin (BLG) is the main milk allergen. Knockout of the BLG gene using genomic editing technologies completely solves the problem of creating hypoallergenic dairy products. There are known works on directed modifications of the BLG gene in cattle using ZFN and TALEN technologies, but not using the CRISPR/Cas9 system. In the first GM cloned cow (SCNT) obtained in 2011 using the ZFN editing method, a biallelic modification of the BLG gene was obtained with a small deletion without failure of the reading frame and without the formation of functional knockout alleles (Yu et al., 2011). Later, the same method was used to obtain a cow that does not contain BLG in milk (Sun et al., 2018).

In experiments on cattle zygotes, site-specific mutations of the BLG gene were shown to be highly effective using the ZFN method (30-80% of embryos from survivors). For precise modification, single-stranded DNA matrices with homology arms flanking the integrable sequence were used. Analysis of microinjected embryos showed the effectiveness of site-specific modifications up to 33% for ZFN and 46% for TALEN blastocysts. Sequencing showed that the modification of the target BLG allele can reach 100%; this means that by using the method of genomic editing with microinjection of cattle zygotes, it is possible

to obtain a non-mosaic animal with pre-developed biallel modifications in the F0 generation (Wei et al., 2015). In 2018, a live bull and cow with a BLG gene knockout were obtained by microinjection of zygotes using the TALEN editor due to the deliberate deletion of nine nucleotides at the very beginning of BLG protein synthesis and the generated stop codon, which caused the gene knockout (Wei et al., 2018).

Using TALEN technology applied to fetal fibroblasts, the cattle transgenic for human serum albumin (HSA) with a knockout of the BLG gene were obtained. The concentration of HSA synthesized instead of endogenous  $\beta$ -lactoglobulin in the milk of mono-allelic cows reached 2.3 g/l, the protein had the correct folding. The HSA content in the milk of homozygous cows was significantly higher – 3.3-3.5 g/l (Luo et al., 2016). Goats were also obtained not only with the knockout of the BLG gene, but also with the genes of human milk proteins integrated into its locus – lactoferrin (Cui et al., 2015; Song et al., 2016) and alpha-lactalbumin (Zhu et al., 2016).

No reports of obtaining cattle with a modified *BLG* gene using the CRISPR/Cas9 system have yet been found. BLG-knockout goats were created in 2017 by microinjection of zygotes with CRISPR/Cas9 components (Zhou et al., 2017). We created a plasmid matrix containing homology arms to the regions of the BLG gene of cattle, into which the DNA sequence of the target protein can be cloned. In addition, a genetic construct containing the green fluorescent protein (EGFP) gene under the cytomegalovirus (CMV) promoter was created for integration by homologous recombination into the bovine BLG locus during joint microinjection with site-specific components of the CRISPR/Cas9 system. Precise insertion (substitution) of a transgene with relatively short (up to 1 kb) homology arms to the BLG gene will ensure its expression under the control of full-length endogenous regulatory sequences.

The purpose of the work was to select the sequences of guide RNAs to the selected fragments of the BLG gene, taking into account the definite allelic variant of the BLG gene in cattle; to develop a PCR-based analysis of genetic modifications for knockout gene local insertions or deletions, large deletion, and a mechanism realized by the non-homologous end joining (NHEJ); integration of the transgene made a homology directed repair mechanism (HDR).

## Material and methods

All the work was performed in All-Russian Research Institute of Physiology, Biochemistry and Animal Nutrition. The sequence of the BLG gene of cattle (*Bos taurus*) was taken from the GenBank database, entry X14710 (<https://www.ncbi.nlm.nih.gov/nuccore/48,127>). Selection of primers and restriction analysis were performed in the Vector NTI program.

Sequences for guide RNA were selected using on-line CHOP CHOP programs (<http://chopchop.cbu.uib.no/>), CRISPR direct (<http://crispr.dbcls.jp/>), CRISPOR V. 4.8 (<http://crispor.tefor.net/>). The pX 330-U6-Chimeric\_BB-Cb-hSpCas9 (Addgene plasmid # 42230) plasmid was used to obtain the CRISPR/Cas9 components (Cong et al., 2013). The sequences of oligonucleotides, their description, and primer sequences for evaluating deletions of various sizes and embedding the gene structure by homologous recombination are shown in tables 1 and 2. All primers, oligonucleotides, and plasmid DNA sequences were ordered from "Syntol" (<http://www.syntol.ru>).

**Table 1.** Oligonucleotides for creating components of the CRISPR/Cas9 system

Designation	5'-3' sequence	application
bg51f	<b>caccggagatgtcgtggccgcca</b>	pX330-51b
bg51r	<b>aaactggcggccagcgacatctcc</b>	
bg52f	<b>caccgacttggtactccttgcca</b>	pX330-52b
bg52r	<b>aaactggccaaggagtaccaagtc</b>	
bg31f	<b>caccgatcctgacgtccccgcca</b>	pX330-31b
bg31r	<b>aaactggcggggagcgtcaggatc</b>	
bg32f	<b>caccggggagcgtcaggatgggac</b>	pX330-32b
bg32r	<b>aaactcccatcctgacgtcccc</b>	

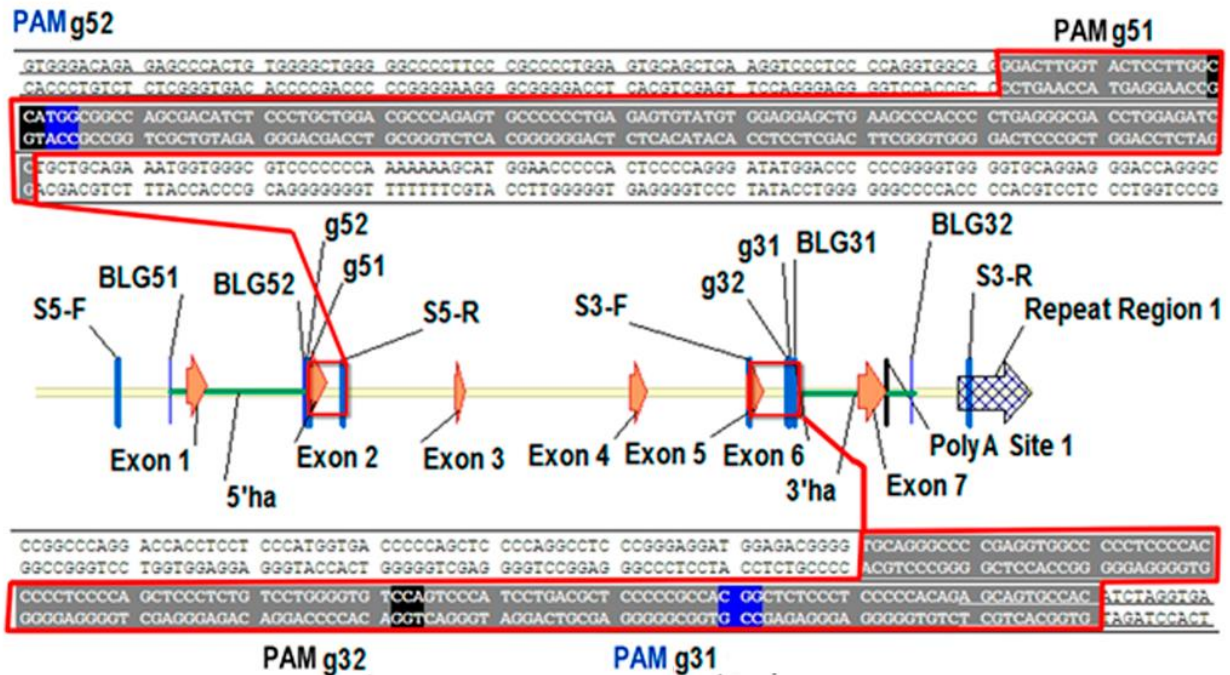
**Table 2.** Primers for PCR analysis of BLG gene modifications

Designation	5'-3' sequence	application
T5L	tccaaatgttccacgtgtg	5'utr < 5'HA, HDR
T5R	ataagcagccttgggtggac	
T3L	ggccctggagaaattcgaca	5'endcmv, HDR
T3R	gaaccctaccctatgtgtg	
HA5F	cttggcctcgaatggaagaa	3'end bGHpolyA to HDR
HA5R	tggaaagtccctattggcgt	
HA3F	Ttgtctgagttaggtgtcatt	3'utr > 3'HA, HDR
HA3R	Gactgaacactccaagttaa	

## Results and discussion

To perform modifications of the BLG gene (knockout by local indels or large deletion), sequences for guide RNAs (sgRNAs) were selected. The search was carried out in three on-line programs, the main selection criteria were a high rating and minimal off-target influence of possible gRNAs. To increase the probability of a large deletion, two gRNAs were selected for the 5'- and 3'-targeted regions of the gene.

Target sequences for gRNAs to form double-stranded sections in the 5' region of the BLG gene were selected in the region limited by the BLG52 / S5-R primers: 120 nucleotides were taken for analysis after the 5' homology arm. This fragment is completely part of the 2nd exon. Target sequences in the 3' region of the BLG gene were selected in the region bounded by S3-F / BLG31 primers: 120 nucleotides were taken at the end of the 5th intron and the beginning of the 6th exon (Figure 1).



**Fig.1.** Fragments of 5'- and 3'-sequences of double-stranded DNA of the BLG gene (screenshot of the Vector NTI program window). Gray background – search area, 120 bp fragments 5'HA and 3'HA are underlined. PAM motifs (5'-20b-NGG-3') for selected targets are indicated: black block - for gRNA51, gRNA32 (TGG on the lower DNA chain); highlighted with a frame - for gRNA52 (TGG) and gRNA31 (CGG) on the upper chain. The potential deletion for the gRNA51 – gRNA52 pair is 6bp, for the gRNA31 – gRNA32 pair, it is 19 bp.

*Preparation of plasmids pX330-b51, pX330-b52, pX330-b31, and pX330-b32 encoding Cas9 and gRNAs to the corresponding sites of the BLG gene.* Oligonucleotides for gRNA production were processed according to the Cloning Protocol (Menzorov et al., 2016) with some modifications and cloned into the pX330 plasmid.

1. All oligonucleotides (table 1) were diluted with water (deionized water, with a resistivity of more than 18 MOhm·cm) in the volume specified in the passport for each of them to a concentration of 100 pM/μL.
2. Obtaining a short double-stranded inserts with sticky ends (table 3).

**Table 3.** Composition of mixtures for the formation of double-stranded inserts with sticky ends

No	Component	Mixture to form an insert, μL			
		bg51	bg52	bg31	bg32
1	H <sub>2</sub> O	13	13	13	13
2	bg51f	2	-	-	-
3	bg51r	2	-	-	-
4	bg52f	-	2	-	-
5	bg52r	-	2	-	-
6	bg31f	-	-	2	-
7	bg31r	-	-	2	-
8	bg32f	-	-	-	2
9	bg32r	-	-	-	2
10	T4PNK (5 u/μL)	1	1	1	1
11	10x T4 DNA ligase buffer	2	2	2	2
12	Minera loil	20	20	20	20
	In total:	40	40	40	40

The mixtures were prepared in test tubes for PCR, incubated at 37 °C for 40-50 minutes, kept at 95 °C for 5 minutes and slowly cooled to room temperature.

3. The reaction of the restriction-ligation. Mixtures of the following composition were prepared (Table 4).

The restriction-ligation reaction was performed in a PCR amplifier in the following mode: 1) 37 °C (restriction) – 5 minutes; 2) 20 °C (ligation) – 5 minutes: a total of 38 cycles.

**Table 4.** Composition of ligase mixtures for producing plasmids on the basis of the pX330

№	Component (conc.)	The mixture for the formation of plasmids, $\mu\text{L}$			
		pX330-b51	pX330-b52	pX330-b31	pX330-b32
1	H <sub>2</sub> O	9	9	9	9
2	5x T4 DNA ligase buffer	4	4	4	4
3	BSA* (10 mg/ml)	1,5	1,5	1,5	1,5
4	pX330 (150 ng/ $\mu\text{L}$ )	2	2	2	2
5	BbsI (10 u/ $\mu\text{L}$ )	1	1	1	1
6	T4 DNA ligase	1	1	1	1
7	bg51	1,5	-	-	-
8	bg52	-	1,5	-	-
9	bg31	-	-	1,5	-
10	bg32	-	-	-	1,5
11	Mineral oil	20	20	20	20
	In total:	40	40	40	40

\*BSA – *bovineserumalbumin*

4. *E. coli* Dh5a cells transformed by the ligase mixture were seeded on solid LB-Am<sup>r</sup> medium. Grown clones were reseeded.  
5. For PCR analysis, DNA from transformed clones was isolated using a lysing mixture with proteinase K. The compositions of PCR mixtures are shown in table 5.

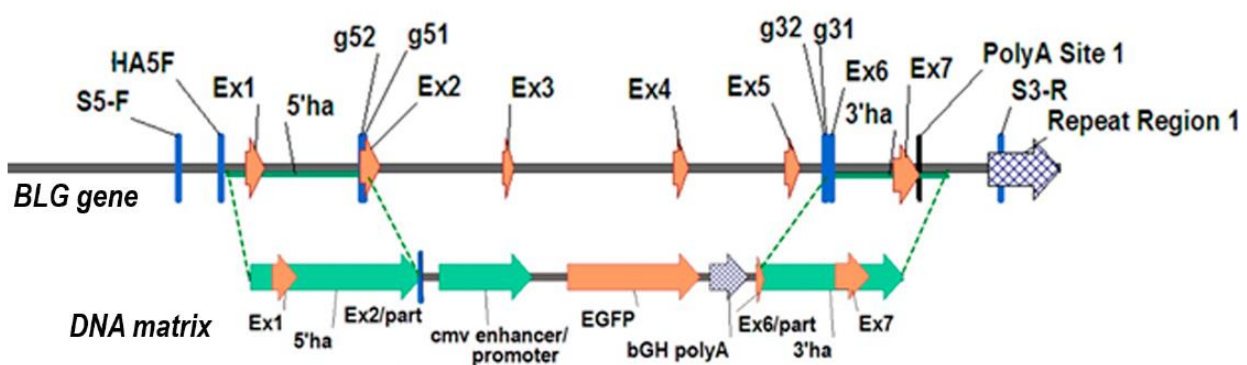
**Table 5.** Composition of PCR mixtures for screening of pX330-based plasmids (per test tube).

No	Component (conc.)	The PCR mixture for analysis of plasmids, $\mu\text{L}$			
		pX330-b51	pX330-b52	pX330-b31	pX330-b32
1	Taq buf $\times 10$	2	2	2	2
2	dNTP $\times 10$ (2 mM)	1,8	1,8	1,8	1,8
3	ENH-R (3 pM/ $\mu\text{L}$ )	1	1	1	1
4	bg51f (3 pM/ $\mu\text{L}$ )	1	-	-	-
5	bg52f (3 pM/ $\mu\text{L}$ )	-	1	-	-
6	bg31f (3 pM/ $\mu\text{L}$ )	-	-	1	-
7	bg32f (3 pM/ $\mu\text{L}$ )	-	-	-	1
8	H <sub>2</sub> O	12,9	12,9	12,9	12,9
9	DNA from resp. clone's	1	1	1	1
10	TaqPol (5 u/ $\mu\text{L}$ )	0,3	0,3	0,3	0,3
11	Mineral oil	20	20	20	20
	In total:	40	40	40	40

PCR amplification conditions: denaturation 94°C - 30 sec (in the first cycle - 2 min.), annealing of primers 62°C - 30 sec., elongation 72°C - 1 min. (in the last cycle - 3 min); a total of 27 cycles.

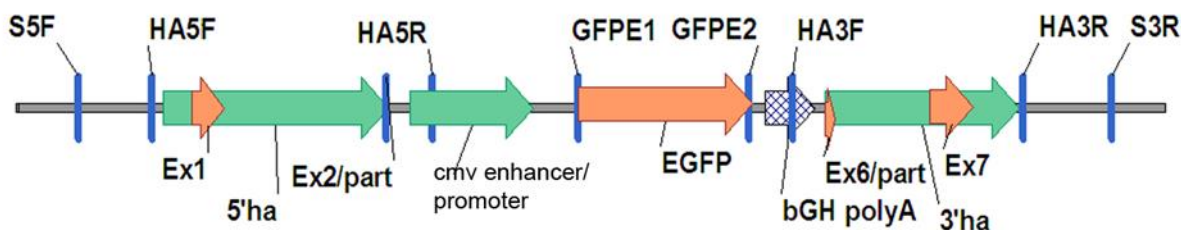
6. A suitable clone was developed, plasmid DNA was isolated, and plasmid concentrations were determined after its linearization with XbaI restriction enzyme. As a result, plasmids pX330-b51, -b52, -b31 and -b32 encoding Cas9 and *grn* to the corresponding sites of the BLG gene were obtained.

**Analysis of BLG gene modifications.** The pBLGcmvEGFP plasmid in circular or linear form (by cutting out the gene structure with EcoRI and BglII restriction enzymes) can be used as a DNA matrix for homologous recombination with the BLG gene using CRISPR/Cas9 components (figure 2).



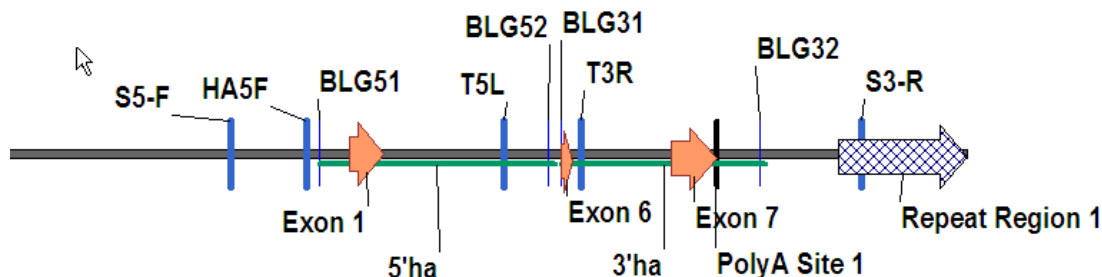
**Fig. 2.** Scheme of homologous recombination of the BLGcmvEGFP gene construct with the BLG gene as a result of the CRISPR/Cas9 system. Here are g51, g52, g31, g32 targets for gRNAs; 5'ha and 3'ha homology arms; gene structural elements and constructs; primers.

As a result of embedding the gene construct, the locus of the BLG gene will be modified, as shown in figure 3. To confirm the fact of homologous recombination, the HA5F /HA5R and HA3F/ HA3R pairs were selected (tables 2, 6).



**Fig. 3.** Modified as a result of embedding the BLGcmvEGFP gene construct via the HDR pathway, the BLG gene locus. Here are the structural elements of the gene and the built-in gene construct; "nest" primers for screening.

Another variant of possible genetic modifications resulting from the operation of the created components of the CRISPR/Cas9 system is the repair of double - stranded sections by the NHEJ mechanism (example in figure 4). Options for detecting possible small and large deletions are shown in table 5.



**Fig. 4.** A variant of BLG gene repair by non-homologous end junction (NHEJ). A large deletion g51-g32.

**Table 6.** PCR analysis of BLG gene modifications obtained using components of the CRISPR/Cas9 system and the HDR matrix

Païroprimers	The size of the PCR amplicate, bp		Remarks, detection
	WT	Modification	
T5L / T5R	490	< 490 ( $\Delta$ G51/G52)	Small deletions, detection: electrophoresis in polyacrylamide gel
T3L / T3R	397	< 397 ( $\Delta$ G31/G33)	
T5L / T3R	3598	$\approx$ 320 для $\Delta$ G51(G52)/G31(G32)	
HA5F/ HA3R	3621	$\approx$ 350 для $\Delta$ G51(G52)/G31(G32)	Large deletions, detection: agarose gel electrophoresis
HA5F /HA5R	-	1179 (5'HA,HDR )	
HA3F/ HA3R	-	972 (3'HA, HDR)	

$\Delta$  - deletion

## Conclusions

The method of microinjection of zygotes is simple and reliable, especially in combination with CRISPR/Cas9 technology – for obtaining genetically modified and transgenic animals. The efficiency and simplicity of the CRISPR/Cas9 method allows it to be used for obtaining genetically modified farm animals. There are different variations in the use of this technology: if the first work was performed using plasmid forms, involving microinjection of solutions of DNA components directly into the zygote pronucleus, other options immediately appeared. This is the use of mRNA and the protein form of endonuclease Cas9 in combination with sgRNAs, intended for cytoplasmic and pronuclear injections. Despite their higher efficiency, the variant of plasmid forms of CRISPR/Cas9 components remains more preferable for some researchers and laboratories due to the low cost of components, the relative stability of plasmids that do not require deep freezing conditions.

Based on the commercially available pX330 plasmid, plasmids encoding Cas9 endonuclease and gRNAs to the corresponding sites of the BLG gene were obtained. A system for evaluating potential genetic modifications of the BLG gene was developed using PCR and analysis of its products in agarose and polyacrylamide gels.

A recombinant pTZhaBLG plasmid containing 5' and 3' arms of homology to the BLG gene of cattle was created. Based on this model, the pBLGcmvEGFP plasmid was created containing the green fluorescent protein gene under its own regulatory sequences, flanked by homology arms to the BLG gene sequences to evaluate the effectiveness of transgene integration under in vitro culture conditions.

Genome editing technologies will allow to improve and develop new breeds of farm animals in order to obtain both full-fledged food products with new qualities, and to obtain a source of biologically active proteins for pharmacological purposes from milk. The use of CRISPR/Cas9 technology in scientific and practical research can dramatically accelerate the production of such animals. Our work provides a ready-made set of CRISPR/Cas9 components in plasmid form for microinjection into the

pronucleus of cattle zygotes in order to knock out the BLG gene, as well as a ready-made strategy for analyzing its possible modifications.

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## Authors' contribution

KE developed a research program, conducted a comprehensive study, conducted work on the search for genetic sequences, selection of primers and restriction analysis in the VECTOR NTI program, worked with online programs, analyzed the results of sequencing, participated in the implementation of genetic engineering methods, and compiled a manuscript. EV participated in a comprehensive study, performed genetic engineering and molecular biology methods, analyzed the data obtained, and participated in the drafting of the manuscript. All authors approved the final version of the manuscript.

## Declaration of conflicting interests

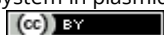
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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