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THE DEVELOPMENT OF THE PCR TEST SYSTEM FOR IDENTIFICATION OF RNA-2 OF BNYVV FOR ENVIRONMENTAL ASSESSMENT OF PHYTOSANITARY STATE*

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Проведено біоінформативний аналіз нуклеотидних послідовностей РНК-2 ізолятів вірусу некротичного пожовтіння жилок буряку (ВНПЖБ). Встановлено консервативні послідовності гена, що кодує протеїн Р75 ВНПЖБ, та розроблено дизайн праймерів, специфічних до нуклеотидних послідовностей геному для ідентифікації протеїну Р75 РНК-2 ВНПЖБ. Синтезовано специфічні олігонуклеотидні праймери Forward 5'- CTTTGGCAGGATTAGGCTCG -3', Reverse 5'- CACTCGGGACTATCACCAGG -3'. Розроблену систему було випробувано та підтверджено ефективність її використання на моделі кДНК українського ізоляту ВНПЖБ. Оптимізовано систему ідентифікації РНК-2 за температурними показниками відпалу праймерів і встановлено, що оптимальна температура варіює в межах 50–62°С. Отримані результати дають змогу ідентифікувати РНК-2 ВНПЖБ у зразках цукрових буряків та створюють передумови для розробки кількісних методів діагностики та ідентифікації ВНПЖБ.

Ключові слова: *вірус некротичного пожовтіння жилок буряку (ВНПЖБ), РНК-2, ген, ПЛР, дизайн, праймер, ідентифікація.*

Beet necrotic yellow vein virus (BNYVV) is the type member of the Benyvirus genus, the causal agent of sugarbeet rhizomania. Rhizomania disease of sugar beet was first reported in Italy in 1952 by Canova. BNYVV has been reported in Europe (Austria, Belgium, Bulgaria, France, Germany, Iran, Hungary,

Greece, Czech Republic, Kazakhstan, Kyrgyzstan, Netherlands, Poland, Romania, Russia, Slovakia, Spain, Sweden, Switzerland, Yugoslavia, Great Britain, Asia (China, Japan, Syria) and North America [1–4]. In Ukraine BNYVV is included in the register of quarantine viruses.

Genome of BNYVV consists of 4–5 particles of (+) RNA. The RNA-2, 4612 nucleotide in length [excl. the poly (A)-tail], has 6 open reading frames (ORF) which code genes of encapsidation, cell-to-cell movement and suppression of posttranscriptional gene silencing (PTGS). RNA-2 is necessary for replication. The first 5`ORF codes for a 21 kDa CP with rather weak UAG termination codon [5]. RNA-2 codes triple gene block (TGB)-encoded proteins (TGBp1, TGBp2, TGBp3) which is necessary for cell-to-cell movement. Cysteine-rich protein P14 is expressed by subgenomic RNA [6]. Protein P75 is involved in vector transmission by *Polymyxa betae* [7].

Since the infection of sugar beet with BNYVV decreases the yield, it is important to prevent the spread of the virus, so it is necessary to carry out timely virus identification. The aim of our research was to develop diagnostic system of BNYVV based on polymerase chain reaction for the identification protein P75 of RNA-2.

MATERIALS AND METHODS

For searching nucleotide sequences of RNA-2 and for bioinformative analysis a database of NCBI (National Center for Biotechnological Information) was used [8]. The bioinformatics genome analysis was performed using the software «MultAline» (Multiple se-

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quence alignment) [9]. Design of primers was developed using software «Primer3» [10].

Extraction of total RNA was performed using a commercial kit «RiboSorb» (AmpliSens, Russia), reverse transcription reaction was performed using a commercial kit «Reverta-L-100» (AmpliSens, Russia), according to recommendations.

The reaction mixture for the polymerase chain reaction (PCR), a volume of 15 ml contained 1x PCR buffer with 1.5 mM $MgCl_2$ (AmpliSens, Russia), 0.2 mM deoxynucleoside triphosphate (dNTP) (AmpliSens, Russia), 1 pkmol each of oligonucleotide primers, 10–40 ng cDNA, 0.5 U Taq-polymerase (AmpliSens, Russia). The amplification reaction of DNA was performed in thermocycler «Tertsyk» TP4-PCR 01.

After amplification PCR products were separated by horizontal electrophoresis in 1.5% agarose gel, which was prepared using TBE buffer with a concentration of 0.5 mg/ml ethidium bromide. PCR results were visualized with UV transilluminator rays (T-312-C), photographed by a digital camera Sony (DSLR A-500).

RESULTS AND DISCUSSION

For primer design, specific to the nucleotide sequences of the genome of BNYVV, the bioinformative analysis was carried out, the first step of which was screening of conservative gene sequences of P75 using genetic information database (GenBank). Specific conserved nucleotide sequence of the P75 gene was determined, which was used as template for oligonucleotide primers for the synthesis of virus-specific fragment (Figure 1). On the matrix consensus sequence of P75 design of primers with optimal characteristics was created: Forward 5'- CTTTGGCAGGATTAGGCTCG-3', Reverse 5'- CACTCGGGACTATCACCAGG -3', P75-F and P75-R respectively. Primer design with optimum characteristic was made with optimum parameters of GC composition and similar temperature annealing of two primers.

The calculated optimum temperature primers annealing for Forward primer is 58.47°C and for Reverse primer is 59.25°C, percentage deoxyguanosine-5'-phosphate and deoxycytidine-5'-phosphate for Forward primer is 55% and for Reverse is 60%.

Fig. 1. Localization of primers hybridization sites on the DNA consensus sequence of the P75 gene matrix of BNYVV

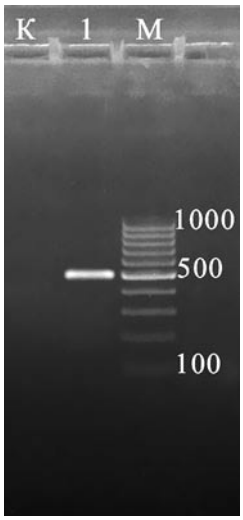

Fig. 2. The electrophoregram of PCR product analysis for the identification of RNA-2 of BNYVV: K – negative control of PCR analysis, 1 – amplification product of P75 gene, M (GeneRuler 10 bp DNA Lader 0241) – marker of fragment lengths (base pairs)

The PCR was performed using cDNA of Ukrainian isolate of BNYVV under the following conditions: initial denaturation 5 min – 94°C; 30 cycles: denaturation 30 sec – 94°C, annealing of primers 30 sec – 60°C, elongation 30 sec – 72°C, 72°C final synthesis – 7 min.

As a result of the visualization of PCR amplification product fragments of size – 490 nucleotides pairs were revealed. An availability on the electrophoregram expected reaction product indicates that the developed system for the identification of RNA-2 of BNYVV is effective (Fig. 2).

On the next stage of the study we had to pick up the operating temperature annealing of primers. We made a series of reactions with temperature annealing of primers from 50°C to 64°C. PCR was performed under the following conditions: initial denaturation of 5 min – 94°C; 20 cycles: denaturation 30 sec – 94°C annealing of primers with 30 sec – 50–64°C, 30 sec elongation – 72°C, 72°C final synthesis – 7 min.

Although primers annealing temperature conditions did not significantly affect on the efficiency of amplification reactions, we have found that more optimal temperature is in the range of 50–62°C. Under these conditions nonspecific amplification products were not observed and the amount of amplicons was sufficient for clear visualization in agarose gel (Fig. 3).

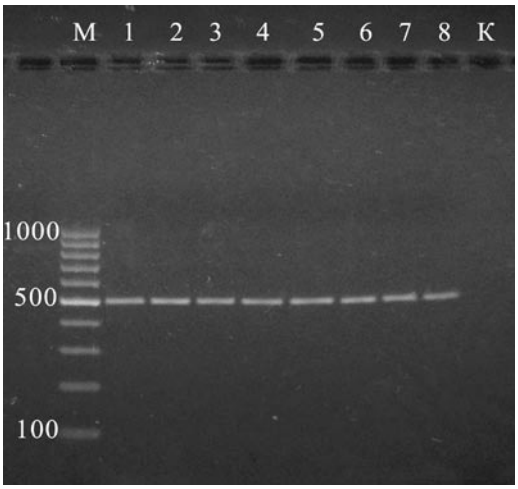

Fig. 3. The electrophoregram of the temperature optimization of primers annealing for the identification of gene P75: M (GeneRuler 10 bp DNA Lader 0241), 1 – 50°C, 2 – 52°C, 3 – 54°C, 4 – 56°C, 5 – 58°C, 6 – 60°C, 7 – 62°C, 8 – 64°C, K – negative control of PCR analysis

CONCLUSIONS

a) A bioinformative analysis of nucleotide sequences of RNA-2 of world isolates of BNYVV was carried out. Conserved sequences of the gene encoding protein P75 of BNYVV were shown and implemented design primers for identification of RNA-2 of BNYVV was made. The primers with optimal characteristics such as: Forward 5'-CTTTGGCAGGATTAGGCTCG-3', Reverse 5'- CACTCGGGACTATCACCAGG -3' were synthesized.

b) The developed system was tested and confirmed the effectiveness of its use on Ukrainian model cDNA isolate of BNYVV.

c) The annealing temperature of primers was optimized. The optimal temperature for identification of BNYVV was in the range of 50–62°C.

In our further studies we are planning to determine the nucleotide sequences of RNA-2 of P75 protein and establish phylogenetic relationship with world isolates.

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