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DEVELOPMENT OF PRIMER SETS FOR DETECTION OF RASPBERRY LEAF BLOTCH VIRUS AND RASPBERRY LEAF MOTTLE VIRUS BY MULTIPLEX RT-PCR

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ABSTRACT

More than 20 pathogenic viruses infect raspberry plants from Rubus genus (family Rosaceae). Raspberry leaf blotch virus (RLMV) and Raspberry leaf mottle virus (RLBV) are the most distributed viruses of commercially cultivated raspberries. The influence of these viruses on the raspberry crop yield is large and losses count in 30–50%. The efficient set of PCR primers were developed to detect the infected stock planting material or plants carrying infection in orchards. Capsid protein (CP) gene of RLMV and nucleocapsid (NP) gene of RLBV were considered for primer design. The specificity analysis of primers confirmed absence of any off-target complementary with the nucleotide collection of NCBI. Eighty-five samples of raspberry were tested by developed primers. Four cultivars of pomological garden and one raspberry plant of wild population were positive for RLMV and RLBV, respectively. The sequencing analysis of RLMV CP and RLBV NP revealed high homology level with Bosnia and Herzegovina isolates sequences from NCBI.

Key words: raspberry, virus, detection, primer set, capsid protein, nucleocapsid protein

INTRODUCTION

Raspberry is a promising berry crop, which is widely cultivated in Europe and America. Only 1.7% of the world yield is harvested in Asia. The leader in the raspberry production is Russia, where the average yield is 137.3 thousand tons [1]. The statistical data on raspberry harvest in Kazakhstan is not available, also the information about cultivated area is limited. Most of the planting material is imported from abroad and is not analyzed for the presence of viral infections. In addition to affecting the quantity and quality of the raspberry, imported viruses can infect many valuable species belonging to *Rosaceae* and *Solanaceae*. More than 20 pathogenic viruses infect raspberry plants from Rubus genus (family *Rosaceae*) [2]. *Raspberry leaf blotch virus* and *Raspberry leaf mottle virus* are the most distributed viruses of commercially cultivated raspberries. The influence of these viruses on the raspberry crop yield is large and losses count in 30–50%. Also, the infected plants are more sensitive to other biotic and abiotic stress comparing to healthy ones. The viral infection reduces the quality of planting material and increase the probability of global distribution of viruses.

Raspberry leaf blotch virus is a negative sense ssRNA virus, about 12.2 kb in lengths, belongs to genus *Emaravirus*, family *Fimoviridae*. The segmented genome of RLBV consists from four linear RNAs (RNA1, RNA2, RNA3, RNA4). The virus is associated with the long-known, severe raspberry leaf blotch disorder (RLBD). The virus is transmitted by the raspberry leaf and bud mite (Phyllocoptes gracilis) [3]. RLBD induces the large yellow blotches or rings on leaves, distortion of leaf margins, twisting of the leaves, necrosis and reduced vigour of the severely affected plants [4]. The main symptoms of RLBD have been previously observed on raspberries and tayberries (black berry × raspberry hybrid) in Netherlands [5], Germany [6], France [7], Norway [8], Scotland [9–10], Serbia [11], Finland [12], Bulgaria [13], and Poland [14].

Raspberry leaf mottle virus is a representative of the *Closterovirus* genus, Closteroviridae family. RLBV is a single stranded positive strand RNA virus with large genome and is associated with raspberry mosaic disease in red raspberry [15] and also has been reported infecting blackberry [16]. RLMV is transmitted by the *Amphorophora agathonica* in a semi-persistent manner. Nowadays, two RLMV isolates have been described in North America and Europe. The high variability in the amino acid and nucleotide sequences of the coat protein and the heat shock protein 70 homolog was revealed [17–18].

Due to the high error-prone nature of viral RNA polymerases and the frequent recombination events, RNA viruses show the significant level of genetic diversity even at the species level. This is particularly confirmed for viruses with large genomes such as closteroviruses. Therefore, new emerging strains or isolates demand more specific sets of primers for detection.

In this work we have developed new set of primers to detect RLBV and RLMV in plant material. The investigations related to RLBD and RLMV monitoring in the raspberry plants growing in Kazakhstan were not conducted yet. For the first time we have detected viruses in the cultivated and wild raspberry.

MATERIALS AND METHODS

Plant material

The samples of leaves were collected from cultivated and wild raspberry in summer of 2021. In the work, cultivars ‘Blesk’, ‘Kolinskaya rannyaya’, ‘11–3–36’, ‘Selekciya’,

and 'Dal'nyaya' were collected in Pomological Garden (Institute of fruits and vegetables). From 5 to 15 samples for each cultivar were analyzed. The wild raspberry leaves were collected in Medeu region (Ile Alatau). Fifty individual wild plants have been tested.

Primer design

To develop specific primers for RLMV and RLBV, all available genome sequences for coat protein and nucleocapsid protein, respectively, have been retrieved from NCBI. Muscle, ClustlW, and MAFFT methods were used to align the sequences with advanced settings for selecting conservative regions in UGENE and MegAlign Pro as described in [19]. Selected promising regions were analyzed in MPprimer and SeqBuilder Pro software for primer selection. Each pair of primers for each target was examined for the formation of dimers with the other primers of the multiplex systems. The developed primers were also tested for specificity in NCBI-Blast and Blast-Primer. The sizes of amplification products in multiplex systems differ from each other by at least 30 nucleotides for visual interpretation.

Detection of viruses

RNA isolation from 85 samples of raspberry were conducted using TRI-reagent according to manufacture protocol (Molecular research center, USA). Reverse transcription was performed in 20 ul of reaction mix containing 0.5 mM oligo-dT plus 0.5 mM random hexamer primer, 50U RevertAid, 1 ul total RNA, 1x RT buffer and 0,5 mM dNTP. cDNA synthesis included first step of RNA denaturation for 10 min at 65 °C and transcription for 60 min at 42C in second step. RNA samples were incubated on ice for 3 min before second step.

PCR detection of RLMV and RLBV were performed with specific primers to each virus. PCR reaction mix included 1 ul cDNA, 2.5 U DreamTaq polymerase (Thermo Scientific Fisher, USA), 1x Dream buffer, 0,5 mM dNTP, 0,5 mM of each developed primer. The amplification program consists of denaturation at 96^0 C for 3 min followed by 30 cycles of DNA denaturation for 30 sec at 96^0 C, annealing for 30 sec at 56^0 C followed by elongation for 1 min at 72^0 C. The final elongation continued 3 min at 72C.

Analysis of PCR products was conducted in 2% agarose gel by electrophoresis in Tris-acetate-buffer.

RESULTS

The design of high specific primers to detect RLMV and RLBV was based on analysis of all sequences of RLMV CP and RLBV NP retrieved from NCBI, respectively (Supplementary 1). Only two full genome sequences (17481 nt; GenBank Accession No. NC_008585.1; 17481 nt; GenBank Accession No. DQ357218.1) and two full CP sequences (597 nt; GenBank Accession No. EF114209.1; 597 nt; GenBank Accession No. DQ016612.1) of RLMV were determined, therefore 18 incomplete nucleotide sequences of CP were considered to reveal variable and conservative regions. As a result, four genome regions with relative conservatively were determined after multiple alignment of twenty-two complete and incomplete sequences of CP and two full genome sequences (Figure 1). For CP detection of RLMV, forward primer -TAGCGTACTTGTACTGTTC and reverse primer- ACGTCATGAAGGGAGAA were developed with amplification of 163 bp product.

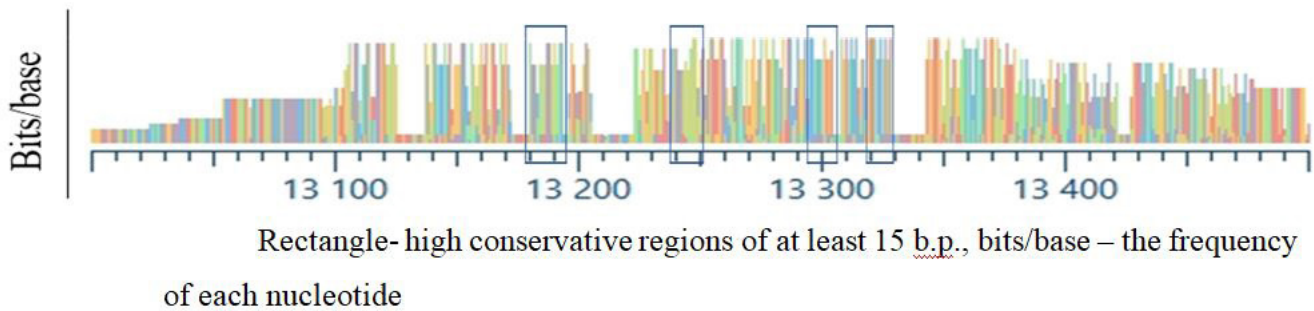


Figure 1 – The consensus sequence of analyzed complete and incomplete gene sequences of CP and full genome sequences of RLMV

Sixty eighth nucleotide sequences, including three full genome sequences of RNA3 of RLBV (1365 nt; GenBank Accession No. FR823301.1; 1365 nt; GenBank Accession No. NC_029559.1; 1362 nt; GenBank Accession No. MK433584.1) were analyzed to select regions for primer design. The multiple alignment analysis revealed long conservative regions in the gene NP, which were considered to develop specific primers (Figure 2). We have identified 7 promising regions of RNA3 which

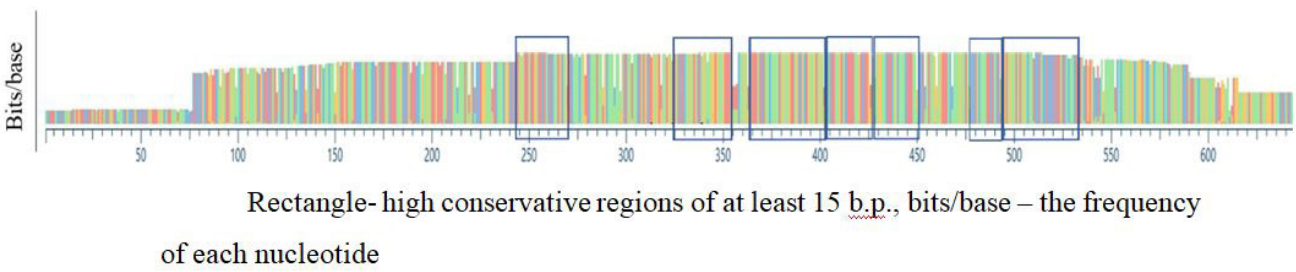


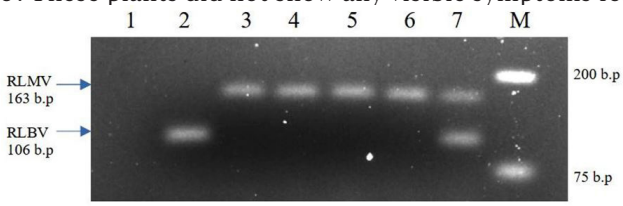
Figure 2 – The consensus sequence of analyzed complete NP sequences including full sequences of RNA3 of RLBV

allowed to detect all known isolates from NCBI in silico. Also, forward primer — TACACTTGTAGCATGTTTGG and reverse primer- CCAACCCTTGTCAATTTTGAT were developed for detection of NP of RLBV. PCR product of 106 bp is amplified in infected by RLBV plants.

The developed primers were analyzed in Primer-Blast (NCBI) considering on-target and off-target specificity. Twenty-four and sixty eighth isolates were identified by RLMV and RLBV primers, respectively. Off-target sequences in nucleotide collection of NCBI were not detected.

The annealing temperature in PCR was adjusted to 56^{0}C, also 54^{0}C and 58^{0}C were tested without successful results. 54^{0}C led to appearance of weak non-specific amplicons. In case of 58^{0}C, low level of viral RNA, which could be at early stage of infection or in case of latent infection, is not detectable.

Primers developed in this study were used for detection of viruses in 85 raspberry plants from Pomological garden and wild populations during vegetation period. The leaves from plants with or without symptoms of viral diseases were sampled and analyzed. For every cultivar, at least 5 plants were selected. We have identified RLMV in 'Kolinskaya rannyaya', 'Selekciya', 'Dal'nyaya', and '11–3–36' out of 5 cultivars (Figure 3). RLMV isolates from infected plants were assigned as isolate K.1 from 'Kolinskaya rannyaya', isolate S.3 from 'Selekciya', isolate D.2 from 'Dal'nyaya', and isolate 11.1 from '11–3–36'. These plants did not show any visible symptoms for



1- negative control (health plant), 2 – wild raspberry plant, 3- 'Kolinskaya rannyaya', 4 - 'Selekciya', 5- 'Dal'nyaya', 6 - '11-3-36', 7 – positive control (isolate 14 and isolate S.3), M - GeneRuler 1 kb Plus DNA Ladder.

Figure 3 – Detection of RLMV and RLBV in plant samples

viral infection and before planting were propagated *in vitro* as promising genetic pool for selection.

As shown in the current work, the raspberry plants in the field are infected by RLMV. The spreading of infection has possibly occurred in the field or via infected *in vitro* propagated stock planting material. *In vitro* propagated plant material was not analyzed in this work. Therefore, regular monitoring of stock planting material and orchards is required.

This is for first time, raspberry cultivars and wild plants in Kazakhstan were tested for viral infection. Also, one sample out of 50 wild plants was positive for RLBV, figure3. RLBV isolate detected in wild raspberry plant was assigned as isolate 14. The symptoms of RLBV in wild raspberry plant were as yellow blotches on leaves, distortion of leaf margins, twisting of the leaves, and necrosis as shown on figure 4.

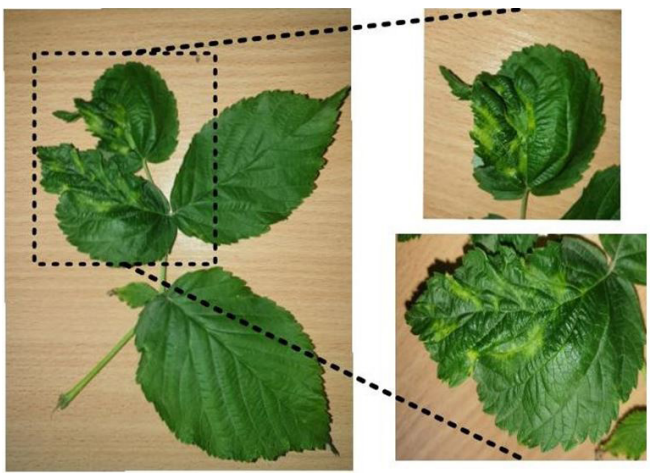


Figure 4 – The leaf from wild raspberry plant infected by RLBV, isolate 14

The partial sequences of CP and NP genes of detected isolates of RLMV and RLBV, respectively, were sequenced to identify the virus and compare with isolates from NCBI, table 1.

The nucleotide sequences of partial CP flanked

Table 1 Nucleotide sequences of isolates of RLMV and RLBV

Isolate	Virus	Plant source	Sequences 5'- 3'
isolate K.1	RLMV	'Kolinskaya rannyaya'	tagcgtacttgtactgttctgacacgatttttggatggagtagacgcactggtgtcgacataaagacgt-tacttcgaggtttcgagtctcggaccgcggcacctcctgggatgatccccgccgacgccgctttggaag-gttgcaggttctcccttcataacgt
isolate S.3	RLMV	'Selekciya'	tagcgtacttgtactgttctgacacgattattggatggagtagacccactggtgtcgacataatgaccttact-tagaggcattgagtctcgtacgcacggcacctcctgagatgatcccggccgacgccgctaagcaaggttg-caggttctcccttcatgacgt
isolate D.2	RLMV	'Dal'nyaya'	tagcgtacttgtactgttctgacacgattatgggatggagtagaccactgttgtcgacataatgccctaact-tagaggcattgagtctcgtacgcacggcacctcctgagatgatcccggccgacgcggctaagcaaggttg-caggttctcctttcatgacgt
isolate 11.1	RLMV	'11-3-36'	tagcgtacttgtactgttccgacacgattattggatggagtagaccactgttgtcgacttaatgccctaact-tagaggcattgagtctcgtacgcacggcacctcctgaaatgatcccggccgacgcggctaagcaaggttg-caggttct cctttcatga cgt
isolate 14	RLBV	wild raspberry plant	cccttgtcaattttgatcaattgaccacatcaactgatgcattcaacataaaagagtgtatttccacaagt-tactttcaactccaaacatgctacaagtgta

by primers of local RLMV isolates shared identity in ranges of 97.0–99.0%. Also, the closest isolates in GenBank were revealed from Bosnia and Herzegovina with 94,29% homology and 100% coverage. The sequencing results of partial NP of RLBV revealed the best match with isolates from again Bosnia and Herzegovina with 94,74% homology and 98% coverage.

DISCUSSION

Viruses are obligate parasite which utilize the host energy for reproduction. Viruses are known to infect wide range of living organisms such as animals, plants, fungi and bacteria. Our crops are under permanent threat to be infected by pathogens including viruses and viroids. The consequences of plant diseases caused by viruses count in tons of crop losses. Viruses induce symptoms which range from mild to severe by altering physiology of plants. Except the crop yield, viral pathogens greatly influence on crop quality.

Because of the fast evolution of viruses, the control of their distribution demands accurate methods and technics. Investigation of viral genomes and genes will allow to develop more precise molecular tests for pathogen detection. The one among other sensitive and reliable methods is PCR based amplification by specific primers. The different sets of primers to detect RLMV and RLBV were developed earlier, but primer design includes only European and American isolates not considering isolates from Asia due to the lack of data [20]. Therefore, these sets were not validated on isolates from others regions of the world. The developed primers in this work allow to detect RLMV and RLBV distributed in raspberry field in Kazakhstan. The primers were designed to conservative regions of genome considering different isolates and strains which are available in NCBI. We have considered CP gene of RLMV and NP gene of RLBV to develop PCR primers because they are the most declared sequences in Nucleotide Database of NCBI. For considering, complete and incomplete sequences were analyzed. The mismatches in last 3 nucleotides of 3'-terminus of primers and no more than 2 mismatches in other part of primers were allowed.

The field plants of different cultivars of raspberry from Pomological garden were tested by developed primers. Four of five cultivars were positive for RLMV infection. These cultivars are perspective genetic pool for selection and have never been tested before on the presence of any viral pathogens. The infected stock planting material could lead to spreading viral infection and decreasing yield of fruits. Also, we have detected RLBV in wild plant of raspberry, the plants were collected in the Northern Tien Shan mountains near to Almaty city. This is the first report of RLBV detection in wild plants, and we have not found any researches conducted under investigation of spreading RLBV in wild raspberries. The first reports of RLMV were recently described in 2020 in Serbia and in 2021 in Poland [21–22]. Also, RLMV was detected in America and other countries of Europe [23]. Kazakhstan is not an origin of raspberry, most cultivars obtained from abroad selection and propagated in commercial fields. The dependence on abroad planting material leads to the threat of spreading new pathogens infecting broad range of crops. Therefore, the regular monitoring of stock planting material and orchards by high- sensitive test-systems is required.

CONCLUSIONS

In this work, the set of primers for *Raspberry leaf blotch virus* and *Raspberry leaf mottle virus* simultaneous detection were developed. The specificity of primers was confirmed in silico and by sequencing the obtained amplicon from testing raspberry plant material collected in Pomological garden and in wild population. We have identified RLMV in 'Kolinskaya rannyaya', 'Selekciya', 'Dal'nyaya', and '11–3–36' cultivars and RLBV in wild raspberry plant for the first time. The researches upon distribution of RLBV in wild population of raspberry have not described before. The developed set of primers can be used in monitoring stock planting material and orchards of raspberry.

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РАЗРАБОТКА НАБОРА ПРАЙМЕРОВ ДЛЯ ДЕТЕКЦИИ ВИРУСОВ *RASPBERRY LEAF BLOTCH VIRUS* И *RASPBERRY LEAF MOTTLE VIRUS* МЕТОДОМ МУЛТИПЛЕКСНОГО ОТ-ПЦР

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АБСТРАКТ

Более 20 патогенных вирусов способны заражать растения малины, принадлежащей к роду Rubus. Наиболее распространенными вирусами у малины, возделываемой для коммерческих целей, являются Raspberry leaf blotch virus (RLMV) и Raspberry leaf mottle virus (RLBV). Потери урожая от данных вирусов могут составлять 30–50%. Для обнаружения вирусных патогенов в посадочном растительном материале, а также в растениях садов в данном исследовании был разработан эффективный набор праймеров. Для дизайна праймеров были использованы последовательности генов, кодирующих капсидный белок (CP) для RLMV и нуклеокапсид (NP) для RLBV. А В результате анализа специфичности праймеров не было выявлено неспецифичного связывания с какой-либо нуклеотидной последовательностью базы данных NCBI. Разработанные праймеры были использованы для обнаружения вирусов у 85 образцов малины. 4 сорта малины из помологического сада и один образец дикой малины были заражены вирусами RLMV и RLBV, соответственно. Результаты секвенирования капсидного белка RLMV и нуклеокапсида RLBV показали, что наибольшая гомология была обнаружена с изолятами из Боснии и Герцеговины.

Ключевые слова: малина, вирус, детекция, набор праймеров, капсидный белок, нуклеокапсид

RASPBERRY LEAF BLOTCH VIRUS ЖӘНЕ *RASPBERRY LEAF MOTTLE VIRUS* ВИРУСТАРЫН МУЛЬТИПЛЕКСТІ КТ-ПТР ӘДІСІМЕН АНЫҚТАУҒА АРНАЛҒАН ПРАЙМЕРЛЕР ЖИЫНТЫҒЫН ҚҰРАСТЫРУ

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ТҮЙІН

20-дан астам патогендік вирустар Rubus тұқымдасына жататын таңқурай өсімдіктерін залалдануына апаруы мүмкін. Коммерциялық мақсатта өсірілетін таңқурайдағы көптеп таралатын вирустарға-Raspberry leaf blotch virus (RLMV) және Raspberry leaf mottle virus (RLBV) жатады. Бұл вирустар салдарынан түсімнің жоғалуы 30–50% дейін жетуі мүмкін. Отырғызуға бағытталған өсімдік материалында, сондай-ақ бақша өсімдіктерінде вирустық қоздырғыштарды анықтау үшін осы зерттеу жұмысында праймерлердің тиімді жиынтығы жасалды. Праймерлердің дизайнын құрастыруда RLMV үшін (CP) капсидті ақуызды және RLBV үшін (NP) нуклеокапсидті кодтайтын гендер тізбегі қолданылды. Праймерлердің ерекшелігін талдау нәтижесінде NCBI мәліметтер базасының нуклеотидтер тізбектерімен нақты емес байланыс түрі анықталған жоқ. Жасалған праймерлер таңқурайдың 85 үлгісінде вирустарды анықтау үшін қолданылды. Помологиялық бақтағы таңқурайдың 4 түрі және жабайы таңқурайдың бір үлгісі сәйкесінше RLMV және RLBV вирустарымен жұқтырылды. RLMV капсид ақуызының және RLBV нуклеокапсидінің секвенирлеу нәтижесі ең жоғары гомология Босния мен Герцеговинаның изоляттарымен табылғаның көрсетті.

Түйінді сөздер: таңқурай, вирус, детекция, праймерлер жиынтығы, капсид ақуызы, нуклеокапсид