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Molecular Biology

Establishing Taxonomy of Pathogenic Bacteria, Causative Agents of Haricot Bacterioses Spread in Different Regions of Georgia by 16S rDNA Fragments

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ABSTRACT. The haricot crop is greatly damaged by bacterial diseases, two of which stand out in Georgia by their harmfulness and wide spread: brown spot and angular leaf spot of haricot caused by the pathogens *Xanthomonas phaseoli* and *Pseudomonas phaseolicola* respectively. In order to determine the taxonomy of pathogenic bacteria causing haricot bacterial disease - brown spot in different regions of Georgia DNA have been isolated and purified. To obtain 16S ribosomal DNA fragments polymerase chain reaction has been carried out with two preliminarily specific primers designed by us, constructed of 20 nucleotides: 5' TGG CGG ACG GGT GAG GAA TA 3' (forward) and 5' CGT CAT CCC CAC CTT CCT CC 3' (reverse). Selected 16S ribosomal DNA fragments were sequenced. Sequence of PCR fragments and their analysis, using computer program "BLASTA", allowed to identify two phytopathogenic strains 1466 and 1475 as *Xanthomonas axonopodis* pv. *phaseoli* . A global alignment of 16S rDNA fragments between the strains 1466 and 1475 by means of computer program Lalign has shown 95.8% identity of the fragments. *© 2013 Bull. Georg. Natl. Acad. Sci.*

Key words: *bacterial DNA, 16S ribosomal DNA, PCR amplification, DNA sequencing.*

Haricot is the most important legume used in human food especially in Georgia. Several diseases caused by bacteria, fungi and viruses are limiting factors of haricot yield. According to existing data, 6 kinds of bacterial diseases are known, two of which stand out in Georgia by their harmfulness and wide spread: brown spot and angular leaf spot of haricot caused by the pathogens *Xanthomonas phaseoli* and *Pseudomonas phaseolicola* respectively [1].

To develop an appropriate disease management strategy, the identification and the genetic diversity of the pathogen populations must be assessed. Current detection and identification procedures consist in biochemical tests [2] and molecular diagnostics by PCR [3].

The genetic diversity of different *Xanthomonas* isolates have been previously characterized by RAPD [4], restriction fragment length polymorphism (RFLP) [5] and amplified fragment length polymorphism analyses (AFLP) [6].

In prokaryotes, the 16S ribosomal DNA) genes are essential and occur in at least one copy in a ge-

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1466 1475 1482 1514 1517 1709 C- 1703

Fig. 1. Electrophoregram of 16S ribosomal DNA fragments of phytopathogenic bacteria, causing haricol brown spot, in Georgia on 1%-agarose gel (C- sample without DNA).

nome [7]. The universality of the genes makes them an ideal target for phylogenetic studies and taxonomic classification of bacteria [8].

The aim of this work is taxonomic identification of phytopathogenic strains, causing haricot brown spot by using 16S rDNA fragments as a marker, and comparison of partial sequences of the 16S rDNA genes.

Materials and Methods

Objects of the study were seventeen isolates (1438, 1466, 1475, 1482, 1513, 1514, 1517, 1617, 1620, 1621, 1467, 1468, 1703, 1708, 1709, 1712, 1713, 1806, 1807, 1814, 1829, 1832, 1853, 1840, 1839, 1858) causing haricot bacterial disease, kindly provided by Dr. Naila Giorgobiani, Kanchaveli Institute of Plant Protection of the Agricultural University of Georgia [1].

For DNA extraction, bacterial cells were grown in 5 ml in Luria–Bertani (LB) with 10% glycerol v/v (suggested by Maringoni et al [9] to avoid xanthane formation) for 48h at 27°C. Cells were centrifuged for 2 min at 13000 x g, the pellet was resuspended in 1.5 ml of lysing buffer (0.**5**% SDS; 50 mM Tris-HCl pH 7.5-8.0; 400 mM EDTA; 1 mg proteinase K) and incubated at 65 °C in water bath for 3-5 h, then deproteinized with 700 μl of chloroform/isoamyl alcohol (24:1) v/v, centrifuged 10 min at 13000 x g. To precipitate DNA, 700 μl of cold isopropanol was added and centrifuged. Precipitated DNA was washed in 70% ethanol, dried at room temperature and suspended in 50 μl of TE buffer10 mM Tris.HCl, 1 mM EDTA, pH 8.0. The samples were electrophoresed on 0.8% agarose gel, stained with ethidium bromide and photographed under UV light.

Primers for 16S rDNA amplification were selected and designed from database of phytopathogenic bacteria **cpgr.tigr.org** based on *Xanthomonas* ribosomal DNA gene sequence using computer program oligo 4.1.

Amplification of 16S ribosomal DNA fragments was carried out using primers 5' TGG CGG ACG GGT GAG GAA TA 3'(forward) and 5' CGT CAT CCC CAC CTT CCT CC 3' (reverse) by PCR(Thermocycler, Techne TC412). All PCRs were performed in a total volume of 25 μl using 50 ng DNA, 25 mM $MgCl_2$, 100 μM dNTPs, 0.5 mM each primer and 0.05U/ μl *Taq* polymerase (Sigma). Cycling conditions were: one cycle of 94°C for 1 min and 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 1 min. Obtained PCR products were studied by horizontal electrophoresis on agarose gel [10]. 16S rDNA gene sequences were conducted commercially at The Laboratory Services Division of the University of Guelph, (ON, Canada). Obtained sequencing results were analyzed, i.e. resemblances of nucleotide sequences of 16S ribosomal DNA with data base analogues were established by computer program BLASTA. The 16S rDNA sequences of different strains of *Xanthomonas phaseoli* were aligned by using **sequence alignment** program (Clustal W, Lalign).

Table 1. Resemblance of analyzed 16S rDNA sequences with the analogues available in database of computer program "BLAST"

Strain	Fragments	Fragment size, bp	BLAST results	Identity, %
1466	16S rDNA	453	*Xanthomonas axonopodis pv. phaseoli* strain IAC13755 16S ribosomal RNA gene, partial sequence	92
			Xanthomonas axonopodis pv. punicae strain AP-4 16S ribosomal RNA gene, partial sequence	90
			Xanthomonas axonopodis pv. punicae strain AP-3 16S ribosomal RNA gene, partial sequence	90
			Xanthomonas axonopodis strain KSI 1432 16S ribosomal RNA gene, partial sequence	90
			Xanthomonas axonopodis strain KSI 1432 16S ribosomal RNA gene, partial sequence	86
			Xanthomonas sp. EECC-449 16S ribosomal RNA gene, partial sequence	86
			Xanthomonas sp. EECC-446 16S ribosomal RNA gene, partial sequence	82
			Xanthomonas sp. EECC-365 16S ribosomal RNA gene, partial sequence	89
1475	16S rDNA	454	*Xanthomonas* sp. EECC-263 16S ribosomal RNA gene, partial sequence	90
			Xanthomonas axonopodis pv. phaseoli strain IAC13755 16S ribosomal RNA gene, partial sequence	89%
			Xanthomonas axonopodis pv. *punicae* strain 82 16S ribosomal RNA gene, partial sequence	89%
			Xanthomonas axonopodis pv. *punicae* strain 81 16S ribosomal RNA gene, partial sequence	89%
			Xanthomonas *axonopodis pv. punicae* strain 80 16S ribosomal RNA gene, partial sequence	85%
			Xanthomonas axonopodis pv. punicae strain 15 IARI 16S ribosomal RNA gene, partial sequence	85%
			Xanthomonas sp. EECC-365 16S ribosomal RNA gene, partial sequence	85%
			Xanthomonas sp. EECC-263 16S ribosomal RNA gene, partial sequence	85%
			Xanthomonas sp. EECC-201 16S ribosomal RNA gene, partial sequence	85%

Results and Discussion

Amplification of 16S ribosomal DNA has been conducted by PCR. DNA of pathogenic bacterial strains (1438, 1466, 1475, 1482, 1513, 1514, 1517, 1617, 1620, 1621, 1467, 1468, 1703, 1708, 1709, 1712 and 1713), causative agents of haricot bacterioses, isolated from different regions of Georgia were applied as matrices for amplification.

The following strains: 1466, 1475 1482, 1514, 1517, 1709 and 1703 gave fragments of 16S ribosomal DNA.

As is seen from the electrophoregram (Fig.1), the fragments of 16S ribosomal DNA obtained by PCR are of equal size on agarose gel. The tested restriction endonuclease Xba I was ineffective. Selected 16S rDNA fragments were sequenced. Successful

Table 2. **Sequence alignment of the strains 1466 and 1475 by computer program Lalign.**

xan1466 453 bp .

xan1475 454 bp

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using matrix file: /usr/molbio/share/fasta3/dna.mat, gap open/ext: -
14/-4
  95.8% identity in 454 aa overlap;           Global score: 1863

               10        20        30        40        50        60
xan146 AGGAATACATCGGAATCTACTCTTTCGTGGNNNNNNNNNNNGGGATAACTACTGGAAACTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
xan147 AGGAATACATCGGAATCTACTCTTTCGTGGNNNNNNNNNNNGGGATAACTACTGGAAACTT
               10        20        30        40        50        60

               70        80        90       100       110       120
xan146 ACGCTAATATTGCATACGACCTACGGGTGAAAGCGGAGGACCTTCGGGCTTCGCGAAATT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
xan147 ACGCTAATATTGCATACGACCTACGGGTGAAAGCGGAGGACCTTCGGGCTTCGCGAAATT
               70        80        90       100       110       120

              130       140       150       160       170       180
xan146 GNNNNNNNNNNNAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
xan147 GNNNNNNNNNNNAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAG
              130       140       150       160       170       180

                190       200       210       220       230
xan146 CGACGATCCGTAGCTNNNCTGAGAGGATGATCATTTTCCCCGGAACTGAGACACGGTCA
        :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
xan147 GCGACGATCCGTAGCTNNNCTGAGAGGATGATCATTTTCCCCGGAACTGAGACACGGTCA
              190       200       210       220       230       240

     240       250       260       270       280       290
xan146 GACTCCTACGGGAGGCAGCAGTGGGGAATAATAGGTGGCGGGCGCAAGCCCGATCCAGCC
       ::::::::::::::::::::::::::::::    :     ::::::::::::::::::::
xan147 GACTCCTACGGGAGGCAGCAGTGGGGAATAGGGCGCAAGCGGCGCAAGCCCGATCCAGCC
              250       260       270       280       290       300

     300       310       320       330       340       350
xan146 ATNNNNNGTAAATGAAGAAGGCCTNNNTTGTAAAGCCCTTTTGTTGGGAAAGAAAAGCAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::
xan147 ATNNNNNGTAAATGAAGAAGGCCTNNNTTGTAAAGCCCTTTTGTTGGGAAAAGCGTTACT
              310       320       330       340       350       360

     360       370       380       390       400       410
xan146 TCGGTTAATACCCGATTGTTCTGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
xan147 TCGGTTAATACCCGATTGTTCTGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGC
              370       380       390       400       410       420


 420       430       440       450
xan146 CAGCAGNNNNCGGTAATACNNGGTGCGGGTGGGG
       ::::::::::::::::::::::::::::::::::
xan147 CAGCAGNNNNCGGTAATACNNGGTGCGGGTGGGG
                430       440       450
```

sequences results were obtained for fragments, belonging to strains – 1466 and 1475.

Resemblance of obtained 16S rDNA sequences with the analogues available in database of computer program “BLASTA” is presented in Table 1.

As seen from the Table 1, 16S rDNA fragments of the strain 1466 reveal 92%, and of the strain 1475 – 90% of identity with that of *Xanthomonas axonopodis pv. phaseoli.*

A global alignment of 16S rDNA fragments between the strains 1466 and 1475 by means of computer program Lalign, version 2.2u [11] has shown 95.8% identity of the fragments (Table 2).

Thus, two strains 1466 and 1475 isolated from brown leaf diseased haricot are identified as *Xanthomonas axonopodis pv. phaseoli* with 95.8% identity of 16S rDNA.

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