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Abstract. The throughput and single-base resolution of RNA-Sequencing (RNA-Seq) have contributed to a dramatic change in diagnostics of viruses and other plant pathogens. A transcriptome represents all RNA molecules, including the coding mRNAs as well as the noncoding rRNA, tRNA, etc. A distinct advantage of RNA-Seq is that cDNA fragments are directly sequenced and the reads can be compared to available reference genome sequences. This approach allows the simultaneous and hypothesis-free identification of all pathogens in the plant. We conducted surveys for potato (*Solanum tuberosum* L.) -associated phytopathogenic bacteria in 56 original and GenBank RNA-seq data sets for potato breeding material. Bacteria of genera *Pseudomonas, Burkholderia, Ralstonia, Xanthomonas*, *Agrobacterium*, and species of family Enterobacteriaceae were most frequently detected in RNA sets from the studied plants. RNA-seq reads identified as *Xanthomonas* spp. were within *X. vesicatoria,* and some other species. *Xanthomonas* spp. covered up to 9,1% of all reads and included the major clades of these bacteria known as pathogens of solanaceous crops, but potato. Bacteria of genus *Xanthomonas* infect different plant species under artificial inoculation, suggesting that they are shared among wild plants and crops. Our studies indicated that a larger number of solanaceous plants can be occupied by specific *Xanthomonas* pathovars as endophytes or latent pathogens. Revealing bacteria distribution in the plant breeding material using RNA-seq data improves our knowledge on the ecology of plant pathogens.

1. Introduction

Potato plant (*Solanum tuberosum* L.) is one of major staple food crop. In 2018, world production of potatoes was 368 million tons, with China (27% of the total yield) on the first place. Russia was on the third place (after India). It is an essential crop in northern and eastern Europe, with the highest consumption per capita in the world, but the most rapid growth of production over the past few decades has occurred in southern and eastern Asia [1]. Potatoes are sensitive to many pathogenic microorganisms, including some bacteria. Potato bacterial diseases are responsible for heavy losses of the yield in the fields (up to 40%) and especially during the storage. Seed potato must be routinely tested before planting for large number of pathogenic bacteria to avoid crop loss. List of potato bacterial diseases includes bacterial wilt /brown rot (*Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1996); blackleg and bacterial soft rot (*Pectobacterium* spp. Hauben et al. 1999; *Dickeya* spp.

Samson et al. 2005), ring rot (*Clavibacter michiganensis* subsp. *sepedonicus* (Spieckermann and Kotthoff 1914) Davis et al. 1984); and common scab (*Streptomyces* spp. Lambert and Loria 1989) [2]. Potatoes contain toxic compounds known as glycoalkaloids, of which the most prevalent are solanine, solamargine, and chaconine. These compounds, which protect the potato plant from its predators and pathogens, are generally concentrated in its green parts [3]. Comparing to potato, the close relative, tomato (Solanum Lycopersicon L) is damaged additionally by pathogenic bacteria from genera *Pseudomonas* and *Xanthomonas*, including *Pseudomonas viridiflava* (Burkholder) Dowson; *P. syringae* van Hall pv. *tomato* (Okabe) Young, Dye, & Wilkie; *P. syringae* pv. *syringae* van Hall; *P. cichorii* (Swingle) Stapp *P. corrugata* Roberts & Scarlett, *P. mediterranea* Catara et al., *Xanthomonas euvesicatoria* Jones et al., *X. gardneri* (ex Šutic) Jones et al., *X. perforans* Jones et al., *X. vesicatoria* (ex Doidge) Vauterin et al.; *X. arboricola* Vauterin et al. 1995 , and *X. campestris* pv. *raphani* (Pammel 1895) Dowson 1939 [4, 5]. There were no reports about any noticeable damage for potato crop from bacteria of genus *Xanthomonas*, and potential threat of these harmful for other solanaceous crops phytopathogens stays unknown.

The throughput and single-base resolution of RNA-Sequencing (RNA-Seq) have contributed to a dramatic change in diagnostics of viruses and other plant pathogens. A transcriptome represents all RNA molecules, including the coding mRNAs as well as the noncoding rRNA, tRNA, etc. RNA-seq uses next-generation sequencing (NGS) to reveal the presence and quantity of ribonucleic acid (RNA) in a biological sample. A distinct advantage of RNA-Seq is that cDNA fragments are directly sequenced and the reads can be compared to available reference genome sequences [6]. In addition to messenger RNA (mRNA) transcripts, RNA-Seq can look at different populations of RNA to include total RNA, small RNA, such as microRNA (miRNA), transfer RNA (tRNA), and ribosomal profiling [6]. RNA-Seq is often used to identify plant viruses, but the obtained data can provide information about presence of other unexpected microorganisms, including latent phytopathogenic or endosymbiotic bacteria.

2. Materials and methods

2.1. Plant material

In vitro plantlets of the potato variety “La Strada” (Cygnet Potato Breeders Ltd.) were grown under artificial light at 27 °C. Three bulked leaf samples (10 plants each) were collected for RNA extraction.

2.2. RNA and DNA extraction

RNA was isolated using a phenol method [7] from each sample. The samples were treated with DNase. Quality of total RNAs was verified on an Agilent 2100 Bioanalyzer (Agilent) and based on the rRNA ratio 25S/18S, RNA Integrity Number, and the absence of smear. Potato DNA was extracted using a CTAB-based protocol. Pooled sample was mechanically homogenized, and 100 mg were mixed with a 0.1% sodium pyrophosphate. The homogenate was mixed with lysozyme solution (100 µl / ml) and 10% SDS, incubated at 37° C for 30 min. 2% STAB was added to the mixture and incubated for 30 min at 65°C. When the mixture was cooled, chloroform was added, vortexed, and precipitated at 12000 rpm for 5 min. DNA was precipitated with isopropanol, washed twice with 75% ethanol, and dissolved in water.

2.3. RNA-Seq

Illumina library preparation and RNA sequencing was done at the BioSpark Co. (TechnoSpark, Troitsk, Moscow) using TruSeq RNA. Illumina libraries were quantified by qPCR. Samples were sequenced on two Illumina HiSeq2000 lanes (51-cycle v3 SE). Analysis of the RNA-Seq reads was made with Taxonomer [8].

2.4. NCBI's Sequence Read Archive analysis

Fifty-three data sets from NCBI's Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra/) were downloaded. They represented samples from China, India, Poland, Germany, USA, and South Africa.

2.5. Classic and quantitative PCR analysis, DNA sequencing, bacteria isolation

RNAs samples were reverse-transcribed to cDNAs with Reverse Transcriptase (New England BioLabs). cDNAs were diluted and used as template in 25-µl PCR reactions containing the Eurogen (Moscow) Master Mix (qPCRmix-HS) and 150 nM of forward, reverse primers and probers designed for hrpB7 gene sequences to target each of the four bacterial spot pathogens infecting plants of genus *Solanum*: *X. euvesicatoria*, *X. gardneri*, *X. perforans*, and *X. vesicatoria*. PCR analysis for *X. campestris* pv. *raphani* and for *X. arboricola* was done as described before by Tsyganokova et al. [10] and Kyrova et al. [11].

PCR reactions were performed on an StepOne instrument (Thermo Fisher Scientific Co.). PCR conditions were: denaturation at 95 °C for 10 min, followed by 44 cycles at 95 °C for 30s, 58 °C for 30s, and 72 °C for 30s. The resulting PCR products were separated by 1.5% agarose gel electrophoresis in TBE buffer gel and visualized by ethidium bromide staining. 1 kb DNA Ladder marker (Evrogen Co, Moscow) was used to evaluate the size of the bands. Selected PCR fragments were sequenced by Eurogen Co. (Moscow). Routine methods of xanthomonads isolation from plant material were employed as recommended [12]. Primers specific for hrpB7 gene (RST65 [5′-GTC GTC GTT ACG GCA AGG TGG TCG-3′] and RST69 [5′-TCG CCC AGC GTC ATC AGG CCA TC-3′]) [13] were used to amplify a 420-bp fragment from strains of bacterial spot pathogens described on solanaceous plants. Protocol including (i) pre-denaturation 95.0°C for 5 min (1 cycle); (ii) 30 cycles of 95.0°C for 30 s, 63.0°C for 30 s, and 72.0°C for 45 s; and (iii) additional synthesis at 72.0°C for 5 min followed by 4.0°C ∞ (1 cycle) was used to amplify the DNA. Amplicons were purified using QIAquick kit (Qiagen, CA), and send to Eurogen Co. (Moscow) by Sanger sequencing. Sequences of hrpB7 gene from related *Xanthomonas* spp. from the National Center of Biotechnology Information (NCBI) database were used for comparison after alignment using the ClustalW feature in MEGA X [14]. The maximum-likelihood tree, with 1,000 bootstrap samples, based on the aligned sequences was created in MEGA X.

3. Results

3.1. RNA-seq analysis

A distinct advantage of RNA-Seq is that cDNA fragments are directly sequenced and the reads can be compared to available reference genome sequences [6, 8]. We conducted surveys for potato (Solanum tuberosum L.) -associated phytopathogenic bacteria in the three original and 53 GenBank RNA-seq data sets for potato breeding material. Bacteria of genera *Pseudomonas, Burkholderia, Ralstonia, Xanthomonas, Agrobacterium,* and species of Enterobacteriaceae were most frequently detected in the studied RNA-seq sets from potato plants.

RNA-seq reads identified as *Xanthomonas* spp. were within groups of *X. vesicatoria, X. euvesicatoria, X. perforans, X. gardneri, X. fuscans* subsp. *aurantifolii, X. axonopodis* pv. *punicae*, *X. translucens* pv. *translucens*, *X. oryzae* pv. *oryzae, X. sacchari, X. citri* pv. *mangiferaeindicae, X. campestris* pv. *raphani, X.* sp. Nyagatare, *X. vasicola*, and *X. citri* pv. *citri* (Table 1).

Table 1. Reads assigned to *Xanthomonas* spp. in 56 RNA-seq data sets obtained for breeding material of potato (*Solanum tuberosum* L.) plants.

Expected host plant	Species	Range of Reads, %
Solanaceae (tomato, pepper)	*Xanthomonas vesicatoria*	0.02 – 6.48
	X. euvesicatoria	0.02 – 2.01
	X. perforans	0.01 – 1.72
	X. gardneri	0.01 – 0.48
Rutaceae (Citrus)	*X. fuscans* subsp. *aurantifolii*	0.01 – 0.04
	X. citri pv. *citri.*	0.01 – 0.03
Expected host plant	Species	Range of Reads, %
Lythraceae	X. axonopodis pv. punicae	0.01 – 0.04
Anacardiaceae (Mango)	*X. citri* pv. *mangiferaeindicae*	0.01 – 0.02
Brassicaceae	*X. campestris* pv. *raphani*	0.01 – 0.02
Leguminaceae	*X.* sp. Nyagatare,	0.01 – 0.02
Poaceae	*X. vasicola*	0.01 – 0.02
	X. oryzae pv. *oryzae*	0.01 – 0.02
	X. translucens pv. *translucens*	0.01 – 0.02
	X. sacchari	0.01 – 0.02

Reads classified as *Xanthomonas* spp. pooled together covered up to 9,1% of all reads and included the major clades of these bacteria known as pathogens of solanaceous crops, but identified for the first time in potato.

Bacteria of genus *Xanthomonas* infect different plant species under artificial inoculation, suggesting that they are shared among wild plants and crops.

3.2. PCR analysis and bacteria identification

Specific PCR analysis of the sequenced potato samples of cv. La Strada was positive for two species (*X. vesicatoria* and *X. arboricola*) only, and negative for *X. euvesicatoria*, *X. gardneri*, *X. perforans*, and *X. campestris* pv. *raphanin.* Fragments of individual plants 3 x 4 mm in size were aseptically cut from PCR positive plants. The plant tissue was transferred to sterile tap water and macerated using a sterile scalpel. A small amount of the suspension was streaked on nutrient agar (NA) (BBL, Becton Dickinson and Co., MD, USA) plates for individual colonies. Isolated bacteria were purified on NA by streaking and inspecting colonies in 7 days of growth at 28°C, and used for hrpB7 gene sequencing.

Identity of obtained PCR products was confirmed by DNA sequencing with the same primers. Twenty *Xanthomonas* spp. isolates similar to *X. vesicatoria* in hrpB7 gene sequence (figure 1) were selected from over 100 bacterial isolates obtained from the La Strada plants. Purified and identified isolates were stored in sterile 30% glycerol solution at −80°C for further study.

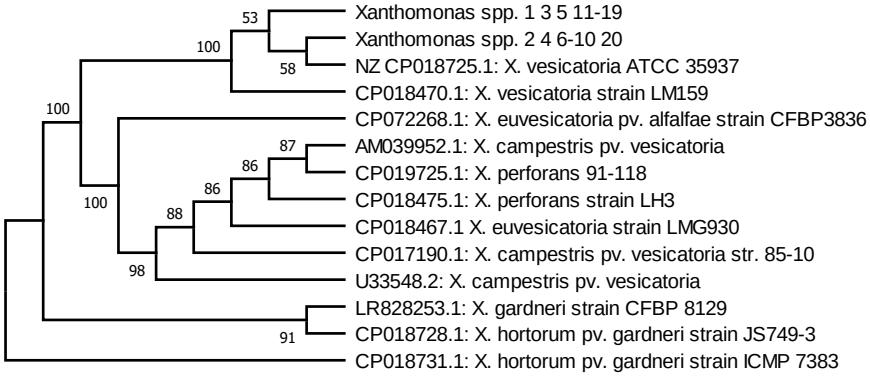

Figure 1. Maximum Parsimony analysis of hrpB7 gene sequence for 20 isolates of *Xanthomonas* spp. (1-20) and reference strains of GeneBank. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches There were a total of 450 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [14].

4. Conclusion

The *Xanthomonas* species complex from solanaceous plants also includes strains classified as *X. alfalfae, X. axonopodis*, and others, which may be further classified into numerous pathovars based on their host range [15, 16]. A larger number of solanaceous plants carry specific *Xanthomonas* pathovars as endophytes or latent pathogens [17, 18]. It was found for the first time that potato plants can be occupied by bacteria of genus *Xanthomonas* as latent pathogens or endophytes. The role of them in subsequent diseases and interaction with other pathogenic bacteria must be established. Revealing bacteria distribution in the plant breeding material using RNA-seq data improves our knowledge on the ecology of plant pathogens.

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