RNA-Seq of potato plants reveals a complex of new latent bacterial plant pathogens

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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 pathogens 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 probes 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 ′GTC GTC GTT ACG GCA AGG TGG TCG′–3′ and RST69 ′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. puniceae, 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       |
| Lythraceae                              | *X. axonopodis pv. punicae*    | 0.01 – 0.04       |
| Anacardiaceae (Mango)                   | *X. citri pv. mangiferaeindicca* | 0.01 – 0.02     |
| Brassicaceae                            | *X. campestris pv. raphani*   | 0.01 – 0.02       |
| Leguminaceae                            | *X. sp. Nyagatara,*            | 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 hrbB7 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 hrbB7 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.

References
[1] Potato production in 2018: UN Food and Agriculture Organization, Statistics Division (FAOSTAT) 2019
[2] Weingartner D P and Hooker W J Diseases of Potato (Solanum tuberosum L) (Common Names of Plant Diseases) Ed R Stevenson Walter Available at: https://www.apsnet.org/edcenter/resources/commonnames
[3] Friedman M and Levin C E 2009 Analysis and Biological Activities of Potato Glycoalkaloids, Calystegine Alkaloids, Phenolic Compounds, and Anthocyanins (Advances in Potato Chemistry and Technology) pp 127–161 DOI: https://doi.org/10.1016/B978-0-12-374349-7.00006-4
[4] Jones J P, Zitter T A, Momol T M and Miller S A Diseases of Tomato (Solanum lycopersicum L) (Common Names of Plant Diseases) Available at: www.apsnet.org/edcenter/resources/commonnames
[5] Vauterin L, Rademaker J and Swings J 2000 Synopsis on the taxonomy of the genus Xanthomonas (Phytopathology vol 90) pp 677–682
[6] Kimbrel J A, Di Y, Cumbie J S and Chang J H 2011 RNA-Seq for Plant Pathogenic Bacteria (Genes vol 2) pp 689–705 DOI: https://doi.org/10.3390/genes2040689
[7] Baeblle S, Krecic-Stres H, Rotter A and et al 2009 PVYNTN elicits a diverse gene expression response in different potato genotypes in the first 12 h after inoculation (Mol Plant Pathol vol 10) pp 263–275
[8] Flygare S, Simmon K, Miller C and et al 2016 Taxonomer: an interactive metagenomics analysis portal for universal pathogen detection and host mRNA expression profiling (Genome Biol vol 17) p 111 DOI: https://doi.org/10.1186/s13059-016-0969-1
[9] Strayer A L, Jeyaprakash A, Minsavage G V and et al 2016 A Multiplex Real-Time PCR Assay
Differentiates Four Xanthomonas Species Associated with Bacterial Spot of Tomato (Plant Disease vol 100) pp 1660–1668

[10] Tsygankova S V, Ignatov A N, Boulygina E S and et al 2004 Genetic relationships among strains of Xanthomonas campestris pv. campestris revealed by novel rep-PCR primers (European J Plant Pathol vol 110) pp 845–853

[11] Kyrova E, Egorova M and Ignatov A 2020 Species of the genus Xanthomonas infecting cereals and oilseeds in the Russian Federation and its diagnostics (InBIO Web of Conferences vol 18) p 00017

[12] Jones J B, Pohronezny K L, Stall R E and Jones J P 1986 Survival of Xanthomonas campestris pv. vesicatoria in Florida on tomato crop residue, weeds, seeds, and volunteer tomato plants (Phytopathology vol 76) pp 430–434

[13] Strayer A L, Jeyaprakash A, Minsavage G V, Timilsina S, Vallad G E, Jones J B, Paret M L 2016 A multiplex real-time PCR assay differentiates four Xanthomonas species associated with bacterial spot of tomato (Plant disease vol 100) pp 1660–1668

[14] Kumar S, Stecher G, Li M, Knyaz C and Tamura K 2018 MEGA X: molecular evolutionary genetics analysis across computing platforms (Molecular biology and evolution vol 35) pp 1547–1549

[15] Huang C L, Pu P H, Huang H J and et al 2015 Ecological genomics in Xanthomonas: the nature of genetic adaptation with homologous recombination and host shifts (BMC genomics vol 16) pp 1–3

[16] Timilsina S, Jibrin M O, Potnis N and et al 2015 Multilocus sequence analysis of xanthomonads causing bacterial spot of tomato and pepper plants reveals strains generated by recombination among species and recent global spread of Xanthomonas gardneri (Appl Envir Microbiol vol 81) pp 1520–1529

[17] Newberry E A, Bhandari R, Minsavage G V and et al 2019 Independent evolution with the gene flux originating from multiple Xanthomonas species explains genomic heterogeneity in Xanthomonas perforans (Appl Envir Microbiol vol 85) p e00885–19

[18] Kyrova E, Dzhalilov F and Ignatov A 2020 The role of epiphytic populations in pathogenesis of the genus Xanthomonas bacteria (InBIO Web of Conferences 2020 vol 23) p 03010