Long-Read Genome Sequence Resources of *Xanthomonas citri* pv. *punicae* Strain Bagalkot Causing Pomegranate Bacterial Blight

Dattatraya Hegde Radhika,¹ Raghavendra Gunnaiah,^{1,†} Ashwini Lamani,¹ Dadapeer Peerjade,² and Rudrappa Chandrashekhhar Jagadeesha¹

¹ Department of Biotechnology and Crop Improvement, College of Horticulture, Bagalkot, University of Horticultural Sciences, Bagalkot, India

² Directorate of Research, University of Horticultural Sciences, Bagalkot, India

Funding

This project was funded by Department of Science and Technology, Science and Engineering Research Board, Government of India (project number ECR/2017/000914).

Abstract

Xanthomonas citri pv. *punicae* causing bacterial blight is a devastating disease of pomegranate in India and Pakistan. Most xanthomonads use the type III secretion system to inject transcription activator-like effector (TALE) proteins into the host cell. TALEs bind to the effector-binding elements in the promoter of host susceptibility genes, triggering disease development. PacBio single-molecule real-time long-read sequencing technology was used to identify the TALE-encoding genes, which is otherwise not possible using next-generation short-read sequencers. In all, 1.74 Gb of raw data containing 368,980 subreads, with an average read length of 4,724 bp and longest read length of 77,471, were generated. Subreads were assembled into 15 scaffolds generating approximately 5.4 Mb (348×) of genome. *X. citri* pv. *punicae* exhibited close lineage with *X. citri* pv. *citri* with 98.78% average nucleotide identity. Of the 4,263 protein-coding genes, 11 non-TALE type III effectors and 2 TALE-encoding genes were identified.

Bacterial blight, caused by the Gram-negative bacterium *Xanthomonas citri* pv. *punicae* (Constantin et al. 2016; Hingorani and Singh 1960), previously referred to as *X. axonopodis* pv. *punicae* (Hingorani and Singh 1960; Vauterin et al. 1995), is a major constraint in pomegranate cultivation, leading to 60 to 80% yield loss (Ramesh and Ram 1991; Sharma et al. 2017). Bacterial blight affects the aboveground plant parts, including leaf, fruit, and stem. Early water-soaked lesions to late necrotic blighting symptoms are initially observed on the leaves. Isolated water-soaked lesions on the fruit coalesce to form Y-, X-, and L-shaped cracks and split fruit. Under severe and prolonged disease conditions, canker and breakage of the stem can lead to deterioration of an entire orchard (Sharma et al. 2017). Although the disease was first reported in India, its prevalence has been reported in Pakistan (Akhtar and Bhatti 1992), South Africa (Petersen et al. 2010), and Turkey (Icoz et al. 2014). Host resistance breeding is believed to be the best bacterial blight management practice in pomegranate; however, no resistance sources are reported to date. Identification of pathogenicity factors and characterization of the host response mechanism is crucial for developing host resistance in elite cultivars.

Many *Xanthomonas* spp. secrete transcription activator-like effector (TALE) proteins through the type III secretion system (Bogdanove et al. 2010; White et al. 2009). TALEs bind to the specific TALE-binding elements in the promoter region of host genes and trigger the expression of multiple disease susceptibility genes (Zhang et al. 2015). The DNA-binding

[†]Corresponding author: R. Gunnaiah; raghavendra.gunnaiah@uhsbagalkot.edu.in

The author(s) declare no conflict of interest.

Accepted for publication 10 March 2021.

Copyright © 2021 The Author(s). This is an open access article distributed under the CC BY-NC-ND 4.0 International license.

Table 1. PacBio sequencing, assembly, and annotation statistics of *Xanthomonas citri* pv. *punicae* genome

Attributes ^a	Value
Raw subreads	368,980
Raw bases	1,743,387,061
Raw longest subread length (bp)	77,471
Raw subread N ₅₀ (bp)	5,961
Total assembly (bp)	5,436,898
GC (%)	63.94
Assembly N ₅₀ (bp)	5,158,969
Longest scaffold (bp)	5,158,969
Total BUSCO groups searched in	221
Complete BUSCOs	216
Complete and single-copy BUSCOs	205
Complete and duplicated BUSCOs	11
Fragmented BUSCOs	1
Missing BUSCOs	4
Predicted genes	4,757
Coding genes	4,652
Protein coding genes	4,263
Total RNA-coding genes	105
Pseudo-genes	389
Type III secretion system effectors	11
TALEs	2
Pseudo-TALEs	4

^a BUSCOs = benchmarking universal single-copy orthologous groups and TALEs = transcription activator-like effectors.

specificity of TALEs is determined by a central domain of near-perfect 33- to 35-amino-acid repeats with two variable amino acids at positions 12 and 13, termed as repeat-variable di-residues (Xu et al. 2017). Genes encoding TALEs occur in multiple copies and contain multiple simple, near-perfect repeats within each gene, which makes it challenging to accurately assemble the repeats through short-read sequencing technologies (Sharma et al. 2012). Long-read sequencing of *X. citri* pv. *punicae* strain Bagalkot isolated from Bagalkot, a major pomegranate-growing area in Karnataka, India, was carried out, and genes encoding non-TALE type III effectors and TALEs were identified by PacBio single-molecule real-time (SMRT) sequencing.

X. citri pv. *punicae* was isolated from bacterial-blight-infected pomegranate leaves and cultured on nutrient glucose (NG) agar (pH 7 ± 0.2) media at 28 ± 2°C for 2 days. Pathogenicity of the isolated culture was reconfirmed by challenge inoculation on the blight-susceptible pomegranate plants. DNA was isolated from the *X. citri* pv. *punicae* cultured on NG broth. Identity of the *X. citri* pv. *punicae* with other isolates of *X. citri* pv. *punicae* was confirmed by sequencing a partial 16S ribosomal RNA (rRNA) gene and sequence similarity analysis. *X. citri* pv. *punicae* 16S rRNA gene MN971672.1 exhibited 100% identity with the 16S rRNA gene of most of the reported *X. citri* pv. *punicae* isolates from India (Kumar et al. 2020). For SMRT sequencing, high-quality *X. citri* pv. *punicae* DNA was fragmented by exonuclease treatment and an average insert size of 8,455 bp was processed with the SMRTbell template prep kit v1.0 (Pacific Biosciences, India) using the manufacturer's protocol and sequenced on a PacBio Sequel at AgriGenome Labs Pvt. Ltd., Kochi, India. Raw polymerase reads were partitioned into 368,980 subreads with longest, mean, and N₅₀ subread lengths of 77,471, 4,724, and 5,961 bp, respectively, constituting 348x genome coverage (Table 1). Long subreads were assembled into 15 contigs with Flye (v.2.5) assembler (Mikheenko and Kolmogorov 2019). MUMmer algorithm-based average nucleotide identity (ANI_m) of the newly assembled *X. citri* pv. *punicae* genome with 36 representative genomes of *Xanthomonas* spp. (downloaded from NCBI) was calculated using JSpecies v. 1.2.1. (Richter and Rosselló-Móra 2009). The completeness of the final assembly was evaluated by benchmarking universal single-copy orthologous (BUSCO v.4.1.4) software (Simão et al. 2015). NCBI Prokaryotic Genome Annotation Pipeline (PGAP) version 4.9 was used for functional annotation of genes. AnnoTALE 1.4.1 was used to detect and annotate TALE genes (Grau et al. 2016).

The *X. citri* pv. *punicae* genome was assembled into a total genome length of approximately 5.4 Mb with a total G+C content of 63.94% (Table 1). The N₅₀ and L₅₀ contig lengths

were 5,158,969 and 1 bp, respectively. The longest contig 8 (5,158,969 bp) exhibited 99.96 and 99.98% identity with the short-read assembled reference genome LMG859 (NZ_CAGJ000000000.1) and hybrid assembly (Illumina Miseq and Nanopore) (CP030163.1). Contig 9 (Plasmid pUnnamed1, 28823 bp) and contig 11 (Plasmid pUnnamed2, 48249 bp) were identical to the plasmids of previously sequenced *X. citri* pv. *punicae*, with 98.33 and 99.12% identity to CP030179.1 and CP030170.1, respectively. Contig 8 also exhibited close lineage with the *Xanthomonas* representative genome *X. citri* pv. *citri* (98.78% ANIm; NZ_CP009016.1) which causes citrus canker disease in citrus. Of the 4,757 genes predicted, 4,652 are coding sequences (4,263 proteins, and 389 pseudogenes) and 105 are RNA genes (three each of 5S RNA, 16S RNA, and 23S RNA; 61 transfer RNAs; and 35 noncoding RNA) (Table 1). Fewer pseudogenes were detected in the PacBio sequenced assembly compared with the other assemblies available in the NCBI database. Of the 1,152 BUSCO groups (BUSCOs) searched, PacBio assembly displayed 1,139 complete BUSCOs (approximately 98.9%) without any duplicates, six fragmented BUSCOs, and seven missing BUSCOs, exemplifying the accuracy of bacterial genome sequencing and assembly. Eleven non-TALE type III effector proteins and one type VI secretion system were identified through automated genome annotation using PGAP. Furthermore, two novel TALE effectors belonging to the class I and class II TALE families and four pseudo-TALEs were identified for the first time in the *X. citri* pv. *punicae* genome using AnnoTALE. Interestingly, two pseudo-TALEs were identified in Plasmid pUnnamed2. Short-read assembly of *X. citri* pv. *punicae* (NZ_CAGJ000000000.1) didn't return any TALEs, and only three TALEs were discovered in the hybrid assembly of *X. citri* pv. *punicae* (CP030163.1). Newly discovered TALE1 and TALE2 were 96.66 and 94.48% identical, respectively, to the *X. oryzae* pv. *oryzae* strain race 4 TALE 7 (MN128606), with >99% coverage. *X. citri* pv. *punicae* TALEs need to be validated and their binding proteins in host target genes need to be identified before starting a resistance breeding program.

The power of long-read PacBio sequencing technology was used to obtain a genome assembly of *X. citri* pv. *punicae* of high depth (348×) and high consensus accuracy that allowed the capture of repetitive regions such as TALEs. Identification of genes encoding type III secretion system effector proteins and TALEs is a first step toward the identification of the corresponding susceptibility genes. With the high-quality pomegranate genome available (MABG000000000.2), TALE-binding DNA elements will be identified and deployed in resistance breeding.

Data Availability

Complete genome assembly and gene annotation data have been deposited at GenBank under the accession number NZ_JAAQOY000000000.1 (BioProject: PRJNA612770).

Acknowledgments

We thank Karnataka Biotechnology and Information Technology Services, Government of Karnataka, India for funding to build laboratory and infrastructure facility.

Literature Cited

- Akhtar, M., and Bhatti, M. R. 1992. Occurrence of bacterial leaf spot of pomegranate in Pakistan. *Pak. J. Agric. Res.* 13:95-97.
- Bogdanove, A. J., Schornack, S., and Lahaye, T. 2010. TAL effectors: Finding plant genes for disease and defense. *Curr. Opin. Plant Biol.* 13:394-401.
- Constantin, E., Cleenwerck, I., Maes, M., Baeyen, S., Van Malderghem, C., De Vos, P., and Cottyn, B. 2016. Genetic characterization of strains named as *Xanthomonas axonopodis* pv. *dieffenbachiae* leads to a taxonomic revision of the *X. axonopodis* species complex. *Plant Pathol.* 65:792-806.
- Grau, J., Reschke, M., Erkes, A., Streubel, J., Morgan, R. D., Wilson, G. G., Koebnik, R., and Boch, J. 2016. AnnoTALE: Bioinformatics tools for identification, annotation, and nomenclature of TALEs from *Xanthomonas* genomic sequences. *Sci. Rep.* 6:21077.
- Hingorani, M. K., and Singh, N. J. 1960. *Xanthomonas punicae* sp. nov. on *Punica granatum* L. *Indian J. Agric. Sci.* 29:45-48.
- Icoz, S. M., Polat, I., Sulu, G., Yilmaz, M., Unlu, A., Soylu, S., Bozkurt, I. A., and Baysal, Ö. 2014. First report of bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae* in Turkey. *Plant Dis.* 98:1427.
- Kumar, A., Sharma, J., Munjal, V., Sakthivel, K., Thalor, S., Mondal, K., Chinchure, S., and Gharate, R. 2020. Polyphasic phenotypic and genetic analysis reveals clonal nature of *Xanthomonas axonopodis* pv. *punicae* causing pomegranate bacterial blight. *Plant Pathol.* 69:347-359.
- Mikheenko, A., and Kolmogorov, M. 2019. Assembly Graph Browser: Interactive visualization of assembly graphs. *Bioinformatics* 35:3476-3478.
- Petersen, Y., Mansvelt, E., Venter, E., and Langenhoven, W. 2010. Detection of *Xanthomonas axonopodis* pv. *punicae* causing bacterial blight on pomegranate in South Africa. *Australas. Plant Pathol.* 39:544-546.
- Ramesh, C., and Ram, K. 1991. Studies on bacterial blight (*Xanthomonas campestris* pv. *punicae*) of pomegranate. *Indian Phytopathol.* 44:370-372.
- Richter, M., and Rosselló-Móra, R. 2009. Shifting the genomic gold standard for the prokaryotic species definition. *Proc. Natl. Acad. Sci. U.S.A.* 106:19126-19131.

- Sharma, J., Sharma, K., Kumar, A., Mondal, K., Thalor, S., Maity, A., Gharate, R., Chinchure, S., and Jadhav, V. 2017. Pomegranate bacterial blight: Symptomatology and rapid inoculation technique for *Xanthomonas axonopodis* pv. *punicae*. J. Plant Pathol. 99:109-119.
- Sharma, V., Midha, S., Ranjan, M., Pinnaka, A. K., and Patil, P. B. 2012. Genome sequence of *Xanthomonas axonopodis* pv. *punicae* strain LMG 859. J. Bacteriol. 194:2395.
- Simão, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., and Zdobnov, E. M. 2015. BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 31:3210-3212.
- Vauterin, L., Hoste, B., Kersters, K., and Swings, J. 1995. Reclassification of *Xanthomonas*. Int. J. Syst. Evol. Microbiol. 45:472-489.
- White, F. F., Potnis, N., Jones, J. B., and Koebnik, R. 2009. The type III effectors of *Xanthomonas*. Mol. Plant Pathol. 10:749-766.
- Xu, Z.-y., Zou, L.-f., Cai, L.-l., Yang, Y.-y., and Cheng, G.-y. 2017. Action modes of transcription activator-like effectors (TALEs) of *Xanthomonas* in plants. J. Integr. Agric. 16:2736-2745.
- Zhang, J., Yin, Z., and White, F. 2015. TAL effectors and the executor R genes. Front. Plant Sci. 6:641.