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16S rRNA partial sequence analysis of *Ralstonia solanacearum* isolated from wilting ginger (*Zingiber officinale*) and potato (*Solanum tuberosum*) crops in Hassan District, Karnataka.

Biotechnology

KEYWORDS : Ralstonia solanacearum, Phytopathogen, 16s ribosomal sequence, Potato, Ginger

Makari Hanumanthappa K	Research and Development Centre, Bharathiar University, Coimbatore -641046, Tamil Nadu, INDIA and Department of Biotechnology, IDSG Government College,Chikmagalur- 577102, Karnataka, INDIA
Palaniswamy M	Department of Microbiology, School of Life Sciences, Karpagam University, Coimbatore -641 021, TamilNadu, INDIA.
Angayarkanni J	Department of Microbial Biotechnology, Bharathiar University, Coimbatore-641046, Tamil Nadu, INDIA
Manjunath Dammalli	Department of Biotechnology, Siddaganga Institute of Technology,Tumkur-572103. Karnataka, INDIA
Vivek Chandramohan	Department of Biotechnology, Siddaganga Institute of Technology,Tumkur-572103. Karnataka, INDIA

ABSTRACT

The aim of the research work is to identify phytopathogenic Ralstonia solanacearum, induced wilting symptoms in potato and ginger crops in Karnataka state. Ralstonia solanacearum is a soil inhabiting Gram-negative bacterium causes wilt leading to devasting lethality in many vegetable crops especially in solanaceous plants. It infects through the roots specifically invading in the host and multiplying in the xylem vessels in potato crops leads to wilting. The bacteria were isolated from infected potato and ginger tubers. The isolates were characterized by TTC, 16s ribosomal sequence analysis was done using 16S rRNA F8 universal primers (16s rRNA F-5'AGAGTTTGATCCTGGCTCAG 3', 16s rRNA R-5'ACG GCTACCTTGTTA3') and phylogenetic analysis were used molecular relatedness of the isolated phytopathogenic bacteria. 16S rRNA sequences were analyses by CLC genomics workbench software for Sequence information, atomic composition, nucleic acid distribution, nucleotide distribution histogram and secondary structure prediction. The findings of the research greatly anticipated for the identification and characterization of the phytopathogenic R. solanacearum.

Introduction

Ralstonia solanacearum, previously known as *Pseudomonas solanacearum*, was originally described by Smith (1986) as the causative agent of bacterial wilt on several hundred plant species, including crops such as potato, tomato, tobacco, banana, peanut, and ginger, and it is responsible for substantial yield losses worldwide [Elphinstone, J and Hayward, A. C. 1991]. This bacterium has wide host range and infection of the bacterium extensively expands to the neighbouring fields and thus resulted in heavy economic losses to the potato and ginger growing farmers in Hassan District, Karnataka State. *R. solanacearum* enters the plant through wounds in the roots from cultivating equipment, nematodes, insects, and through cracks where secondary roots emerge [Agrios G.N, 1997].Bacterial wilt is an important disease of many crops and is caused by various subgroups of the bacterium *Ralstonia solanacearum*, formerly called *Pseudomonas solanacearum*. Historically, strains of *R. solanacearum* were classified into five races based loosely on host range, and into five biovars based on differential ability to produce acid from a panel of carbohydrates [Denny T. P,2006**]**. The applicability of small subunit ribosomal RNA (16S rRNA) seuquences for the bacterial classification has been accepted worldwide. 16s rRNA sequencing technology served as a most result oriented method for fast detection and analysis of bacterial species.*Ralstonia solanacearum* has been isolated from various *solanaceous* plants using SMSA medium and were characterized [Engelbrecht M C 1994, Elphinstone J et al, 1996].The genetic diversity analysis of *Ralstonia solanacearum* was extensively studied using three genetic markers PCR-RFLP, AFLP and 16S rRNA sequence analysis [Stephane Poussier, et al, 2000]. The use of 16S rRNA gene sequences helps to understand taxonomy and phylogeny of specific bacterial housekeeping genetic marker. Based available review of literature, we are proposing use of 16S rRNA sequences as molecular tool to identify phytopathogenic bacteria.

Methodology

Bacterial isolates and pathogenicity test

R. solanacearum bacteria was isolated from infected edible ginger (*Zingiber officinale*) and potato (*Solanum tuberosum*)plants collected in Hassan District, Karnataka, India during the period 2011- 2013 were used for the study (Figure-1). Bacterial ooze was collected in sterilized distilled water from severely infected and wilting plants. Ooze extract was plated on to cassaminoacid Peptone Glucose (CPG) agar with 1% of stock solution of 2,3,5-Triphenyl tetrazolium chloride (TTC)[Kumar A, *et al* 2013, Kelman 1954], and incubated at 28ºC for 48 h. Pathogenicity assay was conducted by injecting bacterial isolates to roots as previously described by Dinesh Singh *et al* (2010).

Genomic DNA extraction and PCR based amplification of 16S rRNA sequences

The genomic DNA of *R solanacearum* was isolated for PCR amplification was done according to the instructional manual provided by Aristogene Biosciences Pvt Ltd, Bangalore, India, (Aristogene PCR Kit, 16S rRNA Sequence amplification Kit, Aristogene Biosciences Pvt Ltd Bangalore).The genomic DNA was isolated by CTAB method and subjected to PCR amplification using 16S rRNA F8U universal primers (16s rRNA F-5'AGA GTTTGATCCTGGCTCAG3';16S rRNA R-5'ACGGCTACCTTGTTA3') PCR amplification of DNA was performed in Effendorf gradient thermal cycler at the suitable conditions for PCR according to the standard procedure [Kumar and M Anandaraj, 2006, Opina, N *et al*, 1997]and the PCR amplified products were separated in agarose gel electrophoresis. Electrophoresed gel was observed for DNA bands on a UV trans-illuminator. The results were documented in Alpha imager Gel Doc system. Eluted DNA samples were subjected to Sequence analysis (Contract Research by Aristogene Biosciences lab, Bangalore) using cycle sequence method and generated sequences were analyzed by BLAST at NCBI.

Sequences analysis

The sequence related analysis for the newly identified sequenced was performed using CLC genomics workbench software [CLC Bio]. 16S rDNA sequences were compared with those available in the GenBank databases using the gapped BLASTN [Altschul X, *et al* 1997] program through the National Center for Biotechnology Information server. Comparisons were performed using the BLOSUM 62 matrix with default parameters

including a gap existence cost of 11, a cost-per-residue gap of 1, and a lambda ratio of 0.85. Every sequence was aligned with the first 10 database sequences giving the highest scores of sequence similarity, and the quality of the database sequences was assessed.

Phylogenetic analysis of unidentified isolates.

For those isolates which were not identified by 16S rDNA sequence analysis, taxonomic relationships were inferred from 16S rDNA sequence comparison. Sequences were obtained from the GenBank database and aligned by using the multisequence alignment program ClustalW [Thompson J D, *et al*, 1994] in the CLC genomics workbench. Phylogenetic relationships were inferred from this alignment by using programs in version 3.4 of the PHYLIP [Felsenstein J, 1993]. A distance matrix was generated using DNADIST under the assumptions of Jukes and Cantor and Kimura. Phylogenetic trees were derived from these matrices using neighbor joining.

Result and Discussion

16S rRNA sequence analysis and bacterial identification. An almost complete 16S rRNA sequence containing fewer than positions was obtained for all of the isolates included in the study; top ten query sequences were available for comparison (Table 1 and Figure 1). For this isolates belonging to the organisms *Ralstonia solanacearum* (Earlier name of *Ralstonia solanacearum* was *Pseudomonas solanacearum).* The Phylogenetic tree was produced using PHYLIP with neighbor joining method, the result are shown in the (Figure 2). The CLC genomics workbench software has generated the following information for the input sequence. Sequence information, Melting temperature - degrees Celsius, Atomic composition and Nucleotide distribution. The results are shown in the (Table 2). Nucleotide Guanine has the maximum number of occurrence (247) and Cytosine being the lowest (163).

From the table it is clear that C+G combination is more (410) compared to A+G (371). The RNA structure prediction results are shown in the (Figure 4 and Figure 5). In that result gives the information about Stem, Multi loop Bulge loop and Hairpin loop.

Table 1: Blast result for newly identified 16S rRNA sequences

Hit	E-value	Score	%Identity	%Gaps
DQ803329	0	707	98	1
DQ300313	, 0	707	98	1
KC894851	0	706	98	1
KC415112	0	706	98	1
KC415111	0	706	98	1
KC934816	0	706	98	1
KC934810	0	706	98	1
KC934808	0	706	98	1
NR_102805	0	706	98	1
JQ782513	0	706	98	1

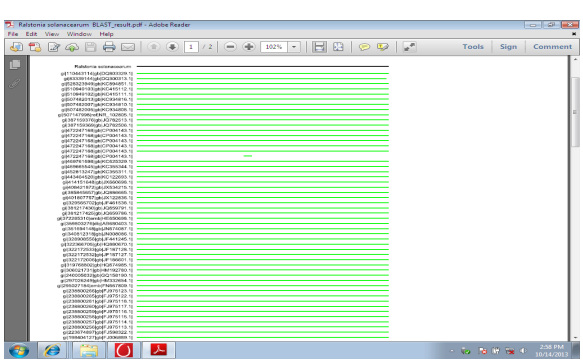

Figure 1: Blast result for newly identified 16S rRNA sequences

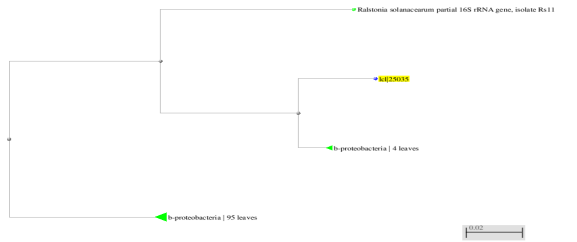

Figure 2: Phylogenetic analysis for newly identified 16S rRNA sequences

Table 3: 16S rRNA Sequences statistics

Sequence information				
Sequence type		rRNA		
Length		781bp		
Organism		Ralstonia solanacearum		
Weight (single-stranded)		253.385 kDa		
Weight (double-stranded)		502.418 kDa		
Melting temperature - degrees Celsius				
[salt] = 0.1M	[salt] = 0.2M	[salt] = 0.3M	[salt] = 0.4M	[salt] = 0.5M
86.42	91.42	94.34	96.42	98.03
Atomic composition				
As single-stranded				
Atom	Count	Frequency		
Hydrogen (H)	9,210	0.354		
Carbon (C)	7,483	0.288		
Nitrogen (N)	3,087	0.119		
Oxygen(O)	5,425	0.209		
Phosphorus(P)	781	0.030		
As double-stranded				
Hydrogen (H)	18,377	0.356		
Carbon (C)	14,839	0.288		
Nitrogen (N)	5,877	0.114		
Oxygen(O)	10,936	0.212		
Phosphorus(P)	1,562	0.030		
Nucleotide distribution				
Nucleotide	Count	Frequency		
Adenine(A)	207	0.265		
Cytosine(C)	163	0.209		
Guanine(G)	247	0.316		
Uracil (U)	164	0.210		
C+G	410	0.525		
A+U	371	0.475		

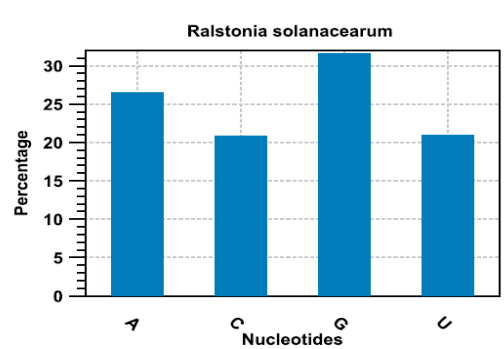

Figure 3: Nucleotide distribution histogram

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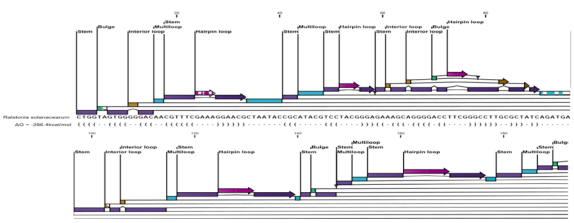

Figure 4: Secondary structure 16s RNA sequence of *Ralstonia solanacearum*
$\Delta G = -286.4\ kcal/mol$The ΔG *is obtained by using the formula*. $\Delta G = \Delta H - T\Delta S$

Figure 5: Secondary structure 16s RNA sequence of *Ralstonia solanacearum*
$\Delta G = -286.4\ kcal/mol$The ΔG *is obtained by using the formula*. $\Delta G = \Delta H - T\Delta S$

Conclusion

Genomic diversity of *Ralstonia solanacearum* can be analyzed using 16S rRNA sequence analysis. Advanced bioinformatics tools can be used for structural analysis and determination of its molecular terms. These findings of this research may helpful for the identification of phytopathogenic *Ralstonia solanacearum bacteria* with advanced molecular tools, new modalities can be framed for its control.

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