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RESEARCH ARTICLE

Identification of virulence factors and type III effectors of phylotype I, Indian *Ralstonia solanacearum* strains Rs-09-161 and Rs-10-244

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Abstract. *Ralstonia solanacearum* is a well-known phytopathogen causing bacterial wilt in a large number of agriculturally important crops. The pathogenicity of *R. solanacearum* is expressed due to the presence of various virulence factors and effector proteins. In this study, various virulence factors and type III effector proteins of *R. solanacearum* that are present in the strains Rs-09-161 and Rs-10-244 were identified through bioinformatics approach and compared with other reference strains. *R. solanacearum* strains, Rs-09-161 and Rs-10-244 belong to the phylotype I, biovar3, and are the only sequenced strains from India infecting solanaceous vegetables. Similarity matrix obtained by comparing the sequences of virulence genes of Rs-09-161 and Rs-10-244 with other reference strains indicated that Rs-09-161 and Rs-10-244 share more than 99% similarity between them and are closely related to GMI1000. The virulence factors in *R. solanacearum* appear to be highly conserved in the *R. solanacearum* species complex. Rs-09-161 has 72 type III effectors whereas Rs-10-244 has 77. Comparison of the complete genes of type III effectors of Rs-09-161, Rs-10-244 and GMI1000 revealed the presence of 60 common effectors within them. Further, Rs-09-161 has two unique effectors and Rs-10-244 has four unique effectors. Phylogenetic trees of RipA, RipG, RipH and RipS effector sequences resulted in the grouping of the isolates based on their phylotypes. Group 1 consists of strains that belong to phylotype I including Rs-09-161 and Rs-10-244. Phylotype III strain CMR15 forms a group closely associated with phylotype I. The strains belonging to phylotypes II and IV have separated to form two different groups.

Keywords. bacterial wilt; type III effector; virulence factor; phylogenetic analysis; *Ralstonia solanacearum*.

Introduction

Ralstonia solanacearum is a phytopathogen, which causes bacterial wilt disease in many crop plants and is responsible for huge losses in agriculturally important crops (Genin and Denny 2012). It has been ranked second in the list of top 10 of the most studied bacterial plant pathogens (Mansfield *et al.* 2012). Due to the extensive diversity that exists among the strains, the organism is now referred as *R. solanacearum* species complex (RSSC) and is divided into four phylotypes (Fegan and Prior 2005). These phylotypes represent their geographical origin: phylotype I (Asia), phylotype II (America), phylotype III (Africa) and phylotype IV (Indonesia). The phylotype IV also includes *R. syzygii* and the banana blood disease bacterium (BDB) (Genin and Denny 2012).

R. solanacearum finds its way into the plant through wounds in the roots and initiates wilting by impairing transport of water in the xylem that ultimately leads to the death of the infected plant (Genin and Denny 2012). Exopolysaccharide (EPS) produced by the bacterium is the primary virulence factor and impairs water transport within its susceptible host (Schell 2000). In addition to EPS, the type II secretory system (T2SS), chemotaxis, swimming, twitching motility and type III secretory system (TTSS) also contribute towards the virulence of the bacterium (Saile *et al.* 1997). The T2SS secretes various plant cell wall degrading enzymes

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(PCWDE) that include cellulolytic and pectinolytic enzymes which promote the colonization of the bacterium in the plant tissue. Chemotaxis and twitching motility also act as important virulence factors and aid in locating and attaching the bacterium to the host roots (Alvarez *et al.* 2010; Genin and Denny 2012).

The TTSS is an essential pathogenicity determinant and is encoded by the hypersensitive response and pathogenicity (*hrp*) regulon in *R. solanacearum* (Boucher *et al.* 1987). The *hrp* regulon is so named because it induces a hypersensitive response (HR) in nonhost or resistant plants and pathogenicity in the susceptible plants (Hueck 1998). A defect in the T3SS results in the loss of the ability to induce both; a hypersensitive response and pathogenicity in plants (Alfano and Collmer 2004). Type III-dependent protein secretion was first identified in the animal pathogen *Yersinia enterocolitica* (Heesemann *et al.* 1984) and later was found to be present in a variety of gram-negative phytopathogenic bacteria (Hueck 1998). The T3SS enables a bacterium to translocate pathogenicity proteins called as 'type III effectors (T3E)' into the cytosol of eukaryotic host cells. The effectors act as toxins and target the host immune system. This translocation is brought about by *hrp* dependant filamentous structure called as *hrp* pili (Van Gijsegem *et al.* 2000, 2002). *In situ* immunogold labelling experiments suggest that the *hrp* pili acts as a needle to provide a protein transport channel for transport of effector proteins into the host cytosol. All the *hrp* mutants that lack the *hrp* pilus protein (*HrpY*) cannot secrete *hrp* substrate proteins like hairpins and effectors (Van Gijsegem *et al.* 2002).

The expression of *R. solanacearum* T3SS is induced in the presence of poor nutritional conditions which mimics that of intracellular spaces in plants (Genin *et al.* 1992). The T3SS is an emerging area of study among many molecular biologists with plant and animal pathogens like *Pseudomonas, Xanthomonas*, *Salmonella* etc. Many T3Es are validated and many are under process in *R. solanacearum* through translocation studies using reporter-based systems like the Cya reporter and HA reporter systems (Cunnac *et al.* 2004; Mukaihara and Tamura 2009; Mukaihara *et al.* 2010; Sole *et al.* 2012).

In India, *R. solanacearum* has been isolated from various agriculturally important crops like eggplant, chilli, ginger, tomato, potato, capsicum etc. (Kumar *et al.* 2004; Chandrashekara *et al.* 2012; Ramesh and Phadke 2012). Even though the bacterial wilt is a severe issue and affects various crops in India, there are only two strains (Rs-09-161 and Rs-10-244) infecting solanaceous vegetables sequenced from India. These strains belong to race 1, biovar 3, phylotype I and based on endoglucanase (*egl*) gene sequence analysis the isolates belong to two different representative subgroups (Ramesh *et al.* 2014a,b). Both the strains are highly pathogenic on tomato and eggplant, cause 100% wilt within 15 days after inoculation (R. Ramesh, G. Achari, S. Gaitonde and T. Asolkar Bacterial wilt in solanaceous vegetables, *unpublished data*).

In RSSC, 16 strains are sequenced from different phylotypes (Salanoubat *et al.* 2002; Gabriel *et al.* 2006; Remenant *et al.* 2010, 2011; Xu *et al.* 2011; Ramesh *et al.* 2014a). Data on T3E of phylotype I strains is majorly contributed by studies on GMI1000 and no information on Indian strains is available. This study aims to identify and analyse various virulence factors and T3Es of *R. solanacearum* strains Rs-09-161 and Rs-10-244 using bioinformatics approach.

Materials and methods

R. solanacearum strains

R. solanacearum strains Rs-09-161 and Rs-10-244 were selected to analyse the virulence factors and T3Es in this study. These are the whole genome sequenced strains and belong to phylotype I from India (Ramesh *et al.* 2014a) and are being maintained in the culture collection of Plant Pathology Lab, ICAR-CCARI, Goa. Annotation of various virulence genes of *R. solanacearum* Rs-09-161 and Rs-10-244 was carried out using Eugene-P, with GMI1000 (phylotype I) as standard reference strain. The general features of all *R. solanacearum* strains used in this study are provided in the table 1.

Analysis of virulence factors

Various virulence genes involved in the colonization and wilting of the host were identified in the strains Rs-09-161 and Rs-10-244 based on the annotation data. These include the genes coding for *EPS* (*epsA, epsB, epsC, epsD, epsE, epsF, epsP* and *epsR*), PCWDE (*PehA, PehB, PehC, Pme, Egl* and *CbhA*), chemotaxis (*CheA* and *CheW*), swimming motility (*FliC* and *FlgM*) and twitching motility (*PilA* and *PilP*). The coding sequences of these virulence genes were compared with representative strains of *R. solanacearum* from different phylotypes, namely GMI 1000 (phylotype I), CFBP2957 (phylotype IIA), Po82 (phylotype IIB), CMR15 (phylotype PIII) and Psi07 (phylotype IV). The nucleotide sequences for virulence factors of Rs-09-161 and Rs-10-244 were retrieved from annotated files and of the reference strains were extracted from NCBI database (http://www.ncbi.nlm.nih.gov). Virulence sequences were submitted to GenBank and accession numbers were obtained (for details see table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). The sequences were aligned pairwise using Clustal W (Thompson *et al.* 1994) and the evolutionary similarity matrix was constructed using MEGA ver. 6 software with p-distance method and bootstrap value of 1000 (Tamura *et al.* 2013).

Table 1. *R. solanacearum* strains used in this study.

Strain	Phylotype	Geographical origin	Isolated from	Genome size (Mb)	Total no. of T3Es	Hypothetical effectors	Accession	Remarks/purpose in this study
Rs-09-161	I	India	Eggplant	5.65	72	3	PRJNA217471	Test strain
Rs-10-244		India	Chili	5.66	77	1	PRJNA236788	Test strain
GMI1000		French Guyana	Tomato	5.811	71	1	PRJNA13	Virulence factors, T3E
RS1000		Japan	Tomato	NA	65	0		T3E
Y45		China	Tobacco	5.712	50	3	PRJNA182081	T3E
FQY_4		China	Bacterial Wilt Nursery	5.805	52	2	PRJNA182081	T3E
CFBP2957	IIA	French West Indies	Tomato	5.683	72	5	PRJEA50685	Virulence factors, T3E
IPO1609	IIB	Netherlands	Potato	5.313	60	5	PRJNA32087	T3E
UW551		Kenya	Geranium	5.895	58	3	PRJNA15601	T3E
Molk2		Philippines	Banana	5.961	76	8	PRJNA32085	T3E
Po82		Mexico	Potato	5.43	75	4	PRJNA66837	Virulence factors, T3E
CMR15	III	Cameroon	Tomato	5.593	68	5	PRJEA50681	Virulence factors, T3E
Psi07	IV	Indonesia	Tomato	5.606	72	7	PRJEA50683	Virulence factors, T3E
BDB R229		Indonesia	Banana	5.159	57	4	PRJNA53877	T3E
R. syzygii R24		Indonesia	Clove	5.424	48	2	PRJNA53879	T3E

Information was compiled based on the published literature and NCBI database.

Identification of T3E

The preliminary identification of T3E genes was carried out by screening the presence of *hrpII* box element (TTCGn16TTCG) in the region 500-bp upstream of the start codon using PatScan where only one mismatch was allowed. The presence of T3SS dependent export pattern in the T3E genes was detected by analysis of 50 amino acid N-terminal domain. The T3E was considered positive for N-terminal domain if it fulfilled at least two of the three criteria mentioned below: (i) serine + proline content should be greater than 30% (ii) leucine content should be lesser than 10% (iii) acidic residues should be absent within the first 12 amino acids.

Prediction of the start codon of the gene was carried out by the multiple sequence alignment of the region located downstream of the *hrpII* box element. The more distal 5′ initiator codon conserved among different strain sequences was considered as the start codon. The predicted T3E genes were also analysed for frame-shift mutations and pseudogenes. T3E genes that had open reading frames disrupted by the insertion of IS element, altered structure ($< 50\%$) of the gene or evidence that the T3E gene product is not translocated by the T3SS were considered as pseudogenes (Peeters *et al.* 2013). The identification of candidate T3Es in the genomes of Rs-09-161 and Rs-10-244 was carried out using 'Scan Your Genome' (Peeters *et al.* 2013).

Analysis of the T3Es

The phylogenetic analysis based on the gene families of T3Es of *R. solanacearum* was studied. The gene families analysed include RipA (*AWR* family), RipG (*GALA* family), RipH (*HLK* family) and RipS (*SKWP* family). The coding sequences of effectors belonging to each gene family were arranged in concatenated manner and compared with other strains. Reference strains used to study the phylogenetic relation of T3E are indicated in table 1. Phylogenetic analysis was performed in MEGA ver. 6.0 (Tamura *et al.* 2013) by using neighbour-joining (NJ) and the algorithm of Jukes and Cantor (1969) with 1000 bootstrap resamplings.

Results and discussion

With the availability of genomic data through whole genome sequencing, it has become interesting to study *R. solanacearum* at the genomic level. We therefore have studied virulence associated genes of Indian isolates Rs-09-161 and Rs-10-244 and compared them with the isolates available globally. This involves identifying the virulence factors and T3Es present in Indian strains and analysing the coding sequences of these genes for generating similarity matrix and phylogenetic trees. The basic architecture of the strains Rs-09-161 and Rs-10-244 and the sizes of the two

replicons; the chromosome and the megaplasmid are similar to that of the previously sequenced strains (Salanoubat *et al*. 2002; Remenant *et al.* 2010). The details are provided in table 2 of electronic supplementary material.

Analysis of virulence factors

The virulence genes in the test strains were identified using GMI1000 (phylotype I) as standard reference strain. The details of virulence genes of *R. solanacearum* strain Rs-09-161 and Rs-10-244, their location in the genome and the probable function are provided in table 1 in electronic supplementary material. The nomenclature of virulence genes is with the prefix RALSO161 and RALSO244 for Rs-09-161 and Rs-10-244, respectively. A similarity matrix obtained by comparing the sequences of virulence genes of Rs-09-161 and Rs-10-244 with other reference strains is given in table 3 in electronic supplementary material. The sequences of Rs-09-161 and Rs-10-244 share more than 99% similarity between them and are closely related to GMI1000.

The main virulence factor of *R. solanacearum*, the EPS is secreted by seven genes, namely *epsA, epsB, epsC, epsD, epsF, epsP* and *epsR*. The gene *epsD* is absent in phylotype I strains and all the EPS contributing genes are located on the megaplasmid. The sequences of CMR15 (phylotype III) and Psi07 (phylotype IV) share more than 90% similarity with Rs-09-161 and Rs-10-244. Both Rs-09-161 and Rs-10-244, have the presence of all six PCWDE genes. The sequences of PCWDE genes of Rs-09-161 and Rs-10-244 share 99% similarity with that of GMI1000. *PehB* is the only gene present on the chromosome and this observation is consistent with the reports of other *R. solanacearum* strains. This shows that along with the major housekeeping genes, some of the essential virulence associated genes are also present on the chromosome (Genin and Denny 2012).

Motility associated genes in *R. solanacearum* help the bacterium to locate and invade the host root for colonization (Meng *et al.* 2011). The genes identified for chemotaxis (*CheA* and *CheW*) and swimming motility (*FliC* and *FlgM*) are located on the megaplasmid whereas twitching motility (*PilA* and *PilP*) are located on the chromosome. The mutants of swimming motility are highly reduced in the degree to cause virulence on tomato plants under natural conditions (Tans-Kersten *et al*. 2001) and of chemotaxis are completely nonchemotactic (Yao and Allen 2006). The swimming motility and chemotaxis associated genes *FliC*, *PilP* and *CheW* are found to be highly conserved among all phylotypes and share more than 95% similarity (table 3 in electronic supplementary material). These are probably the regions which are not evolving or are conserved across the phylotypes. Twitching motility is a trait associated with the type IV pili and plays an important role in autoaggregation and biofilm formation (Kang *et al.* 2002). The *pilP* gene of Rs-10-244 shares 100% similarity with GMI1000 whereas Rs-09-161 is 99% similar. The *PilA* gene shares 89% similarity within the two strains and 91% with GMI1000. *PilA* exhibits diversity in the sequence among the other phylotypes of RSSC and is probably the region which undergoes evolution and thus can be used for designing primers for the strain-wise differentiation. *PilA* has been used to study the genetic diversity in soil bacterium *Myxococcus xanthus* strains and has shown highest polymorphism in comparison to that of other genes used (Vos and Velicer 2006). Further, we observed that there are no major differences between Indian strains and reference strain in the virulence gene sequences except *pilA* gene.

Sequences of virulence associated genes in strains Rs-09-161 and Rs-10-244 are found to be more close to phylotypes III and IV strains. Similar results were also observed by Ramesh *et al.* (2014b), when *egl* and *hrp* gene sequences from phylotype I strains were analysed.

Analysis of T3Es

We have identified 72 T3Es in Rs-09-161 and 77 T3Es in Rs-10-244 (including one multiple copy T3E) based on the identification criteria (table 2). The identified T3Es are assigned the names with prefix Rip (Ralstonia injected protein) as per the newly proposed nomenclature by Peeters *et al.* (2013) and the locus tag of the T3Es is represented by the prefix 161_ and 244_ for Rs-09-161 and Rs-10-244, respectively. The T3E gene RipTPS is present in multiple copies in both the strains. Rs-09-161 has the presence of three candidate effectors (Rs_T3E_Hyp6, Rs_T3E_Hyp7 and Rs_T3E_Hyp15) and three pseudogenes (RipF1, RipAX1 and Rs_T3E_Hyp8). Rs-10-244 has the presence of one candidate effectors (Rs_T3E_Hyp7) and four pseudogenes (RipO1, RipAX2, Rs_T3E_Hyp8 and Rs_T3E_Hyp15). Pseudogenes are nonfunctional genes and its presence can be attributed to the fact that either these genes are been mutated due to the presence of transposable elements within the gene, leading to its disruption or due to errors in sequencing. Comparison of the functional T3Es genes of *R. solanacearum* strains Rs-09-161, Rs-10-244 and GMI1000 revealed 60 common T3Es within the three strains. Rs-09-161 has two unique T3Es (Rs_T3E_Hyp6 and Rs_T3E_Hyp15) and shares 63 common effectors with GMI1000 and 66 T3Es with Rs-10-244. Rs-10-244 bears four unique T3Es (RipC2, RipE2, RipP3 and RipBB) and shares 66 common effectors with GMI1000 (figure 1). Majority of the *R. solanacearum* strains have an average of 70–75 T3E, which is much larger than many other bacterial plant pathogens like *P. syringe* and *Xanthomonas* sp., where it is in the range of 30–40 (Zumaquero *et al*. 2010; Hajri *et al.* 2011). Hence, it is presumed that an ancestor of *R. solanacearum* probably possessed a large number of effectors since the majority

Table 2. Identification of T3Es in *R. solanacearum* strains Rs-09-161 and Rs-10-244.

	Reference LT*	Locus tag Rs-09-161	GC (%)	Length	% Blast	Locus tag Rs-10-244	GC (%)	Length	% Blast
RipA1	RSc2139	161_20690	68.36	3177	99	244_36120	68.32	3204	99
RipA2	RSp0099	161_34090	70.3	3378	99	244_00720	70.44	3384	99
RipA3	RSp0846	161_40770	71.96	3702	99	161_40780	71.96	3717	99
RipA4	RSp0847	161_40790	72.43	3990	99	244_07960	72.5	3990	99
RipA5	RSP1024	161_42360	69.51	2723	99	244_09460	69.43	3726	99
RipB	RSc0245	161_02320	69.25	1584	92	244_18450	69.1	1479	99
RipC1	RSp1239	161_44460	66.87	2832	99	244_11640	66.41	2745	99
RipC2	RCFBP_mp20032	Nil	0	0	0	244_24680	59.83	2490	81
RipD	RSp0304	161_35920	63.61	1932	99	244_02680	63.71	1932	99
RipE1	RSc3369	161_31680	67.84	1278	97	244_47820	68.38	1278	99
RipE2	RCFBP_mp10565	Nil	0	0	0	244_24320	57.64	909	88
RipF1	RSp1555	Pseudogene	0	0	0	244_08360	64.41	2181	99
RipF2	RCFBP_mp30453	Nil	0	0	0	Nil	0	0	0
RipG1	RSp0914	161_41320	65.7	1986	99	244_08450	65.71	1998	99
RipG2	RSp0672	161_39180	69.35	3129	98	244_06280	69.41	3129	99
RipG3	RSp0028	161_33870	68.28	1797	98	244_00270	68.06	1794	99
RipG4	RSc1800	161_17570	71.34	1389	99	244_33040	71.34	1389	99
RipG5	RSc1801	161_17580	69.88	1617	99	244_33050	70.19	1617	99
RipG6	RSc1356	161_13550	68.85	1875	88	244_29420	68.75	1866	88
RipG7	RSc1357	161_13560	64.97	1836	80	244_29430	66.89	1935	98
RipG8	CMR15v4_10224	Nil	0	0	0	Nil	0	0	0
RipH1	RSc1386	161_13830	69.3	2304	99	244_29700	69.32	2298	99
RipH2	RSp0215	161_35090	67.76	2274	98	244_01730	68.28	2229	98
RipH3	RSp0160	161_34560	67.54	2160	99	244_01190	67.68	2160	99
RipH4	RPSI07_mp0161	Nil	0	0	0	Nil	0	0	0
RipI	RSc0041	161_00420	65.5	1206	99	244_16460	64.73	1293	99
RipJ	RSc2132	161_41350	57.41	2712	78	244_36060	58.87	1296	99
RipK	RCFBP_mp10024	Nil	0	0	0	Nil	0	0	0
RipL	RSp0193	161_34900	69.03	4176	98	244_01530	69.06	4173	99
RipM	RSc1475	161_18160	71.5	1755	99	244_33610	71.5	1755	99
RipN	RSp1136	161_43460	66.1	1425	99	244_10610	66.03	1425	99
RipO1	RSp0323	161_36140	61.2	1539	98	Pseudogene	62.36	1047	99
RipO2	RALSY_mp30159	Nil	0	0	0	Nil	0	0	0
RipP1	RSp0826	Nil	0	0	0	244_24210	55.46	1107	100
RipP2	RSp0868	161_08570	59.71	1467	99	244_24510	59.71	1467	99
RipP3	RSY45_33690	Nil	0	0	0	244_46550	61.41	1161	99
RipQ	RSp1277	161_44830	68.85	1557	99	244_12020	69.04	1557	99
RipR	RSp1281	161_44870	70.6	5229	99	244_12060	70.66	5229	99
RipS1	RSc3401	161_32000	69.04	7056	98	Nil	0	0	0
RipS2	RSp1374	161_45440	70.18	7539	99	244_12410	69.99	7458	99
RipS3	RSp0930	161_41420	66.81	6876	99	244_08550	66.85	6876	99
RipS4	RSc1839	161_17940	70.78	7725	99	244_33400	70.78	7725	99
RipS5	RSp0296	161_35870	67.55	7017	99	244_02630	67.5	7017	99
RipS6	RSc2130	161_20590	66.62	2424	99	244_36050	66.35	2547	99
RipS7	RPSI07_mp1267	Nil	0	0	0	Nil	0	0	0

Table 2. (*contd*)

	Reference LT*	Locus tag Rs-09-161	GC (%)	Length	% Blast	Locus tag Rs-10-244	GC (%)	Length	% Blast
RipS8	RPSI07_1850	161_17980	68.77	9303	92	244_3343	68.89	9294	92
RipT	RSc3212	Nil	0	0	0	244_46320	56.62	966	99
RipU	RSp1212	161_44210	62.57	879	0	244_11370	61.84	891	100
RipV1	RSc1349	161_13470	67.95	2013	100	244_29350	68	2013	99
RipV2	RPSI07_1895	Nil	0	0	0	Nil	0	0	0
RipW	RSc2775	161_26420	63.61	1146	95	244_41770	63.59	1140	99
RipX	RSp0877	161_41090	66.76	996	95	244_08260	67.05	1023	98
RipY	RSc0257	161_02390	67.2	2790	99	244_18510	65.36	2844	72
RipZ	RSp1031	161_42440	69.22	4110	99	244_09550	69.12	4113	99
RipAA	RSPI07_2742	161_06050	62.56	804	87	244_22050	62.4	798	87
RipAB	RSp0876	161_41080	65.14	525	99	244_08250	64.76	525	99
RipAC	RSp0875	161_41070	63.59	2958	97	244_08240	62.92	2643	99
RipAD	RSp1601	161_47560	64.11	978	98	244_14660	64.21	978	99
RipAE	RSc0321	161_03080	66.4	1932	99	244_19150	67.02	1932	99
RipAF1	RSp0822	0161_4053	1020	846	95	244_07700	68.78	1041	97
RipAF2	RALSY_20037	Nil	0	0	0	Nil	0	0	0
RipAG	RSc0824	161_08850	59.59	297	98	244_24200	60.6	297	100
RipAH	RSc0895	Nil	0	0	0	244_24720	62.88	291	100
RipAI	RSp0838	161_40690	70.75	612	99	244_07860	71.07	612	99
RipAJ	RSc2101	161_20300	69.15	1005	99	244_35750	69.05	1005	99
RipAK	RSc2359	Nil	0	0	0	244_38200	65.72	2430	99
RipAL	RPSI07_mp0618	161_39680	70	1350	99	244_06790	70	1350	99
RipAM	RSc3272	161_30700	71.82	465	99	244_46830	71.18	465	99
RipAN	RSp0845	161_40760	67.96	4323	99	244_07940	67.82	4323	99
RipAO	RSp0879	161_41110	68.96	1479	97	244_08280	69.57	1479	98
RipAP	CMR15v4_10224	161_44240	67.32	2863	93	244_11400	67.49	2421	93
RipAQ	RSp0885	161_41170	69.6	2112	99	244_08340	69.5	2112	99
RipAR	RSp1236	161_44430	69.53	1815	96	244_11600	69.63	1818	98
RipAS	RSp1384	161_45560	71.86	2802	99	244_12510	71.73	2802	99
RipAT	RSp1388	161_45600	70.65	1755	99	244_12550	70.59	1755	99
RipAU	RSp1460	161_46300	67.19	939	99	244_13250	67.3	939	99
RipAV	RSp0732	161_39740	68.3	2538	99	244_06850	68.7	2538	99
RipAW	RSp1475	161_46450	66.29	1347	99	244_13400	66.44	1347	97
RipAX1	RSc3290	Pseudogene	0	0	0	244_47020	58.68	759	100
RipAX2	RSp0572	161_38220	57.99	657	99	Pseudogene	0	0	0
RipAY	RSp1022	161_42340	64.48	1236	97	244_09440	64.56	1236	97
RipAZ1	RSp1582	161_47400	61.03	834	99	244_14480	60.91	834	99
RipAZ2	RALSY_20407	Nil	0	0	0	Nil	0	0	0
RipBA	RSc0227	161_02140	54.71	594	99	244_18270	55.21	594	98
RipBB	RPSI07_mp0573	Nil	0	0	0	244_05100	61.43	1320	0
RipBC	RCFB_mp30170	Nil	0	0	0	Nil	0	0	0
RipBD	RALSY_20184	Nil	0	0	0	Nil	0	0	0
RipBE	RS1000_RIP10	Nil	0	0	0	Nil	0	0	0
RipBF	RPSI07_2863	Nil	0	0	0	Nil	0	0	0
RipBG	RSMK00763	Nil	0	0	0	Nil	0	0	0

Table 2. (*contd*)

	Reference LT*	Locus tag Rs-09-161	GC (%)	Length	% Blast	Locus tag Rs-10-244	GC (%)	Length	% Blast
RipBH	RPSI07_mp30113	Nil	0	0	0	Nil	0	0	0
RipBI	RCFB_mp30113	Nil	0	0	0	Nil	0	0	0
RipTAL	RSc1815	161_17720	66.04	3726	99	244_33180	66.05	3738	99
RipTPS	RSp0731	161_39730 161_43110	68.68	1785	99	244_06840 244_10270	68.57	1785	99
RS_T3E_Hyp1	RSPsi07_0331	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp2	RSPsi07_1883	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp3	RSPsi07_mp0834	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp4	RSPsi07_mp1047	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp5	RSPsi07_mp1559	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp6	CMR15v4_30001	161_32360	55.45	1311	88	Nil	0	0	0
RS_T3E_Hyp7	RSMK06225	161_32180	55.1	519	0	244_48280	55.1	519	0
RS_T3E_Hyp8	RSMK02655	Pseudogene	0	0	0	Pseudogene	0	0	0
RS_T3E_Hyp9	RRSL_01783	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp10	RSMK02638	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp11	RSMK01187	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp12	RSMK03335	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp13	RSPO_m01098	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp14	BDB mp_40006	Nil	0	0	0	Nil	0	0	0
RS_T3E_Hyp15	RSPsi07_1860	161_35100	52.73	2799	94	Pseudogene	0	0	0
RS_T3E_Hyp16	RSc3174	Nil	0	0	0	Nil	0	0	0

Locus tag of representative strains given in the above table: (RSc/RSp: GMI1000), (RCFBP: CFBP2957), (CMR15: CMR15), (RPSI07: Psi07), (RSMK: Molk2), (BDB: BDB R229), (RSPO: Po82), (RALSY: R24), (RSY45: Y45).

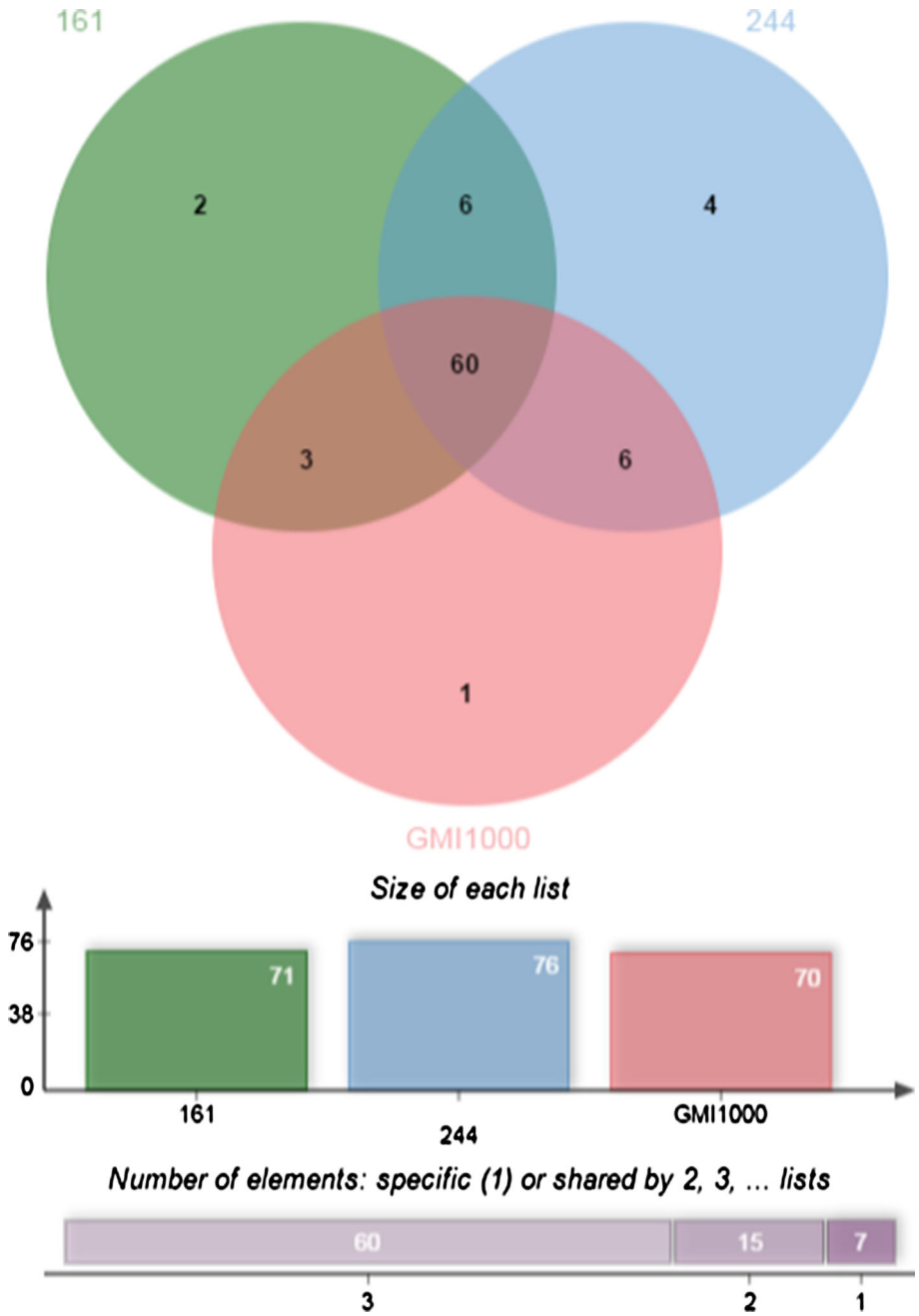

Figure 1. Venn diagram constructed using T3Es complete genes of *R. solanacearum* strain Rs-09-161(green), Rs-10-244 (blue) and GMI1000 (pink). The merged regions display the T3Es shared between the strains. In Rs-09-161 and Rs-10-244, one effector is present in multiple copies which is not indicated in the bar graph.

of the strains possess a high number of effectors. The only exception to this is BDB, which has less number of effectors (Genin and Denny 2012).

A majority of *R. solanacearum* T3Es have been validated through translocation studies (Cunnac *et al.* 2004; Mukaihara *et al.* 2004; Mukaihara and Tamura 2009; Mukaihara *et al.* 2010; Sole *et al.* 2012). Many of the effectors share homology with those of other bacterial plant pathogens like *P. syringae, Xanthomonas* sp. and *Acidovorax* sp. Few of these effectors are present as effector families and have three to eight effectors. These include RipA (*AWR* family), RipG (*GALA* family), RipH (*HLK* family) and the RipS (*SKWP* family) (Poueymiro and Genin 2009; Mukaihara *et al.* 2010; Remigi *et al.* 2011; Sole *et al.* 2012; Peeters *et al.* 2013). These effectors possess certain inherent characters about the sequence such as specific internal repeats within them, which characterizes them to constitute a family. The T3SS

secretes T3Es in a highly specialized manner in the eukaryotic hosts and is used by many plant and animal pathogenic bacteria, as a tool to colonize in their respective hosts. The various motifs or domains present on the T3Es interact with the host cells and benefits the bacteria in colonization. The T3Es are secreted into the cytosol of the host through the *hrp* pili. Unique T3E of Indian strains (Rs-09-161 and Rs-10-244) were analysed for functional/conserved motifs through homology search in NCBI revealed the presence of various motifs. The T3E RipBB present in Rs-10-244 exhibits presence of ankyrin repeats which mediate protein–protein interactions in diverse families of proteins. RipC2 shares homology with haloacid dehalogenase (HAD)-like hydrolases and RipP3 which is also known as PopP3 displays YopJ serine/threonine acetyltransferase activity. The T3E Rs_T3E_Hyp15 present in Rs-09161 displays presence of serine/threonine protein kinase domain within it.

RipA (*AWR* family) effectors include five effectors (RipA1 to RipA5), and both the strains, Rs-09-161 and Rs-10-244 have all the RipA effectors present in them. Among the RipA effectors, RipA1 is present only in phylotype I strains, whereas RipA2 along with RipA4 and RipA5 is present in all the phylotypes of *R. solanacearum* isolates studied till date. RipA5 is also present in multiple copies in some of the phylotype II strain (Molk2, IPO1609, UW551 and Po82). The RipA effectors consist of a conserved region containing the alanine–tryptophan–arginine tryad and can be virulent or avirulent depending on the host with which *R. solanacearum* interacts and RipA2 was found to be a major contributor to the virulence among the AWR family (Sole *et al.* 2012).

RipG (*GALA* family) possesses eight T3Es (RipG1–RipG8) and seven (RipG1–RipG7) are present in both Rs-09-161 and Rs-10-244. The RipG has the presence of leucine rich repeats (LRR) and F-box domain with them. The F-box protein forms a component of E3-ubiquitin ligase complex and is found in eukaryotes. This complex plays an important role in ubiquitination of proteins which leads to the degradation or modification of the activity of the targeted protein (Hua and Vierstra 2011). RipG8 is present only in CMR15 (phylotype III). More isolates from phylotype III needs to be studied to identify if the RipG is specific to phylotype III strains.

RipH (*HLK* family) consists of four effectors (RipH1–RipH4); Rs-09-161 and Rs-10-244 has the presence of RipH1, RipH2 and RipH3 with an average size of ~600 amino acids. RipH4 is found to be present only among phylotype IV strains. The RipH (*HLK* family) is named so because of the presence of histidine–leucine–lysine triad in a conserved C-terminal region. Phylogenetic analysis of the RipH effectors indicates an ancestral strain of *R. solanacearum* most likely had only three RipH effectors and the fourth one has evolved later independently (Chen *et al.* 2014).

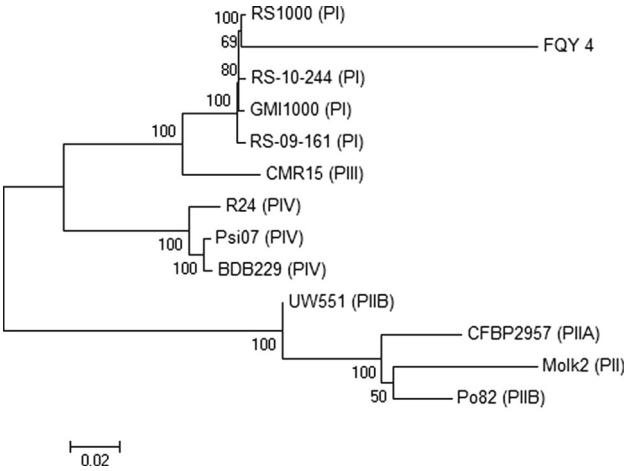

Figure 2. Phylogenetic tree constructed using NJ method based on the coding sequences of effectors of RipA (*AWR* family) of *R. solanacearum* isolates. The isolates are represented with their names followed by the phylotypes in parenthesis. The tree was generated by MEGA-6 (Tamura *et al.* 2013) software using the NJ and the algorithm of Jukes and Cantor (1969) with 1000 bootstrap resamplings. Numbers at each branch indicate bootstrap value. The scale indicates the genetic distance.

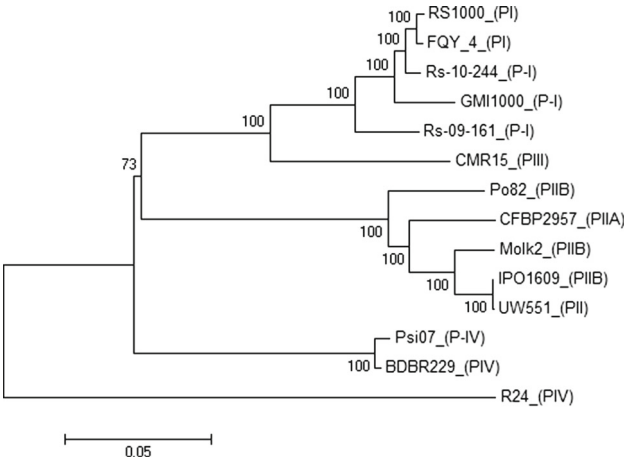

Figure 3. Phylogenetic tree constructed using NJ method based on the coding sequences of effectors of RipG (*GALA* family) of *R. solanacearum* isolates. The isolates are represented with their names followed by the phylotypes in parenthesis. The tree was generated by MEGA-6 (Tamura *et al.* 2013) software using the NJ and the algorithm of Jukes and Cantor (1969) with 1000 bootstrap resamplings. Numbers at each branch indicate bootstrap value. The scale indicates the genetic distance.

RipS (*SKWP* family) has eight effectors (RipS1–RipS8); RipS7 is absent in Rs-09-161 whereas RipS1 and RipS7 are absent in Rs-10-244. RipS7 is absent in all phylotype I strains studied till date and is present in all phylotype IV strains. RipS1 and RipS6 is absent in all phylotype IV isolates. Phylotype II lacks RipS6 and RipS8. The structure of RipS (*SKWP* family) effectors is found to be related to heat/armadillo repeat domain. The RipS proteins exert

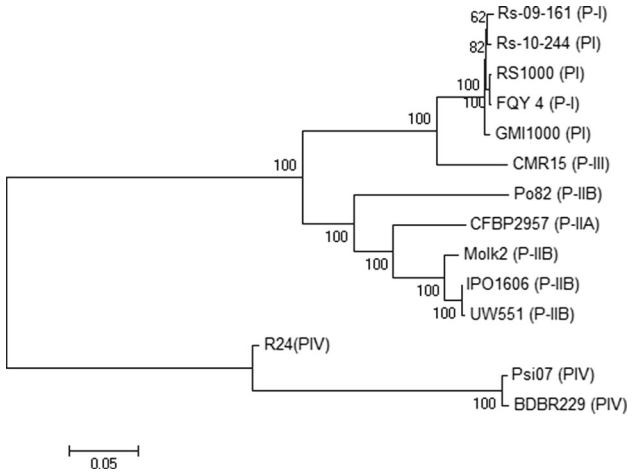

Figure 4. Phylogenetic tree constructed using NJ method based on the coding sequences of effectors of RipH (*HLK* family) of *R. solanacearum* isolates. The isolates are represented with their names followed by the phylotypes in parenthesis. The tree was generated by MEGA-6 (Tamura *et al*. 2013) software using the NJ and the algorithm of Jukes and Cantor (1969) with 1000 bootstrap resamplings. Numbers at each branch indicate bootstrap value. The scale indicates the genetic distance.

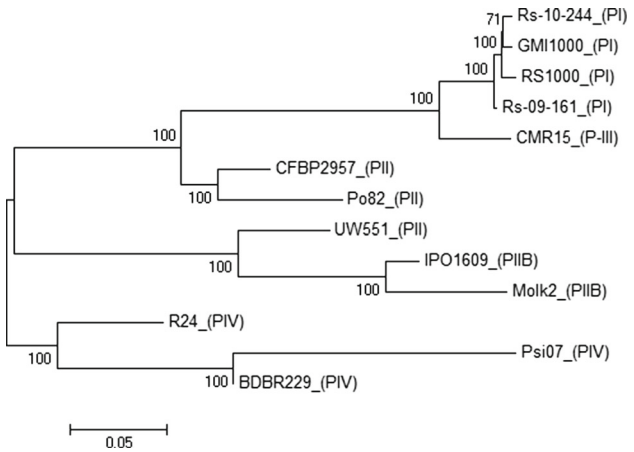

Figure 5. Phylogenetic tree constructed using NJ method based on the coding sequences of effectors of RipS (*SKWP* family) of *R. solanacearum* isolates. The isolates are represented with their names followed by the phylotypes in parenthesis. The tree was generated by MEGA-6 (Tamura *et al*. 2013) software using the NJ and the algorithm of Jukes and Cantor (1969) with 1000 bootstrap resamplings. Numbers at each branch indicate bootstrap value. The scale indicates the genetic distance.

their virulence on their host plant by interaction through the SKWP domain (Mukaihara and Tamura 2009).

Phylogenetic analysis of T3Es

The phylogenetic trees constructed for RipA, RipG, RipH and RipS effectors are depicted in figures 2–5. Effector gene sequences appear to be conserved and thus have revealed grouping of the isolates based on their phylotypes. Group 1 consists of strains that belong to phylotype I including Rs-09-161 and Rs-10-244. Phylotype III strain CMR15 forms a group closely associated with phylotype I. The strains belonging to phylotype II (IIA and IIB) and phylotype IV have separated to form two different groups. This grouping is consistent with the four gene families studied here. Similar results are also observed by Remenant *et al.* (2011) and Peeters *et al.* (2013), where isolates from phylotype I and III have clustered together. It is likely that the isolates from phylotype I and phylotype III did not undergo much evolution and hence form a major group (Remenant *et al.* 2011).

In conclusion, in this study, analysis of virulence genes and T3E genes of *R. solanacearum* strains Rs-09-161 and Rs-10-244 indicated that a majority of the virulence associated genes are present in both the strains. It was observed that all the virulence genes of Rs-09-161 and Rs-10-244 are highly conserved and share high level of similarity except for *pilA* gene, which shares a minimum of 72% similarity. Seventy-two T3E genes were identified in *R. solanacearum* strain Rs-09-161 and 77 in Rs-10-244. Phylogenetic analysis of T3E genes of RipA, RipG, RipH and RipS revealed close association between phylotype I and phylotype III strain of *R. solanacearum*.

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