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ORIGINAL RESEARCH ARTICLE

Development of T3SS Mutants (*hrpB*$^-$ and *hrcV*$^-$) of *Ralstonia solanacearum*, Evaluation of Virulence Attenuation in Brinjal and Tomato—A Pre-requisite to Validate T3Es of *R. solanacearum*

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Abstract *Ralstonia solanacearum* is an important plant pathogen which infects a large number of agriculturally important crops. The Type Three Secretion System (T3SS) plays a major role in its pathogenicity by secreting type III effectors (T3Es) which overthrow the host defence mechanism. The secretion of T3Es is transcriptionally regulated by *hrpB* and its secretion is dependent on the pili formed by *hrcV* gene. In this study, two T3SS mutants of *R. solanacearum* strain Rs-09-161 viz. Rs-HrpB$^-$ and Rs-HrcV$^-$ were developed through insertional mutagenesis. The method of development of insertional mutant is quite simple and reliable. The plasmid integrates through homologous recombination and in vitro studies have proved that the integration was stable for several generations. The mutants are non-pathogenic on its highly susceptible hosts, brinjal and tomato inoculated by soil drench method and by petiole inoculation directly into the vascular system. Further it was observed that the colonisation ability of the mutants was also highly reduced in the susceptible host. These mutants will be useful in validating putative T3E through translocation studies.

Keywords *Ralstonia solanacearum* · Bacterial wilt · Type three secretion system · Mutant

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Introduction

Ralstonia solanacearum is a soil borne phytopathogen which causes bacterial wilt in a large number of agriculturally important crops [1]. Phylogenetic studies of *R. solanacearum* strains reveals the presence of huge diversity among the strains, therefore this group of strains is now referred as *Ralstonia solanacearum* species complex (RSSC) [2]. This species complex is divided into four phylotypes which roughly reflect their area of origin: Phylotype I (Asia), Phylotype II (America), Phylotype III (Africa) and Phylotype IV (Indonesia) [2].

The type three secretion system (T3SS) was initially identified in animal and human pathogens, and is studied in various pathogens like *Pseudomonas aeruginosa, Brucella* spp., etc. [3, 4]. The study of type three secretion system (T3SS) is vital as it plays a crucial role in the pathogenicity of *R. solanacearum*. It is encoded by the *hrp* (hypersensitive response and pathogenicity) regulon and defects in the T3SS impair the induction of hypersensitive response in resistant hosts and pathogenicity in its susceptible hosts [5]. The T3SS injects pathogenicity proteins termed as Type III effectors (T3Es) into the eukaryotic hosts through the *hrp* pili [5]. These effectors are directly translocated into the cytosol of the hosts [6]. Once within the host cells, the effectors promote its colonisation by interacting with various host proteins and subverting the host immunity [7]. The T3SS and the expression of T3Es is controlled by an AraC family transcriptional regulator called as the *hrpB* [2, 8]. Whole genome sequencing of *R. solanacearum* strains have revealed the presence of large number of effectors distributed throughout the genome of the bacteria. *R. solanacearum* model strain GMI1000 possesses 72 T3Es [9]. Recently we have reported 72 and 77 T3Es in *R. solanacearum* strains Rs-09-161 and Rs-10-244 [10] Many

of the T3Es expressed by *R. solanacearum* are validated through translocation studies in GMI1000 and RS1000 with the help of T3SS mutants [5, 11, 12].

In India, bacterial wilt caused by *R. solanacearum* is widespread and severe incidence is well documented across various states of the country. *R. solanacearum* has been isolated from wilted plants of brinjal, tomato, potato, chilli, capsicum, cluster beans, elephant foot yam, water melon, banana, Jute, tobacco, ginger, cardamom, coleus, davana, marigold and sunflower [13–16]. The genetic diversity among the isolated strains is also analysed by REP PCR, ITS-PCR, PCR RFLP, MLST and sequencing of *egl, pga* and *hrp* gene [17, 18]. Though the genetic diversity of the pathogen is widely reported, the molecular aspects and the importance of the T3SS as the main pathogenicity determinant of Indian isolates are not reported. Majority of the work on T3SS is carried out using the strains, GMI1000 and RS1000. Present study aims at development of T3SS mutants of Indian strain of *R. solanacearum* and assessing the reduction in virulence of the mutants on its susceptible hosts. This will provide the basic information and strains/mutants to validate the putative T3Es of Indian strains of *R. solanacearum*.

Materials and Methods

Bacterial Strains and Plasmids

R. solanacearum strain Rs-09-161 maintained at the culture collection of Plant Pathology laboratory, ICAR-CCARI was used in this study to develop mutants. This strain is highly pathogenic on its susceptible hosts (brinjal and tomato), and its whole genome sequence is available [18]. The details about the plasmid used in this study and the clones developed are listed in Table 1. The bacterial strains, vectors and mutants were maintained as 30% glycerol stock at − 80 °C. *R. solanacearum* was routinely grown on BG medium (1% peptone, 0.1% casamino acids, 0.1% yeast extract, 0.5% glucose) at 28 °C. *E. coli* was grown at 37 °C in Luria and Bertani medium. Ampicillin was used in the concentration 50 µg/ml while growing *E. coli* and *R. solanacearum* mutants.

Development of Clones in *E. coli* Vector

The genes of the T3SS viz. *hrpB* and *hrcV* were selected in this study. *HrpB* is the transcriptional regulator of the T3SS and *hrcV* forms the transmembrane component of the *hrp* pili involved in the translocation of the T3Es into the host cells. Primers were designed for the amplification of internal fragments of the genes using Oligo primer analysis software, version 6.4. The sequences of the primers, reaction mixture and standardised PCR conditions are given in Table 2. Mastercycler Pro (Eppendorf, GmBH) was used for product amplification. The amplicons were visualised on 1.0% agarose gel containing 0.5 µg/ml of ethidium bromide and documented with MultiImage Light Cabinet (Alpha Innotech Corporation). The amplified products were purified using GeneJETTM PCR Purification Kit (Fermentas Life Sciences, EU) and quantified using Nano drop-1000, Thermo fisher scientific, USA. The purified fragments were cloned in the vector pTZ57R/T as per manufacturer's protocol (Fermentas TA cloning kit). The positive colonies were identified using blue-white selection. The selected white colonies were confirmed for the presence of insert by colony PCR using the same conditions as amplification of the internal fragment. The recombinant plasmid was isolated using QIAprep Spin Miniprep Kit and clone was confirmed by restriction digestion at 37 °C for 1 h.

Development of *R. solanacearum* Mutants

Overnight grown culture of *R. solanacearum* isolate Rs-09-161 was inoculated into BG medium to an initial OD_{600nm}

Table 1 List of plasmids and strains used in the study

Strains	Relevant genotypes or characteristics	Source/reference
E. coli JM107		
Ec-hrpB	hrpB::pTZ57R/T	This study
Ec-hrcV	hrcV::pTZ57R/T	This study
R. solanacearum		
Rs-09-161	WT	Ramesh et al. [18]
Rs-hrpB$^-$	hrpB::pTZ57R/T mutant	This study
Rs-hrcV$^-$	hrcV:zx:pTZ57R/T mutant	This study
Plasmid		
pTZ57R/T	Fermentas TA cloning vector	Fermentas TA clonning kit
hrpBpTZ57R/T	*HrpB* gene fragment cloned in the TA vector	This study
hrcVpTZ57R/T	*HrcV* gene fragment cloned in the TA vector	This study

Table 2 Details about the primers, PCR conditions and expected size of amplicon

Primer	Reaction mixture	PCR conditions	Size of amplicon
hrpB gene			
Forward: 5′-GCGAGGAAAGTCCGACGACTA-3′ Reverse: 5′-CGCAGCAGGCTGAGGTATTC-3′	20 µL reaction mixture contained 1.0 µM of each primer, 200 µM dNTPs, 1× assay buffer, 10% DMSO, 20 µg BSA, 0.5U Taq DNA polymerase and 50 ng of the DNA template	Initial denaturation of 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 30 s and final extension of 10 min at 72 °C	582 bp
Diagnostic: 5′-CACCAACGACCAGATGC-3′ M13 forward: 5′- GTAAAACGACGGCCAGT-3′	20 µL reaction mixture contained 0.5 µM of each primer, 200 µM dNTPs, 1× assay buffer, 3% DMSO, 20 µg BSA, 0.5U Taq DNA polymerase and 50 ng µl of the DNA template	Initial denaturation of 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 66 °C for 30 s, 72 °C for 1 min 30 s and final extension of 10 min at 72 °C	1628 bp
hrcV gene			
Forward: 5′- GCTGATGGTGCTGCCGCTGCC -3′ Reverse: 5′-GGACAGCTGCCGCACGATCTC -3′	20 µL reaction mixture contained 0.5 µM of each primer, 200 µM dNTPs, 1× assay buffer, 5% DMSO, 20 µg BSA, 0.5U Taq DNA polymerase and 50 ng of the DNA template	Initial denaturation of 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 68 °C for 1 min 15 s and final extension of 10 min at 72 °C	763 bp
Diagnostic: 5′- GGACGAAGTCAAGCGCGAGC -3′ M13 reverse: 5′- CAGGAAACAGCTATGAC-3′	20 µL reaction mixture contained 0.5 µM of each primer, 200 µM dNTPs, 1X assay buffer, 3% DMSO, 20 µg BSA, 0.5U Taq DNA polymerase and 50 ng of the DNA template	Initial denaturation of 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 64 °C for 30 s, 72 °C for 1 min 30 s and final extension of 10 min at 72 °C	1624 bp

of 0.15 and grown at 28 °C at 160 rpm. Actively growing cells were harvested at 0.5 OD_{600nm} and electro-competent cells were prepared under ice cold conditions using standard procedure with minor modifications as follows. A series of centrifugations were carried out at 8000 rpm for 10 min to reconstitute the active cells in equal volume of cold MilliQ water, half volume of MilliQ water, one-fourth volume of 10% glycerol and finally in 1/100th volume of 10% glycerol solution respectively. Electroporation was carried out using MicroPulser (Biorad) at 2 kV using 2 µg of plasmid for hrpBpTZ57R/T and hrcVpTZ57R/T respectively, plated on BG medium with ampicillin.

Confirmation of *R. solanacearum* Mutants

The Genomic DNA was extracted from the antibiotic resistant colonies by the method described by Ramesh et al. [16]. Confirmation of integration of the plasmid and the orientation of the insert at the genomic level was done using specifically designed diagnostic primers and M13 primers for *hrpB* and *hrcV* genes. Diagnostic primers were designed in the region 1000 bp upstream of the internal fragment of *hrpB* and *hrcV* genes. The sequences of the diagnostic primers and standardised PCR conditions are given in Table 2. The confirmed mutants were then maintained as 30% glycerol stock at −80 °C.

Stability Studies of the Rs-HrpB$^-$ and Rs-HrcV$^-$ Mutant

A single colony of Rs-hrpB$^-$ and Rs-hrcV$^-$ was inoculated in 10 ml of BG broth without ampicillin and maintained at room temperature on a shaker for 24 h. After 24 h, 1 ml of the culture aliquot was diluted to 0.1O.D.$_{600nm}$ and 10^{-6} dilution was plated on BG agar with and without ampicillin. The plates were incubated for 48 h and the number of colonies obtained on the two media was counted. Two replications were maintained for each dilution plated on each medium. Next, 100 µl of culture was inoculated from 24 h old day-one tube to a fresh 10 ml BG broth and incubated for 24 h, diluted to 0.1O.D.$_{600nm}$ and 10^{-6} dilution was plated on BG agar with and without ampicillin. The same cycle was repeated for 10 consecutive days and the stability of the plasmid was assessed by plating after every 24 h.

Inoculation of *R. solanacearum* Mutants on Brinjal and Tomato: Virulence Assay

Virulence of the mutants was assayed on brinjal and tomato by soil drench inoculation and petiole inoculation. Highly susceptible brinjal cultivar (*Agassaim*), tomato variety (Pusa Ruby) was used in the studies. Seedlings were raised and maintained in the greenhouse at 30 °C during the day

with 60% relative humidity, 16 h of light and 8 h of dark period. Thirty days old seedlings grown in the potting mixture containing red soil: FYM: sand (2:1:1) were used for inoculation studies. Wild type strain Rs-09-161 was used as a positive control and sterile water was used as a negative control in each of the experiments. In case of soil drench inoculation, 10 ml of inoculum of 0.1 OD_{600nm} (10^8 cfu/ml) prepared from overnight grown bacterial culture was drenched around the root zone [19]. In case of petiole inoculation, the seedlings were inoculated on the third leaf petiole from the shoot apex. The petiole of the seedlings was cut 2 cms away from the stem, 2000 cells of *R. solanacearum* were inoculated on the petiole of tomato and 500 cells on brinjal (standardized in the previous studies). The seedlings were observed for wilt symptoms for 21 days post inoculation.

Colonization of *R. solanacearum* Mutants in Brinjal

Colonisation of *R. solanacearum* wild type strain Rs-09-161 and mutants Rs-hrpB$^-$ and Rs-hrcV$^-$ was assessed in 30 days old highly susceptible brinjal cv. *Agassaim*. The seedlings were raised and maintained in the greenhouse at 30 °C during the day with relative humidity of 60% and a light: dark period of 16:8 h. Overnight grown *R. solanacearum* strains Rs-09-161, Rs-hrpB$^-$ and Rs-hrcV$^-$ were resuspended to 0.1 OD_{600nm} (10^8 cfu/ml) and 10 ml of this inoculum was drenched around the root zone. Three replications were maintained for each strain. Colonisation of *R. solanacearum* was studied on day 2, 4, 6, 8 and 10 after the inoculation in experiment 1 and on day 4 after the inoculation in experiment 2. The root of the inoculated plant was cut into fine pieces of 1–2 mm and re-suspended in 5 ml of sterile water. The tubes were maintained on shaker at 150 rpm for 1 h and later at 4 °C for 30 min under stationary condition. Appropriate dilutions were prepared and 20 µl was spot inoculated on BG agar plates containing antibiotic wherever required. The plates were incubated at room temperature for 14–16 h. Two replications were maintained per sample. *R. solanacearum* colonies were observed under Olympus CX41 microscope at 10X magnification and identified by their twitching motility (Fig. 1).

Result and Discussion

R. solanacearum Rs-09-161 is a highly virulent strain, isolated from the west coast of India [18]. It is pathogenic on agriculturally important crops like brinjal, tomato and causes 100% wilt in 10–15 days after inoculation in the greenhouse studies. Owing to its high pathogenicity and the availability of genome sequence [18], this strain was selected in this study. We aimed to develop T3SS mutants of *R. solanacearum* using a simple and reliable method, check the stability of the mutant in the absence of antibiotic selection and further investigate the virulence of the mutants on its susceptible hosts. The two genes studied here, *hrpB* and *hrcV* play an important role in the pathogenesis caused by the T3SS.

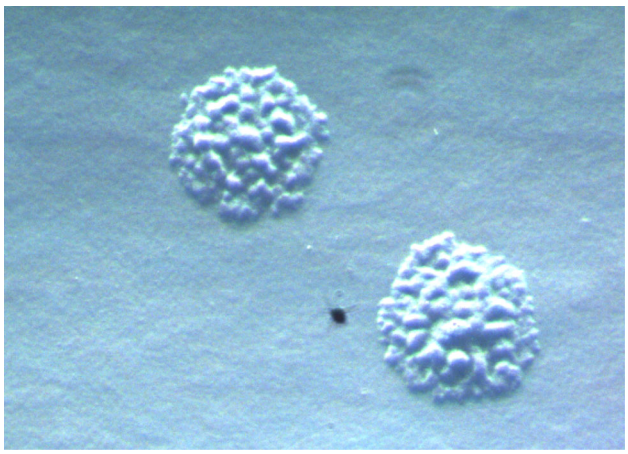

Fig. 1 Twitching motility displayed by the *R. solanacearum* strain Rs-09-161 observed at 10× magnification after 18–20 h. Each raft of motile cells forms an individual colony which can be easily visualised by naked eyes after 48 h

Development of Clones with Internal Fragment of *hrpB* and *HrcV* Gene in *E. coli* Vector

The amplified internal fragments of gene *hrpB* (582 bp) and *hrcV* (763 bp) were used in the concentration of 100 and 131 ng respectively for cloning in the vector pTZ57R/T. Positive clones were selected by blue and white selection. Amplification of internal fragment of *hrpB* gene is depicted in Fig. 2. The presence of *hrpB* and *hrcV* gene insert fragment in the recombined plasmid was confirmed with colony PCR of Ec-hrpB and Ec-hrcV which gave the characteristic amplification bands at 582 bp (Fig. 2) and 763 bp (Fig. 3) respectively. Restriction digestion of the isolated plasmid confirmed the orientation of the insert. The restriction profile of plasmid hrpBpTZ57R/T and plasmid hrcVpTZ57R/T is depicted in Figs. 2 and 3 respectively.

Development of *R. solanacearum* Mutants

Cloning of internal fragment of specific *R. solanacearum* gene in the vector pTZ57R/T generates a recombined plasmid that lacks the oriC for *R. solanacearum and* hence cannot replicate in the host. However, due to the sequence similarity shared by the cloned insert fragment and the target gene, the plasmid integrates itself into the target gene

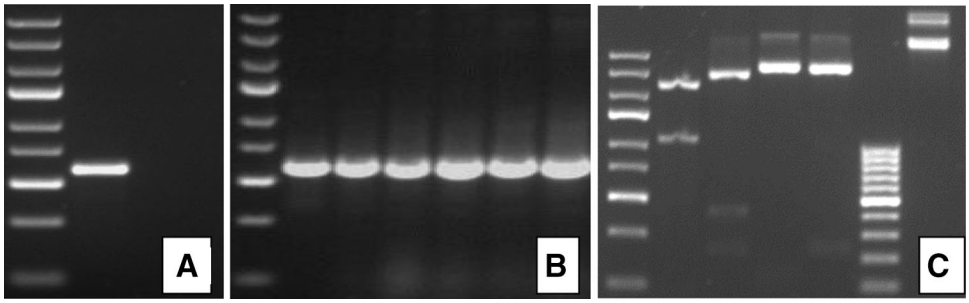

Fig. 2 Cloning of internal fragment of *HrpB* gene in *R. solanacearum* **a** amplification of internal fragment of *HrpB* gene. Lane 1: Ladder (100 bp–5 Kb, GeneRuler Express DNA Ladder), lane 2: 582 bp. **b** Colony PCR for the confirmation of *HrpB* insert in *E.coli* colonies. Lane 1: Ladder (100 bp–5 Kb, GeneRuler Express DNA Ladder), Lane 2–7: 582 bp. **c** Restriction digestion of plasmid hrpBpTZ57R/T which contains the internal fragment of *HrpB* gene cloned into it. Lane 1 contains the 100 bp–5 Kb ladder (GeneRuler Express DNA Ladder); lane 2: *Rsa* I digest with 3 bands at 2333, 1066 and 72 bp; lane 3: *Bam* HI/*Eco* RI double digest with 3 bands of 2847, 403 and 221 bp; lane 4: *Sac* I/*Not* I double digest with 2 bands of 3361 and 110 bp; lane 5: *Eco* RI digest with 2 bands at 3250 and 221 bp; lane 7: 100 bp–1 Kb ladder; lane 7: uncut plasmid

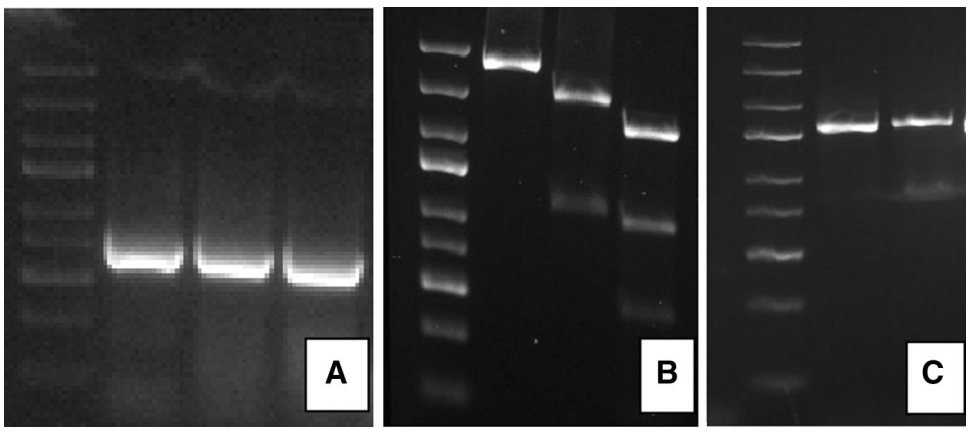

Fig. 3 Mutagenesis of *hrcV* gene in *R. solanacearum* Rs-09-161. **a** Colony PCR for the confirmation of *HrcV* insert in *E. coli* colonies. Lane 1: Ladder (100 bp–5 Kb, GeneRuler Express DNA Ladder), Lane 2–4: 763 bp. **b** Restriction digestion of plasmid hrcVpTZ57R/T which contains the internal fragment of *HrcV* gene cloned into it. Lane 1: 100 bp–5 Kb (GeneRuler Express DNA Ladder) ladder; lane 2: uncut plasmid hrcVpTZ57R/T; lane 3: *Bam* HI/*Eco* RI double digest with 2 bands of 2847, 802 bp; lane 4: *Rsa* I digest with 4 bands at 1987, 1066, 483 and 113 bp. **c** Confirmation of insertional mutagenesis in *R. solanacearum* Rs-09-161. Lane 1: 100 bp–5 kb (GeneRuler Express DNA Ladder) ladder; lane 2: 1628 bp obtained by disruption of *hrpB* gene in Rs-hrpB$^-$; lane 3: 1624 bp obtained by disruption of *hrcV* gene in Rs-hrcV$^-$

by homologous recombination leading to the disruption of the cloned gene. The insert fragment is flanked by M13 primers nucleotide sequence on the either side. The diagnostic primer designed 1000 bp upstream of the gene located in the genome pairs with either of the M13 primers based on the orientation of the plasmid and amplifies the region directing the disruption of the gene at the desired site. Rs-hprB$^-$ was confirmed by the presence of 1628 bps band with M13 forward primer and Rs-hrcV$^-$ was confirmed by 1624 bps band with M13 reverse primer. Amplification of *hrpB* and *hrcV* disrupted gene is depicted in Fig. 3c. This is a simple, time saving and cost efficient method and can be easily used for the development of insertional mutants for a large number of genes.

HrpB is the transcriptional regulator which controls the expression of T3SS and the T3Es [20] and *hrcV* is a transmembrane protein that forms a component of hrp Pili, the needle like apparatus which is involved in translocation of the T3Es into the host cells. The *hrpB* mutant of *R. solanacearum* will fail to express the *hrpB*-dependent T3Es whereas the mutant of *hrcV* gene will fail to secrete the T3Es in the external environment. These mutants will form a base material for validating the secretion of novel T3Es by translocation studies [21]. These factors make them excellent choice of gene for development of T3SS mutant.

Stability of Mutants

The mutants Rs-hrpB$^-$ and Rs-hrcV$^-$ were found to be stable in the absence of antibiotic for a period of 10 days. There was no significant difference in the population of mutants on BG medium with antibiotic and without

Table 3 Population of *R. solanacearum* mutant Rs-hrpB$^-$ and Rs-hrcV$^-$ in the absence of antibiotic selection to study the stability

Days	Rs-hrpB$^-$(log CFU/ml)		Rs-hrcV$^-$(log CFU/ml)	
	BG	BG with ampicillin	BG	BG with ampicillin
1	10.51	10.04	10.58	10.63
2	10.15	9.79	10.48	10.52
3	10.31	9.78	10.45	10.41
4	10.52	10.47	10.50	10.18
5	10.63	10.10	10.43	10.25
6	10.43	9.92	10.34	10.07
7	10.82	10.45	9.80	9.65
8	10.76	10.10	9.70	9.80
9	10.95	10.39	10.01	9.71
10	10.72	10.15	9.90	9.54

antibiotic in each day. Population of Rs-hrpB$^-$ was log 10.72 cfu/ml on BG medium without ampicillin and log 10.15 cfu/ml on BG medium with ampicillin after 10 days. The population of Rs-hrcV$^-$ was log 9.9 cfu/ml on BG medium without ampicillin and log 9.5 cfu/ml on BG with ampicillin after 10 days (Table 3). This indicates the plasmid was stably integrated into the genome and would not lose in the plant where the antibiotic selection is absent.

Virulence of *R. solanacearum* Mutants on Brinjal and Tomato

T3SS in *R. solanacearum* is the main pathogenicity determinant and the mutants of the T3SS are unable to cause the disease [21, 22]. The wild type strain of *R. solanacearum,* Rs-09-161 caused 100% wilt in brinjal seedlings with drench inoculation on day 13 whereas 95% wilt was observed with cut petiole inoculated seedlings on day 8 (Figs. 4, 5, 6). In case of tomato, 100% wilt was

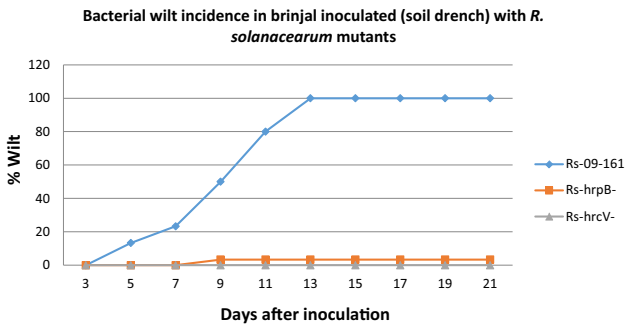

Fig. 4 Virulence assay in brinjal inoculated by soil drench with wild type *R. solanacearum* Rs-09-161 and T3SS mutants Rs-hrpB$^-$ and Rs-hrcV$^-$. Wilt % is mean of 2 experiments conducted separately with 2 and 3 replications in experiment 1 and experiment 2 respectively. Each replication had 5 plants. The plants were observed for 21 days post inoculation

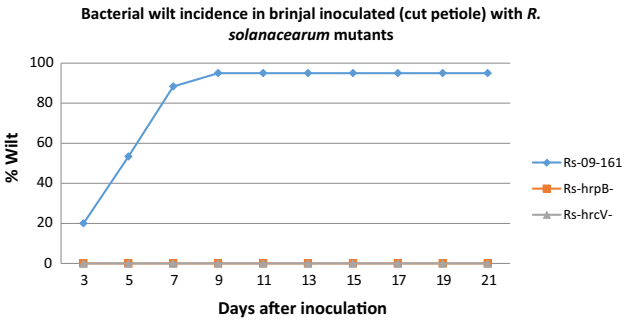

Fig. 5 Virulence assay in brinjal inoculated on cut petiole with wild type *R. solanacearum* Rs-09-161 and T3SS mutants Rs-hrpB$^-$ and Rs-hrcV$^-$. Wilt % is a mean of 2 experiments conducted separately with 2 and 3 replications in experiment 1 and experiment 2 respectively. Each replication had 5 plants. The plants were observed for 21 days post inoculation

observed in 10–11 days when seedlings were inoculated by soil drench or cut petiole (Figs. 6, 7, 8).

Our results are in agreement with this observation as we did not observe any wilt in the brinjal and tomato seedlings inoculated with the mutant Rs-hrcV$^-$ around the root through drench inoculations which follows the conditions of natural infection process as well as in the vascular system through petiole inoculation. In case of Rs-hrpB$^-$ inoculated seedlings, the petiole inoculated seedlings of brinjal and tomato as well as drench inoculated seedlings of tomato did not show any wilt. However, the brinjal seedlings inoculated by drench inoculation resulted in 3.33% wilt which might be an exception as the wilt was observed in only one out of 15 inoculated seedlings in the second experiment.

The expression of the T3SS was considered very crucial during the early stages of infection to suppress the host immunity and gets turned down in the higher cell densities. However, the recent studies using *in planta* transcriptome have proved that the T3SS plays an important role throughout the disease cycle and is not repressed during high cell densities [20, 23]. This can be the probable reason for the reduction in wilt by the Rs-hrpB$^-$ and R-hrcV$^-$ mutants in tomato and brinjal seedlings inoculated through drench and petiole inoculation.

Colonization of *R. solanacearum* mutants in Brinjal

We observed that, the colonization ability of the mutants was highly reduced in brinjal seedlings as compared to the wild type strain even though the inoculum concentration of the three strains was uniform. In the plant colonization experiment, experiment 1 was conducted to standardize the optimum day for studying the colonization of wild type and mutants of *R. solanacearum*. Day four was considered as optimum day for colonization studies as the wild type

Fig. 6 *In planta* assays using *R. solanacearum* strains Rs-09-161, Rs-hrpB^{-} and Rs-hrcV^{-}. **a** 100% wilt caused by wild type Rs-09-161 on its susceptible hosts brinjal cultivar *Agassaim* and tomato variety *Pusa ruby*. **b** Tomato seedlings (*Pusa ruby*) inoculated on the petiole with 2000 cells of *R. solanacearum* strains Rs-hrpB^{-}, Rs-hrcV^{-} and Rs-09-161; Rs-09-161 inoculated plants show complete wilt whereas Rs-hrpB^{-} inoculated do not display any wilt symptom. **c** Brinjal cultivar *Agassaim* inoculated with 10 ml inoculum of 0.1 OD_{600nm} of *R. solanacearum* strains Rs-hrpB^{-}, Rs-hrcV^{-} and Rs-09-161 by drench inoculation; Rs-09-161 displays wilt symptoms whereas no wilt is observed with plants inoculated with Rs-hrpB^{-} and Rs-hrcV^{-}

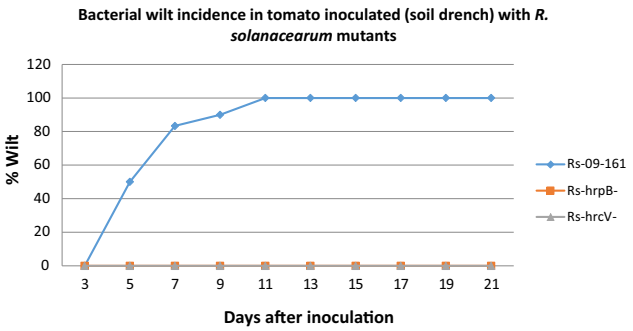

Fig. 7 Virulence assay in tomato inoculated by soil drench with wild type *R. solanacearum* Rs-09-161 and T3SS mutants Rs-hrpB^{-} and Rs-hrcV^{-}. Wilt % is mean of 2 experiments conducted separately with 3 replications each. Each replication had 5 plants. The plants were observed for 21 days post inoculation

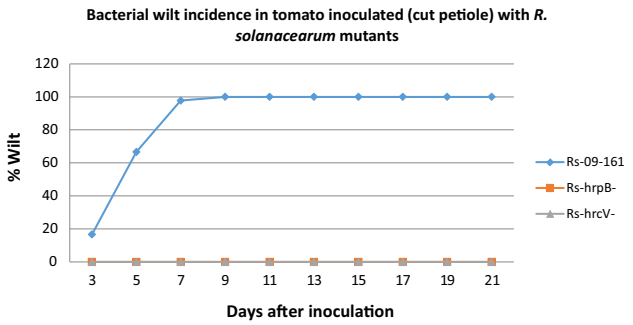

Fig. 8 Virulence assay in tomato inoculated on cut petiole with wild type *R. solanacearum* Rs-09-161 and T3SS mutants Rs-hrpB^{-} and Rs-hrcV^{-}. Wilt % is mean of 3 experiments conducted separately with 2 replications in experiment 1 and 3 replications in experiment 2 and 3 respectively. Each replication had 5 plants. The plants were observed for 21 days post inoculation

showed an average population of log 11 cfu/g and Rs-hrpB^{-} also showed its highest population. The population of wild type Rs-09-161 in the roots of the susceptible brinjal was log 6 cfu/g on day two which gradually increased to log 14 cfu/g till day 6 and in the stem was 4 cfu/g on day two and increased to 16 cfu/g on day eight in experiment 1. On day 10, the wild type inoculated plants had wilted completely so the colonisation was not assessed for them. Rs-hrpB^{-} did not colonise in the susceptible plants and the population was log 1.2 cfu/g on day two, log 3.3 cfu/g on day four and log 1.5 cfu/g on day 10 in the roots and log 1.2 cfu/g on day 10 in the stem respectively in experiment 1. After day four, the population of Rs-hrpB^{-} disappeared which might be due to the reduced virulence mechanism of the Rs-hrpB^{-} mutant against the host. The reduction in colonization was also observed in In-planta assays on tomato, brinjal and *Arabidopsis thaliana* with wild type and hrp^{-} mutant of *R. solanacearum* [21].

In experiment two we observed that, the mutants, Rs-hrpB^{-} and Rs-hrcV^{-} failed to colonise in the plants and their population was less than 10^{8} cfu which is correlated as threshold population for initiating wilt in the susceptible tomato [24] whereas the wild type attained the population of log 10 cfu/g (Table 4). Similar results were also obtained by others wherein the *hrpB* and *hrcV* mutants are weakly invasive, poorly infective and exhibit limited multiplication in the intercellular spaces of the root cortex and vascular system of the inoculated tomato plants and are discussed in Genin and Denny [2].

This is the first report on the development of mutants of the T3SS Indian strains of *R. solanacearum* and the mutants were developed through a simple method. This is an easy, cost effective and reliable method that develops a stable insertional mutant through homologous recombination in the genome of the *R. solanacearum* cells. It is understood from the available literature that the Rs-hrpB^{-} mutant is defective in expression or production of T3Es within the cell and the Rs-hrcV^{-} mutant is defective in the secretion of the produced T3Es in the external environment through the type three secretion pili. These mutants can be used in future to study the putative T3Es and validate their secretion through translocation assays.

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Table 4 Plant colonisation by *R. solanacearum* strains Rs-09-161, Rs-hrpB$^-$ and Rs-hrcV$^-$

Strain	Sample	Population of *R. solanacearum* (Log)					
		Expt 1					Expt 2
		Day 2	Day 4	Day 6	Day 8	Day 10	Day 4
Rs-09-161	Root	6.00	11.25	14.27	ND	ND	10.18
	Stem	4.11	8.89	14.29	16.58	ND	ND
Rs-hrpB$^-$	Root	1.21	3.38	0	0	1.50	5.74
	Stem	0	0	0	0	1.28	ND
Rs-hrcV$^-$	Root	ND	ND	ND	ND	ND	5.65
	Stem	ND	ND	ND	ND	ND	ND

The data obtained is a mean of 3 samples with two replications

ND Not done

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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