Phytopathology Research

RESEARCH Open Access

Ralstonia solanacearum virulence in eggplant seedlings by the leaf-clip inoculation

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Abstract

Ralstonia solanacearum causes a lethal bacterial wilt disease in numerous plants including important vegetable crops such as eggplant and tomato. One of the difficulties in studying virulence of this bacterium in different host plants is the development of an easy and stable pathogenicity assay. Recently we described a leaf-clip inoculation method to study its pathogenicity at the cotyledon stage of tomato seedlings. Hereafter, we demonstrated the leaf-clip inoculation method to be equally efficient for studying *R. solanacearum* pathogenicity in the cotyledon stage of eggplant seedlings. Our study revealed eggplant seedlings to be highly susceptible to *R. solanacearum* as compared to tomato seedlings, illustrated by appearance of disease symptoms in significantly higher number of seedlings. We also tested the virulence of several global transcription regulator mutants of *R. solanacearum* including *hrpB*, *hrpG* and *phcA* in eggplant seedlings. The *phcA* mutant was found to be only moderately virulence deficient in eggplant seedlings but was significantly reduced in virulence in tomato. This is indicative of some host specific responses towards certain pathogenicity functions of *R. solanacearum*, which are markedly different in tomato and eggplant seedlings. Apart from being economical in requiring less labor, time and space, this simple gnotobiotic leaf-clip inoculation method is anticipated to be helpful in further exploring the interaction between *R. solanacearum* and eggplant seedlings at the cotyledon stage.

Keywords: Bacterial wilt, Virulence, Pathogenicity assay, Leaf-clip inoculation, Eggplant

Background

Ralstonia solanacearum causes a lethal bacterial wilt disease in 200 plant species of 53 botanical families including agronomically important crop plants such as tomato, potato, eggplant, olive, banana, peanut, ginger, etc. (Hayward 1991). *R. solanacearum* is a soil borne bacterium. Under natural conditions, this pathogen infects the host plants through root, colonizes in the xylem vessels and then spreads systemically till causing wilting in its hosts (Genin 2010). Tomato and *Arabidopsis* plants are mainly used as model hosts to describe its pathogenicity functions at the molecular level (Vasse et al. 1995; Yang and Ho 1998). The pathogen uses an elaborate sensory and regulatory network to regulate its virulence and pathogenicity functions (Schell 2000; Mole et al. 2007).

Though, the pathogen causes the wilt disease in different hosts, its aggressiveness is not identical (Genin 2010; Genin and Denny 2012). There are plants, referred to as distant hosts, where *R. solanacearum* colonizes but fails to cause any disease symptom (Guidot et al. 2014). Its host range is expanding further with recent findings (Coutinho et al. 2000; Ozaki and Watabe 2009; Jiang et al. 2016; Weibel et al. 2016). Its differential virulence behavior in varied hosts still remains poorly understood. Recent studies of experimental evolution in this bacterium have given crucial insight into the role of transcription regulators in its host adaptation and colonization (Marchetti et al. 2010; Guidot et al. 2014).

Bacterial wilt in eggplant is a common disease in tropical and subtropical regions (Shekhawat et al. 1978; Ramesh 2006; Antony et al. 2015; Sakthivel et al. 2016;

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Singh et al. 2017). Studies regarding *R. solanacearum* pathogenicity in eggplant have mainly focused on identifying resistant cultivars against bacterial wilt and understanding the resistance mechanism of eggplant against the pathogen (Artal et al. 2012; Gopalakrishnan et al. 2014; Pensec et al. 2015; Salgon et al. 2017). The well-known soil drenching and stem inoculation methods are used to study *R. solanacearum* pathogenicity in eggplant (Salgon et al. 2017; Tjou-Tam-Sin et al. 2017). In these approaches, four to five weeks old (3–5 leaves stage) eggplants are inoculated with the pathogen and the inoculated plants remain under surveillance for about 1 month till completion of the pathogenicity assay (Lebeau et al. 2013; Cho et al. 2018). These pathogenicity assays are quite labor intensive and time consuming. In addition, requirement of sufficient space for incubation of large number of inoculated plants in experiments such as screening for resistant cultivars and screening of *R. solanacearum* mutants poses limitation to these protocols. The other complexity with *R. solanacearum* infection of soil-grown host plants is the colonization of unwanted bacteria from the soil in plant root and xylem, which may interfere with *R. solanacearum* pathogenicity assays in the host plants.

Though eggplant is an important vegetable crop, and bacterial wilt is a serious disease of it, there has not been any report describing the virulence functions of *R. solanacearum* in this host plant. There may be several reasons why eggplant has not been used as a model host for this pathogen. As eggplant is closely related to tomato, it might have been thought that the pathogen behavior towards eggplant will be similar to that of tomato. The other possible reason may be that the pathogenicity assays used till date are more consistent and reproducible in tomato than in eggplant. In this present study we have demonstrated that the leaf-clip inoculation method is an easy and stable method for evaluating the virulence of *R. solancearum* in eggplant seedlings, which was ever successfully used to test *R. solanacearum* virulence in tomato seedlings (Kumar et al. 2017). This is an easy and stable method of inoculation in which the juvenile seedlings are maintained in 1.5 or 2.0 mL microfuge tubes. The seedlings are then inoculated with the pathogen by clipping only a part of the cotyledon leaves with a pair of scissors dipped in bacterial suspension. This method of inoculation takes care of the limitations encountered with the inoculation of the soil-grown plants. In this study we have demonstrated that *R. solanacearum* is more aggressive in eggplant seedlings than in tomato seedlings by the leaf-clip inoculation. Eggplant being a different host, as well as from economical viewpoint, understanding eggplant and *R. solanacearum* interaction is of significant importance. In addition, pathogenicity study in different hosts would enable researchers to explore novel host specific pathogenicity functions as well as host specific responses towards pathogenicity functions.

Results

R. solanacearum F1C1 causes wilt disease at the cotyledon stage in eggplant seedlings inoculated by the leaf-clip method

The *R. solanacearum* F1C1 pathogenicity assay at the cotyledon stage in eggplant seedlings (14–15 days old; Devgiri cultivar) was executed by the leaf-clip inoculation method (Fig. 1). From 2 days post inoculation (dpi) onwards, some of the inoculated seedlings started to exhibit disease symptoms. Out of the 40 seedlings inoculated, more than 90% seedlings were dead on 5 dpi and by 7 dpi all the inoculated seedlings were dead due to the disease (Additional file 1: Figure S1). Similar magnitude of F1C1 pathogenicity (Additional file 1: Figure S2) could also be observed in seedlings of the other two eggplant cultivars (viz. DevKiran, Param Hybrid). A consistent 100% death of the inoculated eggplant seedlings was recorded during each infection set-up. It was interesting for us because, in the case of tomato seedlings, some of the inoculated seedlings (~ 10%) did not exhibit the disease symptom (Kumar et al. 2017). This indicated that the eggplant seedlings may be more susceptible to *R. solanacearum* infection than the tomato seedlings. No disease symptoms till 10 dpi could be observed when the eggplant seedlings were inoculated with non-pathogenic bacteria such as *E. coli* and *P. putida* (Additional file 1: Figure S1). The virulence of *R. solanacearum* in eggplant seedlings was studied upon inoculating the seedlings with different titers of the pathogen (10^9 to 10^3 CFU/mL). Similar pathogenicity magnitudes and disease progressions were observed for 10^9 and 10^7 CFU/mL. At a bacterial concentration of 10^5 CFU/mL, number of seedlings died on 8 dpi was close to the number of seedlings died on 5 dpi for 10^7 CFU/mL. At a titer of 10^4 CFU/mL, number of seedlings died was significantly lesser (~ 32%) and for 10^3 CFU/mL only a few seedlings were dead (< 10%). The general observation was that the number of dead seedlings decreased as bacterial titers in the inoculum went below 10^7 CFU/mL, which might be proportional to the number of bacteria deposited initially at the inoculated site (Additional file 1: Figure S3).

Colonization of F1C1 in the inoculated eggplant seedlings was demonstrated by the GUS staining of the infected seedlings as performed previously in tomato seedlings (Kumar et al. 2017). GUS staining was observed along the shoot region of the seedlings suggesting that growth and migration of the bacteria occurred towards the root region from the inoculated site in the cotyledon leaves along with the shoot (Fig. 2). We further confirmed

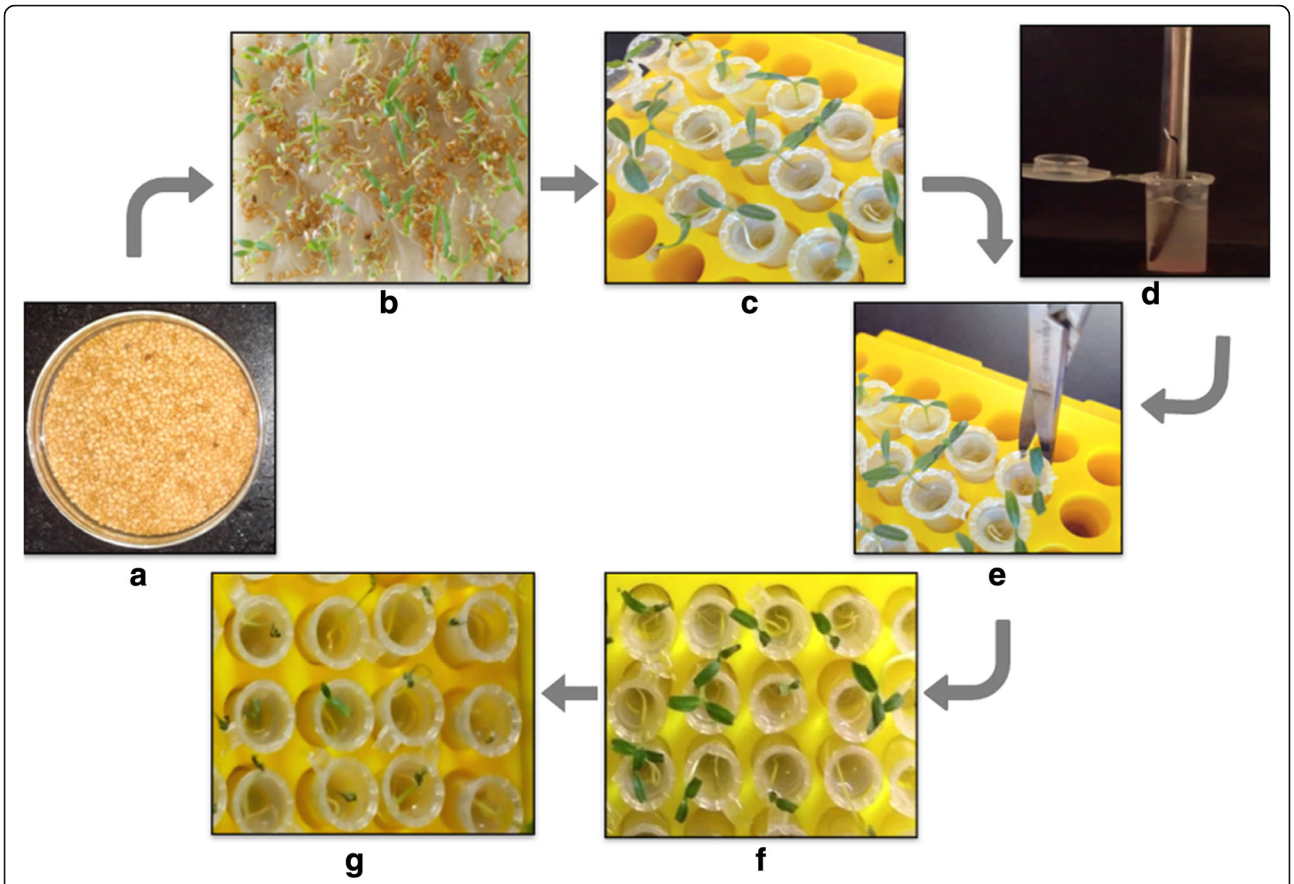

Fig. 1 Picture depicting different steps of the pathogenicity assay of *R. solanacearum* performed in eggplant seedlings by the leaf-clip inoculation method. **a** Surface sterilization of eggplant seeds with 70% ethanol followed by washing twice with sterile distilled water. **b** Germination of the seeds in a wet cotton and tissue paper bed in a growth chamber maintained at 28 °C and 75% relative humidity up to 14–15 days. **c** Transfer of seedlings to sterile 1.5 mL microfuge tubes containing sterile water. **d**, **e** Dipping a pair of scissors in bacterial inoculum and clipping off a portion of both the cotyledon leaves. **f** Transfer of inoculated seedlings into growth chamber (28 °C, 75% RH and 12 h photoperiod) and observation of disease symptoms. **g** Infected seedlings after 10 dpi

the bacterial colonization in the eggplant seedlings using mCherry marked wild type F1C1 (Fig. 3).

The leaf-clip inoculation method is efficient to study the pathogenicity functions of *R. solanacearum* in eggplant seedlings

HrpB, HrpG and PhcA are well established global transcription regulators of many pathogenicity determinants in *R. solanacearum* (Genin et al. 2005; Valls et al. 2006). We inoculated eggplant seedlings with *hrpB*, *hrpG* or *phcA* mutants of F1C1. The *hrpB* mutant was found to be non-pathogenic in eggplant seedlings like that in tomato seedlings. The *hrpG* mutant caused wilt only in ~ 5% whereas the *phcA* mutant inflicted ~ 80% wilting in the inoculated eggplant seedlings (Fig. 4). This suggested that the leaf-clip inoculation method in eggplant seedlings was able to demonstrate marked variation among the three virulence deficient mutants. The low virulence of *hrpG* as well as the moderate virulence phenotype of the *phcA* mutant in eggplant seedlings was surprising (Fig. 4) because *hrpG* mutant was non-pathogenic and *phcA* mutant showed highly reduced virulence on the tomato seedlings by the same leaf inoculation procedure (Kumar et al. 2017). The peculiar behaviour of *phcA* mutant in eggplant seedlings is difficult to ascertain at this moment. However, this further indicated that eggplant seedlings were more susceptible to *R. solanacearum* F1C1 than tomato seedlings. The Kaplan-Meier survival curve has been plotted to indicate survival probabilities with respect to DPIs (Additional file 1: Figure S4). We further studied differential colonization of mCherry tagged *hrpB* and *phcA* mutants of *R. solanacearum* F1C1 strains in eggplant seedlings (Fig. 3). The migration and colonization of *phcA* mutant was observed throughout the seedlings while colonization of the *hrpB* mutant was observed to be largely restricted to the inoculated leaf area. Interestingly, *hrpB* mutant migration in the seedlings could be restored upon co-inoculation with the wild type bacterial strain (Additional file 1: Figure S5).

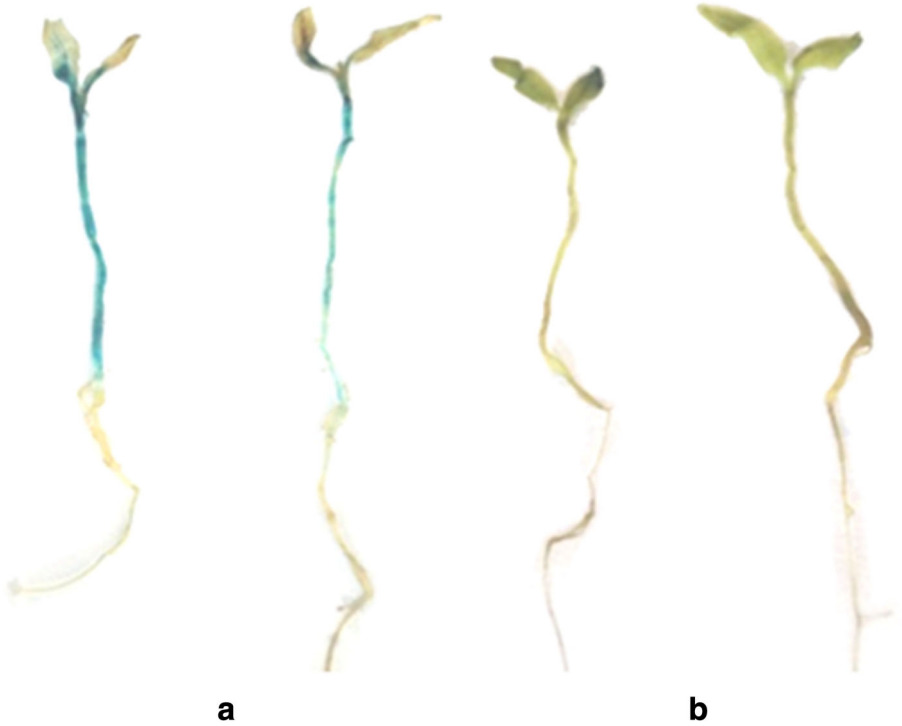

Fig. 2 GUS staining of infected eggplant seedlings after 4 dpi by the leaf-clip method inoculated with *R. solanacearum* TRS1002. Eggplant seedlings were inoculated with the *gus* tagged *R. solanacearum* F1C1 (TRS1002). Seedlings died at 4 dpi were examined by GUS staining. **a** Blue staining in the dead infected seedlings suggested the bacterial colonization. **b** GUS staining of control seedlings, without TRS1002 inoculation, exhibited no blue color

Eggplant seedlings are more susceptible to *R. solanacearum* F1C1 infection than tomato seedlings

We compared pathogenicity of *R. solanacearum* between tomato and eggplant seedlings by using *hrpB*, *hrpG* and *phcA* virulence deficient mutants. For a close comparison between these two hosts, their seedlings were kept in a single microfuge tube and inoculated with different *R. solanacearum* strains including the F1C1 wild type, the derived *hrpB, hrpG* and *phcA* mutants, respectively. We observed faster disease progression in the eggplant seedlings inoculated with F1C1 or the *phcA* mutants than that in the tomato seedlings inoculated with the same strain (Fig. 5). The *hrpB* mutant was found to be non-pathogenic while *hrpG* was highly reduced for pathogenicity in both hosts (Fig. 5 and Fig. 6). Whereas ~ 15% eggplant seedlings, and ~ 10% tomato seedlings were found dead due to *hrpG* inoculation. The Kaplan-Meier survival curve as well as statistical significance was presented (Additional file 1: Figure S6). In the earlier study (Kumar et al. 2017), we had reported *hrpG* mutant as non-pathogenic alike *hrpB* in tomato seedlings by the leaf-clip inoculation. The *hrpG* mutant used in this study is an insertion mutant of *R. solanacearum* F1C1 strain while the *hrpG* mutant studied in Kumar et al. (2017) was a GMI1000 derived deletion mutant. Either the strain difference and/or the way the mutation in *hrpG* created might be responsible for the differential virulence phenotypes of the *hrpG* mutants.

To further ascertain that the eggplant seedlings were more susceptible than tomato seedlings to *R. solanacearum* infection, we inoculated the seedlings with lower concentrations of the pathogen (10^4 and 10^5 CFU/mL), respectively. In both the concentrations, F1C1 caused higher death in eggplant seedlings than that of tomato seedlings. The number of dead eggplant seedlings inoculated with a bacterial concentration of 10^4 CFU/mL was more than that of tomato seedlings inoculated with 10^5 CFU/mL of the pathogen (Additional file 1: Figure S7).

Discussion

In this work we have demonstrated that the leaf-clip inoculation is a stable and consistent method to study *R. solanacearum* pathogenicity in the cotyledon stage of eggplant seedlings. With this inoculation method, several global transcription regulator mutants involved in its pathogenicity viz. *hrpG*, *hrpB* and *phcA* could be differentiated from each other as well as from the wild type strain in regard to their pathogenicity functions in eggplant seedlings. As of now, this is the first study of these *R. solanacearum* pathogenicity functions in eggplant.

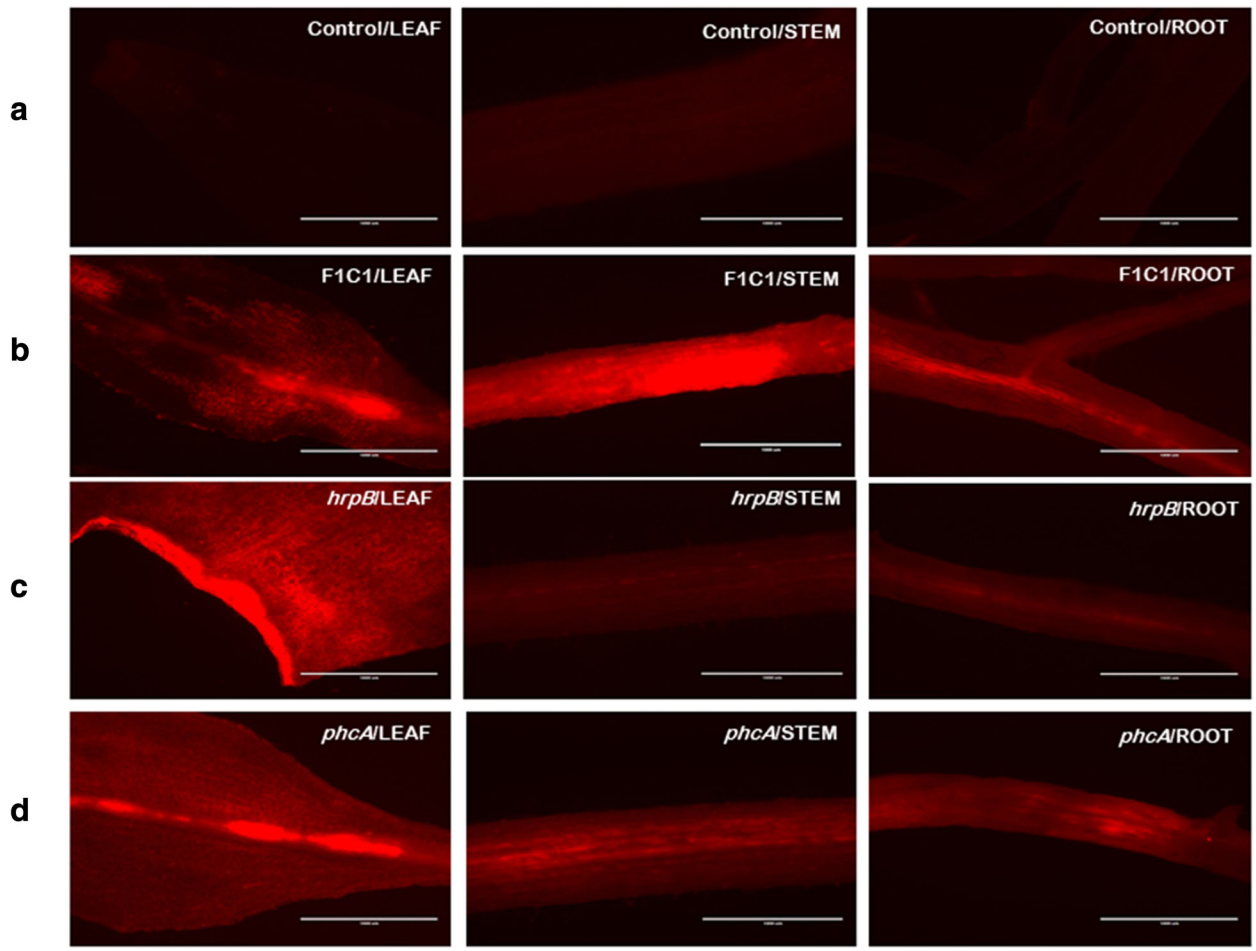

Fig. 3 Fluorescence microscopy of eggplant seedlings after 4 dpi by the leaf-clip method inoculated with **a** Water, **b** mCherry tagged *R. solanacearum* (TRS1016), **c** *hrpB* mutant (TRS1017) and **d** *phcA* mutant (TRS1018). F1C1 *hrpB* mutant was tagged with mCherry (TRS1017) and the *phcA* mutant was tagged with mCherry (TRS1018). TRS1016 (F1C1 wild type tagged with mCherry), TRS1017 and TRS1018 inoculated eggplant seedlings were observed under the fluorescence microscope at 4 dpi. In the case of TRS1016, fluorescence was observed in leaf, stem as well as in root regions, whereas in the case of TRS1017 fluorescence was largely limited to the region in the leaf where inoculation was done and was very faint in stem and root regions, which indicated that the *hrpB* mutant is growth deficient inside the seedling. In the case of TRS1018, fluorescence was observed in leaf, stem and root like TRS1016, which indicated that *phcA* mutant is able to grow and spread better than *hrpB* mutant in eggplant seedlings

It is pertinent to note that *R. solanacearum* pathogenicity in host plants is not so simple. Sometimes the bacterium colonizes its hosts without causing wilting symptom (Van der Linden et al. 2013; Guidot et al. 2014; Zuluaga et al. 2015). Therefore, a stable and consistent pathogenicity assay effective in any particular host is an important requirement for host-pathogen interaction study. The limitation may be a reason for the use of only a few model host plants for this pathogen out of a large number of hosts. Though the leaf-clip method is already established in tomato seedlings (Kumar et al. 2017), its applicability in eggplant seedlings is important for studying *R. solanacearum* pathogenicity functions in this plant. Even though tomato and eggplant are phyllogenetically close, there is significant difference in the germination processes of their seeds: germination of eggplant seeds takes more time than that of tomato seeds (Methods). We found eggplant seedlings to be more susceptible to *R. solanacearum* infection in comparison to tomato seedlings in terms of the duration of disease progression, the number of seedlings killed after inoculation with the pathogen as well as the with-host growth rate of the pathogen. The leaf-clip inoculation method described here is easy, simple and consistent. In future, it may allow a large scale screening of eggplant specific virulence deficient mutants of *R. solanacearum*. It may also be helpful for researchers interested in screening large number of disease resistant eggplant cultivars. The cotyledon leaves are short lived in seedlings unlike true leaves. Therefore, this mode of inoculation has the limitation to be recruited only in the cotyledon stage and can't be done in grown-up stage

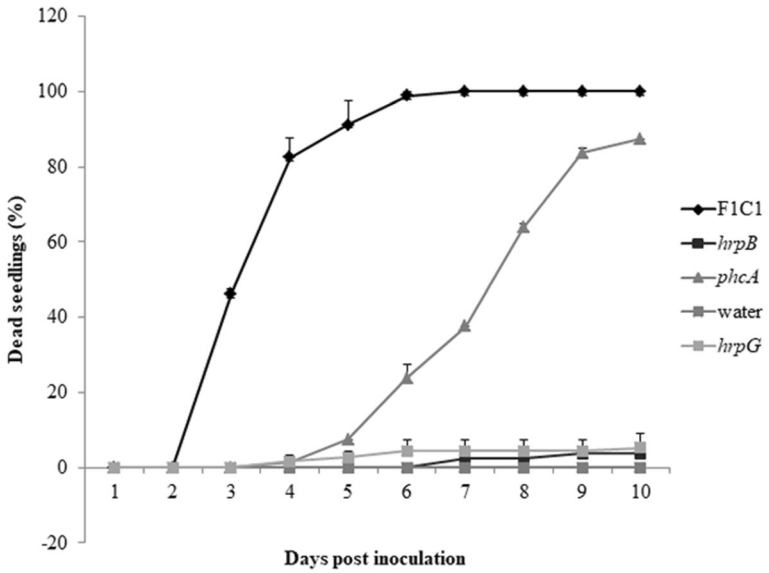

Fig. 4 Virulence of *R. solanacearum* F1C1 wild type, *hrpB, hrpG* and *phcA* mutants in eggplant seedlings tested by the leaf-clip inoculation method. Eggplant seedlings were inoculated by the F1C1 wild type, *hrpB, hrpG* or *phcA* mutants with saturated concentrations (10^9 CFU/mL). X-axis represents dpi and Y-axis represents the percentage of seedlings killed. On 6 dpi, 100% of seedlings were killed upon inoculation with F1C1 wild type, in the case of the *phcA*, till 4 dpi, almost all the seedlings were healthy, but by 9 dpi, more than 80% seedlings were killed. For *hrpB* mutant, by 10 dpi only 3% seedlings exhibited disease symptoms. We observed 5% seedlings death by 10 dpi for *hrpG* mutant. This suggested that *phcA* mutant is virulence deficient compared to wild type ($P < 0.001$; log-rank test) while *hrpB* and *hrpG* mutants are almost non-pathogenic. All data point in the line graph is an average of three independent experiments with two replicates. Error bars are depicting standard errors

of eggplant seedlings. Inoculation by clipping the true leaves will be interesting for a future study.

This study revealed non-pathogenic behavior of the *hrpB* mutant both in eggplant and tomato seedlings. This is in concordance with our earlier findings in tomato seedlings using the same leaf-clip inoculation procedure (Kumar et al. 2017). The type III protein secretion system, whose expression is positively regulated by the HrpB, is fundamental to *R. solanacearum* pathogenicity, and is therefore important for the pathogenicity in eggplant seedlings too. The *hrpG* mutant was observed to cause disease in a few seedlings of tomato as well as in eggplant. This result is different from the earlier study in tomato seedlings (Kumar et al. 2017), where the *hrpG* mutant was non-pathogenic like *hrpB*. In *R. solanacearum* GMI1000, *hrpG* positively regulates the expression of *hrpB*, as well as several other important virulence functions (Valls et al. 2006). So, it can be predicted that *hrpG* mutant would be less pathogenic than *hrpB* mutant. This was indeed found to be true in a recent study that described differential impact of *hrpB* and *hrpG* mutants on root growth of *Arabidopsis thaliana* (Lu et al. 2018). In this regard the disease caused by *hrpG* mutant in a few seedlings of tomato and eggplant in this study seemed intriguing. It need mention that GMI1000 possesses a homologue of *hrpG*, known as *prhG* which regulates the expression of *hrpB* under special circumstances as well as in the absence of *hrpG* (Plener et al. 2010). The genome sequence of F1C1 strain also revealed presence of a *prhG* homologue (unpublished result). It may be possible that *prhG* homologue contributes differently in the expression of genes in the F1C1 strain even when *hrpG* is defective. In addition, the *hrpG* mutant used in this study was observed to elicit a delayed hypersensitive response in tobacco leaves (Additional file 1: Figure S9). Therefore perplexing virulence phenotype of *hrpG* mutant may be attributed to different aspects such as the strain background, inoculation mode, and type of mutation or any other unknown factors. In future, the transcriptomics of the *hrpG* mutant will be of significant interest.

Further, difference between eggplant and tomato seedlings with regard to *R. solanacearum* infection was prominent in the case of *phcA* mutant. The *phcA* mutant exhibited virulence deficiency in tomato seedlings observed in this study is in concordance with earlier results (Kumar et al. 2017). But, for the eggplant seedlings, the *phcA* mutant was observed to be only moderately virulence deficient. PhcA is a known global transcription regulator in *R. solanacearum* and has been described as the largest regulon of the pathogen, which is involved in the regulation of unusually a large number of genes (~ 30% genes in the genome) including important pathogenicity determinants such as exopolysaccharides, extracellular enzymes, motility and type III secretion system (Perrier et al. 2018). *In planta* gene expression

Fig. 5 A representative picture showing differential pathogenicity of *R. solanacearum* between eggplant and tomato seedlings by recruiting their seedlings in a single microfuge tube. Both eggplant (marked with red star) and tomato seedlings (marked with red circle) were kept in one microfuge tube and then inoculated with **a** water **b** F1C1 wild type **c** *phcA* mutant **d** *hrpB* mutant and **e** *hrpG* mutant. Observations were made on each day to score disease in eggplant and tomato seedlings. The picture was taken at 4 dpi. It was distinct that eggplant seedlings were killed earlier than the tomato seedlings in the tube inoculated with F1C1 wild type strain. For inoculation with *phcA* mutant, eggplant seedlings developed disease while tomato seedlings were healthy in the same tube. For *hrpB* and *hrpG* mutants, both eggplant and tomato seedlings were healthy like the water inoculated seedlings. For *hrpG*, few seedlings in both hosts died towards the later dpi. The differential aggression of *R. solanacearum* in tomato and eggplant seedlings by leaf-clip inoculation was very distinct

study in tomato has revealed PhcA as an important regulator for the strategic switch between attachment/spread and growth/virulence in this pathogen (Khokhani et al. 2017). Therefore, its differential virulence behavior in the two hosts indicates that factors associated with PhcA may contribute differently towards the pathogen adaptation inside different hosts. Differential expression of *R. solanacearum* virulence functions in laboratory and in plant environments is already known (Jacobs et al. 2012; Khokhani et al. 2017; Lowe-Power et al. 2018; Mori et al. 2018; Perrier et al. 2018). The disparity in virulence due to *phcA* amidst tomato and eggplant seedlings further demonstrates relevance of leaf-clip inoculation procedure for pathogenicity study in eggplant seedlings. In future, *in planta* gene expression studies in this pathogen with regard to eggplant and tomato seedlings may draw out mechanism of differential virulence of the *phcA* mutant between the two hosts.

Unlike the leaf-clip inoculation method, disease occurrence in the eggplant seedlings by a recently described root inoculation method (Singh et al. 2018) was observed to be inconsistent and time consuming (Unpublished data). But in those seedlings the leaf-clip inoculation method was efficient to study *R. solanacearum* pathogenicity (Unpublished data). We believe that optimization of the root inoculation method will be required in future for efficient pathogenicity study. As root inoculation is a natural mode of infection, therefore inoculation by this mode might necessitate a greater physiological tuning between the host and the pathogen for infection to occur (Singh et al. 2018). It is already reported that root entry mechanism of the pathogen is complex (Tran et al. 2016; Lu et al. 2018). However, in the case of the leaf-clip inoculation, the pathogen is directly deposited at the cut end of the leaf and disease symptom appeared in the inoculated leaves soon after

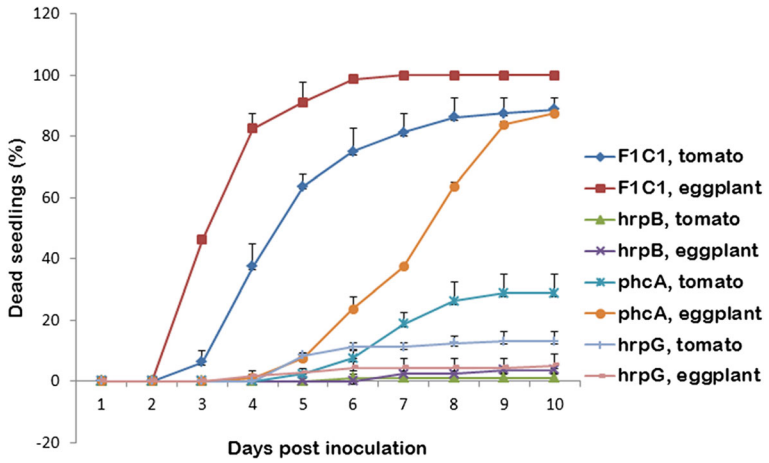

Fig. 6 *R. solanacearum* F1C1 and *phcA* mutant are more aggressive in eggplant than in tomato seedlings inoculated by the leaf-clip method. Tomato and eggplant seedlings in a single microfuge tube were inoculated with F1C1, *hrpB, hrpG* or *phcA* mutants of F1C1 to study *R. solanacearum* aggressiveness in these two host seedlings. Wild type F1C1 exhibited more disease aggressiveness in eggplant seedlings in comparison to tomato ($P < 0.001$; log-rank test). Similarly, for *phcA* mutant inoculation, disease aggressiveness was more in eggplant after 6 dpi than in tomato seedlings ($P < 0.001$; log-rank test). Interestingly, we found more disease aggressiveness in tomato seedlings than in eggplant upon inoculation with *hrpG* mutant. However, *hrpB* was found to be non-pathogenic both in eggplant and tomato seedlings. All data point in the line graph is an average of three independent experiments with two replicates. Error bars are depicting standard errors. Different colors were used to mark the virulence progression of different strains in tomato and eggplant seedlings

pathogenic colonization and growth. Although, it is known that the natural mode of entry of this pathogen is through root regions of its host, looking at the severity of symptoms developed in the cotyledon stage of seedlings by leaf-clip inoculation, entry of *R. solanacearum* into its host through damaged epiphytic regions such as leaves or by other means in natural environments, can't be eliminated. We anticipate this study would open new windows of investigations towards issues related to host specific pathogenic functions and responses in the immediate future.

Conclusions

Here in this work, we are reporting for the first time about susceptibility of the cotyledon stage (~ 14 days old) eggplant seedlings towards bacterial wilt pathogen *R. solanacearum* under gnotobiotic condition. The pathogenicity test conducted via leaf-clip inoculation procedure has indicated it to be an efficient method to study *R. solanacearum* virulence functions in eggplant seedlings too, as was shown for tomato seedlings earlier (Kumar et al. 2017). Our findings further demonstrate higher susceptibility of eggplant seedlings towards *R. solanacearum* (F1C1) virulence than tomato seedlings, when the pathogen was inoculated by the same leaf-clip method. We believe that the efficacy of the *R. solanacearum* leaf-clip inoculation mode in tomato and eggplant seedlings is expected to provide fertile ground for its potential utility in the pathogenicity tests of other hosts in near future. The important virulence regulator, *phcA* (so far the known largest regulon of *R. solanacearum*) that controls plethora of pathogenicity functions downstream seems to have distinct roles in tomato and eggplant seedlings. We anticipate, present study will stir more critical investigations on *R. solanacearum* virulence functions in association with eggplant which would immensely assist in understanding *R. solanacearum* host specific virulence behavior, in coming days.

Methods

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 1. Wild type *R. solanacearum* F1C1 (Kumar 2014), F1C1 derived mutant strains and *Pseudomonas putida* were grown in BG medium (Boucher et al. 1985) (1.0% peptone, 0.1% yeast extract, 0.1% casamino acid, 1.5% agar) supplemented with 0.5% glucose and incubated at 28 °C. *Escherichia coli* was grown in 2% LB medium (Bertani 1951) at 37 °C. Concentrations of antibiotics used were as follows: ampicillin (Amp; 50 μg/mL), spectinomycin (Spc; 50 μg/mL), gentamycin (Gen; 50 μg/mL) and rifampicin (Rif; 50 μg/mL). All chemicals used were bought from Himedia (Mumbai, India).

Germination of eggplant seedlings

Eggplant seeds of respective varieties (viz. Devgiri, Devkiran, Param Hybrid) recruited in this study were surface sterilized with 70% ethanol by submerging for 2 min, followed by washing twice with sterile distilled water, then kept on sterile wet tissue paper and incubated for germination inside a growth chamber (Orbitek, Scigenics, India) maintained at 28 °C, 75% relative humidity

Table 1 Bacterial strains used in this study

Strain	Characteristics	Reference
Ralstonia solanacearum strains		
F1C1	Wild type virulent *R. solanacearum* strain (Phylotype I), isolated from wilted chili plant collected from a nearby field of Tezpur University, Tezpur, India.	Kumar 2014
TRS1002	*rif-1zxx::Tn5gusA11;* Gus + ve, Rif^r, Spc^r, Vir^+, derived after *Tn5gusA11* insertion in an unknown locus in the genome	Kumar 2014
TRS1012	*hrpB::Ω;* Spc^r, HrpB deficient, Vir^-, hypersensitive response deficient (HR^-), derived from F1C1	Singh et al. 2018
TRS1013	*phcA::Ω;* Spc^r, PhcA deficient, exopolysaccharide deficient (EPS^-), hypermotile, derived from F1C1	Singh et al. 2018
TRS1016	Gen^r, mCherry tagged F1C1	Singh et al. 2018
TRS1017	*hrpB::Ω;* Spc^r, Gen^r, HrpB deficient, Vir^-, HR^-, derived from TRS1016	This study
TRS1018	*phcA::Ω;* Spc^r, Gen^r, PhcA deficient, EPS^-, hypermotile, derived from TRS1016	This study
TRS1027	*hrpG::pCZ367;* Amp^r, Gen^r, HrpG deficient, Vir^-, HR^-, derived from F1C1	This study
Other bacterial strains		
MG1655	Wild type *E. coli*	Lab collection
Pseudomonas putida	Isolated from tomato seedling	Lab collection

(RH) with a 12 h photoperiod. The tissue paper bed was kept wet by adding sterile water every day. Germination of eggplant seeds took more than 2 weeks (14–15 days) to reach two leaves cotyledon stage. In the case of tomato, it took only 1 week (6–7 days) (Durga; Ruby variety) to reach two leaves cotyledon stage (Kumar et al. 2017). In this study we referred to the germinated seedlings with only cotyledon leaves (without true leaves) as cotyledon stage seedlings.

Preparation of bacterial inoculum

For inoculum preparation, freshly grown *R. solanacearum* (F1C1) colonies were transfered to 10 mL BG broth and incubated in a shaking incubator (Orbitek, Scigenics, India) at 28 °C, 150 rpm for 24 h. Cultures were resuspended in sterile distilled water to obtain a bacterial concentration of 10^9 CFU/mL after centrifugation at 4000 rpm for 10 min at 28 °C (5804R; Eppendorf, Germany). Inoculum of *P. putida* and *E. coli* were prepared in a similar way except the growth temperature for *E. coli* was 37 °C.

Pathogenicity assay by leaf-clip method

Pathogenicity assay in eggplant seedlings was done by the leaf-clip method as described previously for tomato seedlings (Kumar et al. 2017). The leaf-clip inoculation method used to study *R. solanacearum* pathogenicity in tomato seedlings in our earlier work and here in the eggplant seedlings, was inspired from the work of Kauffman et al. (1973), who studied pathogenicity of *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial leaf blight in rice, in leaves of grown-up host plant. Briefly, 14–15 days old cotyledon stage eggplant seedlings were gently transferred from the germination tray to 1.5 mL microfuge tubes containing 1.0 mL of sterile distilled water (Fig. 1). Then a pair of sterile scissors were dipped in the bacterial suspension (~ 10^9 CFU/mL or other concentrations required) and ~one-third of both the cotyledon leaves from the tips were clipped off in each eggplant seedling, and 6-7 days old tomato seedlings at cotyledon stage were recruited for inoculation in the same way.

In all the pathogenicity experiments, 40 seedlings were inoculated in a set and each experiment was performed three times independently with two replicates. Seedlings inoculated with sterile distilled water were kept as control in all experiments. Inoculated seedlings along with control were transferred to a growth chamber (Orbitek, Scigenics, India) maintained at 28 °C, 75% RH under 12 h photoperiod and observed for disease progression next day onwards till 10 dpi. Statistical analysis of virulence data were done by Kaplan-Meier survival curve (Kaplan and Meier 1958) and log-rank test.

Eggplant seedlings of three different cultivars namely Devkiran (Bangalore), Param Hybrid (Hyderabad) and Devgiri (Kolkata) were tested for susceptibility to *R. solanacearum* F1C1 by the leaf-clip inoculation..

Leaf-clip inoculation of non-pathogenic bacteria such as *P. putida* and *E. coli* in eggplant seedlings was done also as described above.

Inoculation of eggplant and tomato seedlings within a single microfuge tube

One of the difficulties in *R. solanacearum* pathogenicity test is to make close comparison between infected susceptible host plants. A close comparison of *R. solanacearum* F1C1 pathogenicity between eggplant and tomato seedlings was made by keeping the two seedlings in a single microfuge tube. The seedlings were then inoculated with F1C1 and the mutants including *hrpB, hrpG* and *phcA,*

respectively at concentrations ~ 10^9 CFU/mL by the leaf-clip method. The disease progression was recorded till 10 dpi.

Inoculation of eggplant seedlings with different concentration of *R. solanacearum*

To determine the effect of different titers of the pathogen on disease progression, the eggplant seedlings were inoculated with different titers of *R. solanacearum* F1C1 (~ 10^9, 10^7, 10^5, 10^4 and 10^3 CFU/mL). A set of 40 seedlings were used for each dilution inoculation and the experiment was repeated three times independently with two replicates. Inoculated seedlings were analyzed for disease progression till 10 dpi.

Creation of mCherry tagged *hrpB* and *phcA* mutant strains of *R. solanacearum* F1C1 and colonization study in eggplant and tomato seedlings

The transformation protocol used in *R. solanacearum* F1C1 was the same as described previously (Singh et al. 2018). To create mCherry marked *hrpB* and *phc*A mutant of F1C1, genomic DNA of TRS1012 and TRS1013 was used to naturally transform mCherry-marked F1C1, respectively (TRS1016). Both types of transformants were selected on BG agar medium supplemented with gentamycin and spectinomycin. The *hrpB* mutant was found to be deficient in eliciting hypersensitive response when infiltrated inside the leaves of the *Nicotiana tabaccum* (Additional file 1: Figure S8).

Inoculum of mCherry labelled *hrpB* mutant (TRS1017) and *phc*A mutant (TRS1018) of F1C1 were used for leaf inoculation of eggplant seedlings. After 4 dpi, the infected seedlings were surface sterilized as described previously (Kumar et al. 2017) and were observed for red fluorescence under the fluorescence microscope (EVOS FL, Life technologies) at 4× magnification.

Creation of *hrpG* insertion mutant of *R. solanacearum* F1C1

We created *hrpG* mutant by using the insertional vector pCZ367 (Cunnac et al. 2004) which also results in the *lacZ* reporter gene fusion. Taking reference sequence of GMI1000, primers were designed for partial amplification of *hrpG* homologue in F1C1 strain. Forward primer oFhrpG (5′-GCCAAGCTTGCGTACCGAGGCATTCAG TC-3′) incorporated with *Hind*III restriction site and reverse primer oRhrpG (5′-GCCTCTAGATCTTGCGC AGCTTGTAGATGT-3′) incorporated with *Xba*I restriction site at their 5′ ends, respectively were used to amplify approximately 500 bp amplicon of *hrpG* homologue in F1C1. Amplicon was cloned into promoter less, insertional vector pCZ367 and the recombinant *hrpG*::pCZ367 construct was then naturally transformed into F1C1 following the protocol described earlier (Singh et al. 2018). Its integration into the genome of F1C1 was confirmed by performing PCR with forward primer (5′-GCCA AGCTTTCCAATCCATCCAGCTTCGC-3′) designed upstream of the *hrpG* cloned fragment and olacR1 (5′-AAGG GGGATGTGCTGCAAGG-3′) designed downstream of the *lacZ* gene. One of the successful transformants TRS1027 was recruited in the further experiments and was deficient in eliciting hypersensitive response (Additional file 1: Figure S8).

Additional file

Additional file 1: Figure S1. Pathogenicity of F1C1 and non-pathogenic bacteria in eggplant seedlings by the leaf-clip inoculation. **Figure S2.** Pathogenicity of wild type F1C1 in different cultivars of eggplant seedlings. **Figure S3.** Pathogenicity study with different concentrations of wild type F1C1. **Figure S4.** Virulence of F1C1 and *hrpB*, *hrpG* and *phcA* mutants of F1C1. **Figure S5.** Colonization of *hrpB* mutant of F1C1 in eggplant seedlings co-inoculated with wild type F1C1. **Figure S6.** Virulence of F1C1 and *hrpB, hrpG* and *phcA* mutants of F1C1 in eggplant and tomato seedlings. **Figure S7.** Comparative virulence of F1C1 and derivative mutants of F1C1 between eggplant and tomato at 10^5 and 10^4 CFU/mL concentrations. **Figure S8.** Hypersensitive response (HR) assay of wild type F1C1, *hrpB* and *hrpG* mutants of F1C1. **Figure S9.** Delayed hypersensitive response of *hrpG* mutant in tobacco leaf. (DOCX 2374 kb)

Abbreviations

Bp: Base pair; CFU: Colony-forming units; dpi: Days post inoculation; LB: Luria Bertani; RH: Relative humidity

Acknowledgements

We thank Dr. L. Sahoo (IIT Guwahati) for the kind gift of the tobacco plants to test the HR in this study. We are grateful to Drs. S. Genin (LIPM, France), M. Dickinson (University of Nottingham, UK), Prabhu B Patil (IMTECH, India), Gopaljee Jha (NIPGR) for their helpful comments on the manuscript. We are very much grateful to the two anonymous reviewers and to Dr. Hui Li, the Editor of this journal for their kind comments which helped us to improve the manuscript significantly. TP and KK are thankful to UGC, GoI for the BSR and the NET-JRF fellowships, respectively. RS/BRJ and NS are thankful to the DBT, GoI for the MSc fellowship and BET-JRF/SRF fellowships, respectively. PLS/AB are thankful to DBT UExcel grant for the SRF/RA fellowships. Research in SKR lab is funded by UExcel NER grant, DBT twinning, CEFIPRA, and Departmental project grants such as UGC-SAP (DSR II), DST-FIST.

Authors' contributions

TP performed and designed the experiments, analyzed the data, wrote the manuscript; KK performed the experiments, analyzed the data, wrote the manuscript; RS performed the experiments; PLS wrote the manuscript; NS wrote the manuscript; AB wrote the manuscript; BRJ performed the experiments; SKR designed the experiments, analyzed and interpreted the data and wrote the manuscript.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 13 March 2019 Accepted: 5 June 2019
Published online: 28 June 2019

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