Development of a soil inoculation method coupled with blocker-mediated 16S rRNA gene amplicon sequencing reveals the effect of antibacterial T6SS on agrobacteria tumorigenesis and gallobiome composition

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ABSTRACT

The type VI secretion system (T6SS) is deployed by many proteobacteria to secrete effector proteins into bacterial competitors for competition or eukaryotic cells for pathogenesis. Agrobacteria, a group of soil-borne phytopathogens causing crown gall disease on various plant species, deploys the T6SS to attack closely- and distantly-related bacterial species *in vitro* and *in planta*. Current evidence suggests that the T6SS is not essential for pathogenesis under direct inoculation but it remains unknown whether the T6SS influences natural disease incidence or the microbial community within crown galls (i.e., gallobiome). To address these two key questions, we established a soil inoculation method on wounded tomato seedlings that mimics natural infections and developed a bacterial 16S rRNA gene amplicon enrichment sequencing platform. By comparing the *Agrobacterium* wild-type strain C58 with two T6SS mutants, we demonstrate that the T6SS influences both disease occurrence and gallobiome composition. Based on multiple inoculation trials across seasons, all three strains could induce tumors but the mutants had significantly lower disease incidences. The season of inoculation played a more important role than the T6SS in shaping the gallobiome. The influence of T6SS was evident in summer, in which two *Sphingomonas* species and the family Burkhoderiaceae were enriched in the gallobiome induced by the mutants. Further *in vitro* competition and colonization assay demonstrated the T6SS-mediated antagonism to a *Sphingomonas* sp. R1 strain isolated from tomato rhizosphere in this study. In conclusion, this work demonstrates that the *Agrobacterium* T6SS promotes tumorigenesis in infection process and provides competitive advantages in gall-associated microbiota.

IMPORTANCE

The T6SS is widespread among Proteobacteria and used for interbacterial competition by agrobacteria, which are soil inhabitants and opportunistic bacterial pathogens causing crown gall disease in a wide range of plants. Current evidence indicates that the T6SS is not required for gall formation when agrobacteria are inoculated directly on plant wounding sites. However, in natural settings, agrobacteria may need to compete with other bacteria in bulk soil to gain access to plant wounds and influence microbial community inside crown galls. The role of the T6SS in these critical aspects of disease ecology have remained largely unknown. In this study, we successfully

developed a Soil Inoculation method coupled with Blocker-mediated enrichment of Bacterial 16S rRNA gene Amplicon Sequencing, named as SI-BBacSeq, to address these two important questions. We provided evidence that the T6SS promotes disease occurrence and influences crown gall microbiota composition by interbacterial competition.

INTRODUCTION

Many proteobacteria, including pathogens and commensals, deploy the type VI secretion system (T6SS) for antagonism or pathogenesis (1, 2) . The T6SS is a protein translocation apparatus used to inject effectors into target cells, mainly in a contact-dependent manner. Based on the destination and biological function of known effectors (3), the T6SS mainly functions as an antibacterial weapon used by bacteria to inhibit or kill the competing bacterial species, thus providing a competitive advantage and shaping microbiota in their ecological niche (4).

Previous studies on the microbiome associated with animal guts or plants indicated that the T6SS genes are enriched in these communities, suggesting T6SS may be important for niche competition (5-8). Metagenomic analysis of human gut microbiota revealed a role of the T6SS for the domination of the gut symbiont *Bacteroides fragilis* by targeting other members of microbiome *in vitro* and *in vivo* (6, 7). A recent study further showed that a murine pathogen *Citrobacter rodentium* and resident commensal Enterobacteriaceae share the same strategy by using the T6SS for niche competition in the murine gastrointestinal tract (9). The T6SS is also deployed by plant pathogens to gain competitive growth advantage *in planta* as well as for beneficial bacteria to prevent or reduce disease symptoms caused by competing pathogens (10-12). Comparative metagenomic analysis of microbiota between $T6SS^+$ and $T6SS^-$ bacterial strains were also carried out. The gut microbiota of pests infected by *Pseudomonas protegens,* a plant-beneficial bacterium capable of invading insect pests, showed that the T6SS has no significant impact on microbiota diversity at phylum/class level but affects the abundance of Enterobacteriaceae (13). These studies collectively suggest that the T6SS is a potent antibacterial weapon used by invading pathogens or resident bacteria to gain competitive advantage in their ecological niches. However, the knowledge regarding the degree of the T6SS in

101 shaping microbiota and the molecular mechanisms of interbacterial competition in
102 complex microbial community are limited.
103
104 Agrobacteria are a diverse group of bacteria that include members from several
105 genera (14). These plant pathogens are capable of inducing crown gall or hairy root
106 disease on plants by transferring a piece of DNA named T-DNA from bacteria into
107 plants via the type IV secretion system (T4SS) (15). The T6SS is highly conserved in
108 several *Agrobacterium* species and plays a role in interbacterial competition (16-18).
109 Among these *Agrobacterium* species, the T6SS is encoded by a gene cluster
110 consisting of an *imp* operon encoding the main T6SS components and an *hcp* operon
111 encoding the puncturing device and effectors (10, 18, 19). Using a key *Agrobacterium*
112 reference strain C58, which is commonly known as a member of *A. tumefaciens* but
113 recently reclassified as *A. fabrum* (20), we previously discovered that it uses a T6SS
114 DNase effector to gain competitive growth advantage *in vitro* and *in planta* (10).
115 Interestingly, agrobacteria with incompatible effector-immunity (EI) pairs exhibit
116 strong antagonism between species level, while only weak or non-detectable effect
117 within species level (16). Moreover, higher T6SS-mediated killing outcome was
118 observed when nutrient is scarce, as opposed to nutrient rich condition (21). Thus,
119 genetic and environmental factors beyond EI pairs also contributes to interbacterial
120 competition.
121
122 To date, agrobacterial T6SS has been only demonstrated as an antibacterial weapon
123 (16-18). No evidence for its role in promoting virulence when tumor assays were
124 conducted in sterile condition or directly inoculated on the stems of various plant
125 species including tomato plants (19). Considering that agrobacteria may need to
126 compete with other bacteria in bulk soil or rhizosphere to gain access to plant wounds
127 for inciting crown galls, we reasoned that agrobacterial T6SS may influence microbial
128 community and pathogenesis under a more natural setting. To address these questions,
129 we developed a soil inoculation protocol to mimic natural infection on wounded
130 tomato seedlings across seasons for evaluating the impact of agrobacterial T6SS on
131 disease occurrence and crown gall microbiota (termed gallobiome). Moreover, to
132 overcome the challenge of interference of host organelles in the study of plant-
133 associated microbiota, we optimized a blocker-based method for enriching true

134 bacterial reads in 16S rRNA gene amplicon sequencing. Based on results from our
135 infection assays, gallobiome composition analysis, as well as *in vitro* competition and
136 colonization assays, this work indicates that agrobacterial T6SS may provide
137 competitive advantages on plant surface for effective infection, leading to a higher
138 disease incidence.
139
140 **RESULTS**
141
142 **Environmental factors and T6SS affect crown gall disease incidence**
143 The wild type (WT) strain C58 and two C58-derived T6SS mutants with deletion of
144 essential T6SS genes, Δ*tssL* and Δ*tssB* respectively, were used to study the effects of
145 agrobacterial T6SS in tumorigenesis using a soil inoculation method. Nine batches of
146 inoculation experiments across different seasons were conducted in this study (Table
147 1). In total, 139 crown galls were collected, including 70 induced by WT, 41 induced
148 by Δ*tssL*, and 28 induced by Δ*tssB*. The results showed that all three strains are
149 capable of inducing tumors, but the disease incidences of Δ*tssL* and Δ*tssB* were
150 significantly lower than WT (Table 1, Fig. 1A). There was no significant weight
151 difference among the crown galls induced by different strains (Fig. 1B). It is notable
152 that there is an inverse correlation between disease incidence and temperature across
153 seasons for all three strains throughout the year (Fig. 1C, 1D). These results indicated
154 that the presence of a functional T6SS and the month of inoculation both affected the
155 disease incidence under soil inoculation condition.
156
157 **Round I of 16S rRNA gene amplicon sequencing: Initial trial**
158 To determine the gallobiome composition, crown galls with similar weights across
159 seasons were selected for 16S rRNA gene amplicon sequencing. For Round I, six
160 crown gall DNA samples, three induced by WT and three by Δ*tssL* were amplified
161 with two commonly used 16S rRNA gene primer sets V3-4 and V5-7 (Table S1). The
162 Illumina sequencing produced 140,830 and 601,299 reads from V3-4 and V5-7 sets,
163 respectively. However, the majority (99.1% in V3-4 and 93.6% for V5-7) of these
164 reads were derived from plant chloroplast and mitochondria. After removing these
165 host contaminations, only 1,230 and 38,320 bacterial reads remained (Table 2**).**
166

167 **Round II of 16S rRNA gene amplicon sequencing: Optimization**
168 Due to the severe host contamination observed in Round I that used the universal 16S
169 rRNA gene primers, we evaluated the performance of different 16S rRNA gene
170 primers with blockers (3' modified oligonucleotides with C3 spacer) that could
171 prevent the amplification of tomato chloroplast and mitochondrial rRNA genes (22-
172 24).
173
174 Based on the alignment of bacterial, tomato chloroplast and mitochondrial 16S rRNA
175 gene sequences (Fig. S1), we designed the primer sets and cognate blockers against
176 different variable region in 16S rRNA genes (Table S1 and S2). The new primer sets
177 and blockers were tested using the three WT-induced crown gall DNA samples from
178 Round I. After sequencing and data processing, the result indicated that the blockers
179 have variable effectiveness in reducing host contaminations (Table 3 and Fig. 2A**).**
180 For V3-4 and V5-7 primer sets, adding blockers increased the bacterial reads to 5.3-
181 41.5% of total reads, compared to only 0.2 -1.9% of bacterial reads per sample
182 without blockers. For V1-3 and V6-8 primer sets, adding blockers only increased the
183 bacterial reads to 1.6-5.6% of total reads per sample. The reads were grouped into
184 species-level operational taxonomic units (OTUs) with 99% sequence identity. The
185 alpha rarefaction curves based on the OTU counts indicated that, for those crown gall
186 samples, the curves would approach saturation when the sequencing depth was over
187 about 10,000 reads per sample (Fig. 2B). According to the bar plot of bacterial
188 composition at family levels, we could observe higher number of families identified
189 from the same crown galls amplified by adding blockers (Fig. 3A). The result of
190 Principal Coordinate Analysis (PCoA) indicated that adding blockers did not cause
191 significant biases in the inferred microbiota composition (Fig. 3B). However, the use
192 of different primer sets resulted in significant difference in the inferred microbiota
193 composition (Fig. 3C), which has been reported previously (25). There is also
194 significant difference among different crown galls (Fig. 3D).

195 Overall, adding blockers at the PCR step increased bacterial read counts and the
196 resolution of gallobiome. Because the use of V5-7 primers and cognate blockers
197 obtained the highest number and percentage of bacterial reads, V5-7 set-up with
198 blockers were selected for 16 rRNA gene amplicon sequencing of crown galls
199 induced by WT and T6SS mutants.

200

201 **Round III of 16S rRNA gene amplicon sequencing: Impact of the T6SS on**
202 **gallobiome**

203 Among all of 139 crown galls collected, 53 tumors in the range of 0.06 – 0.56 g (24
204 induced by WT, 16 by Δ*tssL,* and 13 by Δ*tssB*) were used for the analysis. We
205 obtained an average of 6,9980 ± 26,452 (mean ± SD) reads per sample, ranged from
206 9,814 to 116,072 reads per sample. Before the analysis, the amplicon sequence
207 variants (ASVs) were clustered into species-level OTUs and the singletons were
208 removed. Based on the alpha rarefaction curves and the minimal read counts of the
209 samples (Fig. S2**)**, diversity analysis was conducted with datasets subsampling at
210 9800 reads per sample. The result of PCoA showed that the gallobiome induced by
211 WT and two T6SS mutant strains were not significantly different (Fig. 4A). Most of
212 the variation between samples were contributed by different seasons (July vs.
213 October/November) (Fig. 4B). After splitting and re-analyzing the dataset based on
214 the month of inoculation, we observed the difference between the gallobiomes
215 associated with WT and two T6SS mutants in July (Fig. 4C**)**. In contrast, no
216 difference was detected in those galls induced in October or November (Fig. 4D and
217 4E).

218

219 The alpha diversity indices including observed OTUs, Shannon index, and Pielou's
220 evenness index had no significant difference between the gallobiomes associated with
221 WT and T6SS mutants (Fig. 5A, 5B and 5C**)**. Interestingly, we found that, in galls
222 induced in July, the WT gallobiome showed significantly higher Faith's phylogenic
223 diversity than the gallobiome associated with Δ*tssL*; and in galls induced in
224 November, the Δ*tssL* gallobiome showed higher diversity than the WT gallobiome
225 (Fig. 5D**)**. The gallobiome associated with Δ*tssB* also exhibited higher diversity than
226 the WT gallobiome but the difference was not statistically significant.
227 While there were variations in bacterial compositions among crown galls, the top 10
228 bacterial families in gallobiomes were quite consistent, including: Rhizobiaceae,
229 Comamonadaceae, Chitinophagaceae, Methyphliaceae, Caulobacteraceae,
230 Sphingomonadaceae, Microbacreriaceae, Xanthobacteraceae, Oxalobacteraceae, and
231 Pseudomonaceae (Fig. 5E). The bacterial family Rhizobiaceae, which *Agrobacterium*
232 belongs to, was found to be highly variable and accounted for 5-85% of the entire

233 community. The most abundant OTU in this Rhizobiaceae dataset was 100% matched
234 to 16S rRNA genes of C58, suggesting that the abundance of this OTU could be
235 referred to the relative abundance of WT or mutant inoculum in crown galls. When
236 the weight of crown galls was plotted against the relative abundance of this OTU in
237 gallobiomes, we found that the abundance of Rhizobiaceae in July was dramatically
238 lower than those in October and November (Fig. 5F), but there was no significant
239 difference observed among different strains. Furthermore, a positive correlation
240 between weight of crown galls and relative abundance of this agrobacterial OTU was
241 observed (Fig. 5G).
242
243 **Sphingomonadaceae and Burkholderiacea were more abundant in the**
244 **gallobiomes induced by the T6SS mutants in July**
245 Next, we analyzed the species-level OTU and family with differential abundance
246 (DA) between gallobiome associated with WT and T6SS mutants in July. Two OTUs
247 belonged to Sphingomonadaceae, named as SphinOTU1 and SphinOTU2 here, and
248 Burkhoderiaceae family were significantly enriched in the gallobiomes induced by
249 T6SS mutants (Fig. 6A). SphinOTU1 was only present in gallobiomes in July but not
250 in October/November, and SpinOTU2 was present in both July and October but not in
251 November (Fig 6B). Neither SpinOTU1 or SpinOTU2 was identified in gallobiomes
252 induced by the WT in July. Burkhoderiaceae was enriched in gallobiomes induced by
253 the T6SS mutants in July, but no consistent enrichment in October and November (Fig
254 6C). Although SphinOTU1 and SphinOTU2 were only present in gallobiomes
255 induced by T6SS mutants in July; at family level, Sphingomonadaceae was not
256 enriched in T6SS-dependent manner in any months (Fig. 6C).
257
258 **T6SS-dependent antagonism between agrobacteria and a *Sphingomonas* sp.**
259 **isolate**
260 The T6SS-dependent differential abundance of two *Sphingomonas* OTUs in
261 gallobiomes motivated us to investigate whether C58 exhibits T6SS antibacterial
262 activity to *Sphingomonas*. A *Sphingomonas* sp. strain R1 isolated from the tomato
263 rhizosphere in one soil inoculation experiment was used for interbacterial competition
264 assays *in planta* and *in vitro*. Each of the agrobacterial strains was mixed at 1:1 ratio
265 with *Sphingomonas* sp. R1 as inoculum and perform soil inoculation on wounded

266 tomato seedlings. Colonization efficiency of those strains was determined by counting
267 colony forming unit (CFU) recovered from wounded stem segments at 10 day-post-
268 inoculation (dpi). The results showed a ~ 0.5 log of reduced CFUs of *Sphingomonas*
269 sp. R1 when co-inoculated with WT as compared to that with Δ*tssL* and Δ*tssB* or R1
270 only (Fig. 7A). Recovered CFUs of agrobacterial WT and T6SS mutants inoculated
271 alone or with *Sphingomonas* sp. R1. are 1 to 1.5 log higher than *Sphingomonas* sp. R1
272 but no difference among agrobacterial WT and T6SS mutants inoculated alone or with
273 *Sphingomonas* sp. R1. These results suggest that agrobacteria have higher competitive
274 colonization efficiency than *Sphingomonas* sp. R1 in part dependent on a functional
275 T6SS. Accordingly, interbacterial competition assay on agar plate also showed T6SS-
276 dependent antibacterial activity to *Sphingomonas* sp. R1 (Fig. 7B). However, when
277 interbacterial competition was carried out *in vitro* on agar plates, only weak
278 antibacterial activity was observed at 1:1 ratio but ~0.5 log reduced CFUs of
279 *Sphingomonas* sp. R1 was detected when competition was carried out at 10:1 ratio of
280 agrobacteria: *Sphingomonas* sp. R1. The results show that the T6SS provides
281 agrobacteria with a higher competitive advantage against *Sphingomonas* sp. R1 on
282 plant wounding site than *in vitro.* We further evaluated whether CFUs of
283 *Sphingomonas* sp. R1 was enriched in crown galls induced by T6SS mutants as
284 compared to those induced by WT via soil inoculation method used for 16S rRNA
285 gene amplicon sequencing. Surprisingly, *Sphingomonas* sp. R1 was not always
286 present in crown galls at 28 dpi, in which R1 was only recovered from one out of
287 three independent experiments by direct induction of crown galls on tomato stem
288 (Fig. S3). The abundance of C58 ranged from 10^4 to 10^7 CFU per gall and no
289 consistent difference could be observed between the WT and the T6SS mutants.
290 Together, these results suggest that C58 exhibits antibacterial activity to inhibit the *in*
291 *vitro* growth and plant colonization of *Sphingomonas* sp. R1. The T6SS may help
292 agrobacteria to gain competitive growth over other competing rhizobacteria on plant
293 surface for effective infection, leading to higher disease incidence.
294

295 **DISCUSSION**

296 In this study, we designed experiments to address whether the T6SS affects
297 agrobacterial tumorigenesis and gallobiome composition. By establishing a soil
298 inoculation platform to evaluate the disease incidence of wounded tomato seedlings

299 across seasons, we revealed that a functional T6SS is positively correlated with disease
300 occurrence but not gall weight. Furthermore, by developing an effective protocol for
301 enrichment of bacterial 16S rRNA genes by utilizing blocker to prevent host
302 contamination, we found that seasons or environmental factors are the major driver in
303 shaping gallobiome composition while T6SS could also influence microbiota at more
304 specific manner.
305
306 The evidences that agrobacterial T6SS promotes tumorigenesis in soil inoculation
307 (Table 1, Fig. 1) but not direct inoculation (19) suggest that the T6SS is not directly
308 involved in virulence. Instead, agrobacteria may deploy the antibacterial weapon to
309 increase the occurrence of infection in the presence of relatively complex microbial
310 communities in rhizosphere (Table S3, (26)). Once galls are induced, the T6SS is not
311 required for gall development since no significant difference in weight was observed
312 between WT and T6SS mutants (Fig. 1B). The presence of a functional T6SS has no
313 impact on the population size of agrobacteria, which is consistent with recent findings
314 that T6SS genes were not identified as fitness genes by Tn-seq screen in crown galls
315 (27). The T6SS of C58 also appears not to be the key factor to shape gallobiome
316 composition, suggesting that agrobacteria may also use other ways for niche
317 competition in galls. It is generally believed that the agrobacteria inciting crown galls
318 and later become residents have privilege to access the specific opines synthesized by
319 transformed plant cells containing the agrobacterial T-DNA (28). This "opine concept"
320 has not been experimentally validated but supported by a study showed that the ability
321 of agrobacteria to trap opines can acquire a competitive advantage over the siblings
322 which cannot utilize opine (29). Therefore, it would be interesting to investigate
323 whether opine synthesis and other fitness genes would impact the crown gall microbiota
324 in which T6SS may synergistically enhance the niche occupation.
325
326 The findings that disease incidence and crown gall weight are reversely correlated
327 with temperature (Fig. 1C) are expected. Early studies demonstrated that crown gall
328 sizes were dramatically decreased when the host plants were inoculated at 28-30°C,
329 while no gall formation at 31°C or above (30, 31). In addition, the T4SS complex and
330 its associated T-pilus are more stable or produced at higher levels at 19°C than 28°C
331 whereas T4SS-mediated plasmid conjugation is deficient at 28°C (32-36). The

332 increased tumor weight when inoculated in fall/winter is also correlated with a higher
333 relative abundance of agrobacterial OTU (Fig. 5G), suggesting the active proliferation
334 of agrobacterial population inside crown galls. Furthermore, our data show that crown
335 galls produced in summer have distinct microbiota as compared to those from
336 fall/winter (Fig. 4B), which echoes previous studies on *Allorhizobium vitis* that crown
337 gall microbiota of grapevine is significantly different in summer (37). Interestingly,
338 while there is no global difference of gallobiome induced by WT or T6SS mutants
339 inoculated in fall/winter, we found that the two *Sphingomonas* OTUs and the family
340 Burkholderiaceae were only present or significantly enriched in the gallobiome
341 induced by T6SS mutants in summer (Fig. 6). By counting viable agrobacterial
342 inoculum, we showed that C58 exhibits a T6SS-mediated antibacterial activity to
343 *Sphingomonas* sp. R1 *in vitro* and in rhizosphere (Fig. 7A, B) but no significant
344 difference of R1 abundance between the galls co-infected with C58 WT or T6SS
345 mutants (Fig. S3). Interestingly, we noticed that the colonization efficiency of those
346 T6SS mutants was more variable as compared to those of WT from the combined data
347 set (Fig. S4). To this end, it remains to be determined whether the absence or reduced
348 abundance of *Sphingomonas* and Burkholderiaceae of gallobiome induced by WT as
349 compared to those by T6SS mutants are direct consequence of antibacterial activity
350 conferred by T6SS. However, current evidence suggests that agrobacteria may use
351 T6SS to gain competitive advantage by its antibacterial activity against other bacterial
352 species residing in the soil or rhizosphere and therefore increases its success in
353 inciting tumors on infected plants.
354
355 Besides Rhizobiaceae that agrobacteria belong to, other bacterial families were also
356 occupied considerable proportions of crown gall microbiota, such as
357 Comamonadaceae, members of which are commonly found in soil and water as well
358 as plant-associated bacteria *Variovorax* shown to be important for root growth of
359 *Arabidopsis* (38, 39). Bacteria belong to Xanthobacteraceae and Pseudomonaceae, the
360 two families highly associated with plants as either pathogens or commensals, also
361 had high relative abundance in the crown galls. Some *Pseudomonas* species such as *P.*
362 *fluorescens* and *P. putida* are capable of utilizing opines (40, 41), which may explain
363 why bacterial species other than the agrobacterial inoculum could also colonize and
364 propagate in high relative abundance in crown galls.

365

366 We found that the predominance of plant DNA in crown gall samples led to the low
367 resolution of endophytic bacterial microbiota survey via standard 16S rRNA gene
368 amplicon sequencing due to the co-amplification of chloroplast and mitochondrial
369 sequences. Although the use of 16S rRNA gene primers containing mismatches to 16S
370 rRNA gene of plant chloroplast or mitochondria could potentially reduce host
371 contamination in endophytic microbiota studies (25, 26), the "bacteria-specific"
372 primers resulted in > 90% host reads of our initial attempt (Round I) (Table 2), which
373 is similar to results of a previous study (22). When adding blockers that could
374 specifically anneal to chloroplast or mitochondrial DNA but not be extended by DNA
375 polymerase during PCR, we successfully reduced the host contamination and obtained
376 sufficient sequencing depth for this study. Our results support the effectiveness of
377 blockers in increasing bacterial reads of endophyte community (22-24). Our newly
378 developed SI-BBacSeq protocol for gallobiome may be applicable to microbiota
379 associated with tomato, or even other plants after modifying the blocker sequences.
380

381 To date, collective plant microbiota studies suggest the enrichment of T6SS genes in
382 microbiota and as a trait for niche competition in microbiota (5, 8). However, the role
383 and impact of T6SS in different plant and microbiome contexts may be different. Our
384 study suggests that the T6SS may provide agrobacteria with competitive advantages
385 at initial stage of infection but likely not critical for proliferation once they established
386 their niche inside the crown galls. Future works that investigate the spatial distribution
387 of agrobacterial cells and other endophytes inside crown galls or plant wounding site,
388 together with temporal and spatial visualization of the T6SS activity, are important for
389 dissecting the roles of T6SS in agrobacterial disease ecology.
390

391 **MATERIALS AND METHODS**

392 **Bacterial strains and growth condition**

393 Bacterial strains and plasmids used in this study are listed in Table S4. *Agrobacterium*
394 strain C58 wild type and two T6SS mutants (i.e., Δ*tssB* and Δ*tssL*) (42) were first
395 streaked on 523 medium agar plates (43) and incubate at 25 °C for 48 hr.
396 *Sphingomonas* sp. R1 was grown on R2A medium without soluble starch and
397 pyruvate (44). *Escherichia coli* was grown on lysogeny broth (LB) medium at 37°C.

398 Freshly grown colony was inoculated into corresponding broth for overnight culture.
399 The antibiotics and concentration used are: 10 μg/ml gentamycin for *E. coli,* 50 μg/ml
400 gentamycin for *Agrobacterium*, and 20 μg/ml kanamycin and 50μg/ml gentamycin for
401 *Sphingomonas* sp. R1.
402
403 **Construction of strains enable antibiotics selection**
404 Primers used for construction are listed in Table S5. The DNA fragment containing
405 gentamycin resistance (Gm^R) gene and green fluorescence protein *gfp*(S65T) were
406 amplified from pRL662::GFP(S65T) with the primer pair "BclI-GFP-GmR-F-25" and
407 "BclI-GFP-GmR-R-25", and then cloned into XbaI digested pJQ-COM. The newly
408 constructed plasmid was named pJQ-com-GmR-GFP, which enabled the generation of
409 Gm^R gene and *gfp* (S65T) knocked in at the upstream of *actC* (*atu5330*) of
410 *Agrobacterium* genome after double crossover. The knock-in strains were generated in
411 *Agrobacterium* C58, Δ*tssB*, and Δ*tssL* background. The knock-in event and excision
412 of pJQ200ks backbone were confirmed by GFP signal, gentamycin resistance, and
413 PCR with the primer pair "3'sacB-5'GmR pJQ200 F" and "3'sacB-5'GmR pJQ200 R",
414 which target the fragment between *sacB* and Gm^R gene on pJQ200ks. The plasmids
415 pRL66::GFP(S65T) and pBBR::mCherry conferring kanamycin resistance were each
416 transformed into *Sphingomonas* sp. R1 through electroporation. The procedure of
417 electroporation, doble crossover via pJQ200ks were performed as described
418 previously (45).
419
420 **Plant material and growth condition**
421 Seeds of *Solanum lycopersicum* (tomato) cultivar Known-You 301 from KNOWN-
422 YOU SEED CO., LTD (Kaohsiung, Taiwan) were germinated and grown in
423 unsterilized potting mixture (Jiffy® Premium Fine Peat Substrate, perlite, and
424 vermiculite, mixed in 4:1:1 ratio, v:v:v) in the greenhouse EL329
425 (N25.043047890856812, E121.61135464167913, Greenhouse building, Academia
426 Sinica, Taipei, Taiwan).
427
428 **Soil inoculation**
429 The soil inoculation method was optimized based on the method used for
430 agrobacterial infection of pea and walnut (46, 47). Overnight (14-16 hr) culture of

431 agrobacterial strain in 12 ml of 523 broth were centrifugated at 6,000 × g for 10 min.
432 The pellets were washed once by using 10 ml of sterile saline (0.9% NaCl in H_2O).
433 The washed pellets were centrifuged and resuspended in sterile saline with OD_{600}
434 adjusted to 1. Bacterial suspension was mixed into unsterilized soil (1 ml suspension
435 per 100 g of soil), which is expected to result in ~10^7 colony-forming units (CFUs)/g
436 soil, the optimized concentration that could incite crown galls but not at 100%.
437 Tomato seedlings with two true leaves (two- to three-week-old) were wounded at the
438 site between primary root and cotyledons by a fire-sterilized sewing needle, planted
439 into the inoculated soil, and grown in the greenhouse. Each strain was inoculated with
440 14 to 20 tomato seedlings, and the inoculation with saline were used as a negative
441 control.
442
443 **Harvest, surface sterilization and storage of crown gall samples**
444 Two months after the inoculation, tomato plants were harvested to examine gall
445 formation on the wounded site. The disease incidence was calculated by dividing the
446 number of plants with visible gall formation by the total number of inoculated plants.
447 The sections with crown gall were cut out and sterilized in 35 ml of sterilization
448 solution (3% NaOCl, 0.01% Tween-20) for 30s, then transferred to 35 ml of 70%
449 ethanol and washed in 35 ml of sterile H_2O for three times (48). The sterilization was
450 evaluated by spreading 100 µl liquid from the third wash onto 523 agar plate for 2
451 days to make sure that there was no growth of bacteria. After sterilization, the crown
452 galls were dissected from each of the plant segments in sterile petri dish, wrapped in
453 sterile aluminum foil, frozen by liquid nitrogen, and stored at -80 ℃ prior to DNA
454 extraction.
455
456 **DNA extraction form crown galls**
457 Crown gall samples were homogenized by pestle and mortar with liquid nitrogen.
458 0.25 g of homogenized tissue were transferred to the beating tube of DNeasy®
459 PowerSoil® Kit (Qiagen, Germany) following the manufacturer's instructions.
460 Concentrations of extracted DNA were determined by NanoDrop™ 1000 (Thermo
461 Fisher Scientific, United State) and Qubit dsDNA HS (Invitrogen, United State).
462
463 **16S rRNA gene amplification and sequencing**

464 Three rounds of amplicon sequencing were conducted in this study. The round I was
465 conducted according to established protocols of soil microbiota studies using 16S
466 rRNA gene variable regions (25). Each primer with a partial adaptor sequence was
467 compatible to Illumina Truseq Combinatorial Dual index system. The first PCR
468 amplifications were carried out in following 25 µl reaction: 5 ng DNA template, 2×
469 KAPA HiFi HotStart DNA Polymerase ReadyMix (Roche, Switzerland), and 0.2 µM
470 for each of the forward and reverse primers. The first PCR program and reaction
471 details for V3-4 and V5-7 are listed in Table S6. The PCR products were purified by
472 Ampure XP beads (Thermo Fisher Scientific Inc, Sweden). The V5-7 PCR products
473 underwent BluePippin (Sage Science) size-selection to remove tomato amplicon. To
474 attach dual indices and full-length Illumina sequencing adapters, a second round of
475 PCR amplifications were employed for 50 µl reactions with purified or size-selection
476 PCR products, 2× KAPA HiFi HotStart DNA Polymerase ReadyMix (Roche,
477 Switzerland), and 0.5 µM for each of the forward and reverse primers. All the library
478 products of second PCR were purified and pooled. Pooled libraries were loaded onto
479 an Illumina MiSeq V3 flow cell (Illumina, United States) and for 2×300 bp paired-
480 end sequencing.
481
482 The round II of amplicon sequencing was conducted to test the effect of adding PCR
483 blockers on reducing plant chloroplast and mitochondrial sequences. Three gall
484 samples (1115-W21, 1115-W22 and 1115-W25) were used as the DNA templates to
485 provide biological replicates. For each DNA sample, four primer pairs targeting
486 different variable regions of 16S rRNA gene were used, including: V1-3, V3-4, V5-7,
487 and V6-8 (Table S1). Each primer contained a partial adaptor sequence of the Illumina
488 Truseq Combinatorial Dual index system. Cognate 3' C3 spacer modified
489 oligonucleotides (blockers) which would block the amplification of tomato
490 chloroplast and mitochondrial 16S rRNA genes were designed according to a previous
491 study (22) and applied to one of the two PCRs for each sample-primer combination
492 (Table S2). Procedures for PCR and Illumina sequencing were based on those used
493 for round I, but the amount of DNA template was increased to 25 ng to reduce PCR
494 cycle time and the annealing temperature was adjusted for blockers. More detailed
495 technical information is provided in Table S6.
496

497 Based on results from the first two rounds of amplicon sequencing, an optimized
498 protocol that utilizes blockers and targets V5-7 regions was used for round III. All 53
499 crown gall samples were included in this final round.
500
501 **Raw read processing and microbiota analysis**
502 The Illumina raw reads in FASTQ format were imported into QIIME2 release 2020.8
503 (49). The primer region of imported sequences was trimmed using DADA2 (50). The
504 denoised and merged paired-end reads were used to construct output feature tables
505 containing amplicon sequence variants (ASVs) ID and counts in different samples.
506 For amplicon sequencing round III, the ASVs were further clustered into species-level
507 OTUs based on 99% sequence identity using the "qiime vsearch cluster-features-de-
508 novo" function (51) and the singletons were removed by using the "qiime feature-
509 table filter-features" function prior to the downstream analysis. The taxonomic
510 information was inferred for representative OTU sequences via pre-trained q2-
511 feature-classifier (52) and classify-sklearn naïve Bayes classifier based on SILVA
512 version 138 SSU Ref NR 99 full-length sequences dataset (53). The sequences
513 assigned as chloroplasts, mitochondria, eukaryotes, archaea, or unknown were
514 removed. After the filtering, a phylogenetic tree of eubacterial sequences was inferred
515 using the q2-fragment-insertion plug-in (54-57) based on the SILVA 128 SEPP
516 reference database. Weighted Unifrac (58) analysis of those datasets was performed
517 based on insertion tree; the distance matrix and principal coordinate analysis (PCoA)
518 plot were generated using QIIME2. For round III, phylogenic and diversity analysis
519 was conducted using "qiime diversity core-metrics-phylogenetic" and the sub-
520 sampling depth was set to 9,800 reads/sample.
521

522 **Differential abundance analysis of OTU**

523 Analysis of composition of microbiomes (ANCOM) was applied to identify OTUs
524 that have significantly different relative abundance in the gallobiome associated with
525 C58 WT and T6SS mutants using the function "qiime composition ancom" in
526 QIIME2. The feature table collapsed into family level was imported and transformed
527 by centred log-ratio transformation. The null hypothesis of ANCOM is that the
528 abundance of a OTU is not different between two study groups. After performing all

529 comparison between each OTU in two study groups, the times that the null hypothesis
530 was rejected is called *w*. The OTUs having *w* values at 70 percentile or higher are
531 considered as significant (59, 60).

532 **Isolation of *Sphingomonas* sp. R1**

533 *Sphingomonas* sp. R1 was isolated from a wounded tomato stem during a soil
534 inoculation assay. After 10 dpi of soil inoculation mentioned above, the 1-cm
535 wounded segment of tomato seedlings were harvested and rinsed by sterile water, and
536 then transferred to 1 ml 0.9% NaCl. After vortex at max speed for 3 minutes, the
537 supernatant was transferred to a new microcentrifuge tube and spread on 523 agar
538 plates via exponential mode of easySpiral Dilute® (Ref. 414 000, INTERSCIENCE,
539 France). After growth at 25°C for two days, ~ 20 colonies were selected and streaked
540 out on 523 agar plates for pure culture. The partial 16S rRNA gene of the isolates was
541 amplified and sequenced by V5-V7 primer set, which was used to BLAST in 16S
542 ribosomal RNA gene sequence database in NCBI for identification of 16 bacterial
543 species (Table S3).

544

545 **Interbacterial competition assay *in vitro* and *in planta***

546 For interbacterial competition *in vitro,* the bacterial suspension was adjusted to OD_{600}
547 equals to 3.0 in 0.9% NaCl (w/v) after overnight culture. For the *in vitro* competition,
548 the attacker strains (C58 WT, Δ*tssL*, or Δ*tssB*) were further diluted into OD_{600} equals
549 to 1, and the prey *Sphingomonas* sp. R1 containing pRL662::GFP was further diluted
550 into OD_{600} equal to 0.3 or 0.1 for mixing with the attacker in equal volume for
551 different density ratio. Two spots of 10 µl mixture were spotted on *Agrobacterium*
552 kill-triggering medium (21). After co-incubated for 16 h in 25°C, the colonies were
553 scraped and resuspended in 1 ml 0.9% NaCl (w/v), and spread via exponential mode
554 of easySpiral Dilute® (Ref. 414 000, INTERSCIENCE, France) on 523 media
555 containing 30 µg/ml gentamycin to recover the prey.

556 For agrobacterial colonization with or without *Sphingomonas* sp. R1, each of the
557 agrobacterial strains (C58 WT, Δ*tssL* or Δ*tssB*) with Gm^R-GFP knock-in was mixed
558 with *Sphingomonas* sp. R1 containing pBBR::mCherry at equal OD_{600} =1.0. The 5 µl
559 mixture was inoculated on the needle-wounded stem of tomato seedling with first pair
560 of true leaves. Tomato seedlings were harvested at 10 days post-inoculation (dpi). For
561 each plant, the 1 cm wounded segment was cut and rinsed by sterile water before
562 being transferred into a sterilized Eppendorf tube containing 1 ml of 0.9% NaCl. After
563 vortex at max speed for 3 minutes, the liquid was considered as the rhizosphere
564 sample. The samples were diluted and plated on media with gentamycin for CFU
565 calculation.

566 When counting the CFU of C58 and *Sphingomonas* sp. R1 in the tumor, the crown
567 galls were collected after growing in the greenhouse for three weeks. The 1-cm
568 tomato stem section containing the gall in middle was cut and homogenized with 1 ml
569 0.9% NaCl (w/v) by sterilized mortar and pestle. The homogenized tissue was diluted
570 10-fold and spread via exponential mode of easySpiral Dilute® on 523 media
571 containing 50 ppm gentamycin to recover the attacker and prey. After incubation for
572 two days at 25°C, the colonies were counted by using the Scan® 500 Automatic
573 colony counter (Ref 436 000, INTERSCIENCE, France; software version 8.6.1) to
574 calculate CFUs.

575 **Statistics and visualization**

576 GraphPad Prism 8 was used to perform t-test on sequencing read counts, one-way
577 ANOVA on CFU counts, two-way ANOVA followed by Turkey's multiple
578 comparison test on crown gall disease incidence and alpha diversity index, Kruskai-
579 Wallis test on tumor weight, Spearman correlation on gall weight and relative
580 abundance of agrobacterial OTU. The alpha-rarefaction curves were plotted using
581 Phyloseq (61) in R version 3.6.2. ADONIS in "qiime diversity beta-group-
582 significance" of QIIME2 (62, 63) was used to test if groups of samples have
583 significantly different microbiota composition. The taxonomic composition plots were
584 generated using qiime2R (v0.99.35, https://github.com/jbisanz/qiime2R) in R studio
585 2.
586

587 **DATA AVAILABILITY**

588 All raw data sets are available in the National Center for Biotechnology Information
589 (NCBI) under BioProject accession PRJNA894311.
590
591 **AUTHOR CONTRIBUTION**
592 Conceptualization: SCW, CHK, EML
593 Funding acquisition: CHK, EML
594 Investigation: SCW, CHK, EML
595 Methodology: SCW, APC, SJC, CHK
596 Project administration: EML
597 Supervision: CHK, EML
598 Validation: SCW, CHK, EML
599 Visualization: SCW, CHK, EML
600 Writing – original draft: SCW, CHK, EML
601 Writing – review & editing: SCW, APC, SJC, CHK, EML
602
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619
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819
820
821

822 **FIGURE LEGENDS**

823 **Figure 1. Tumorigenesis assay of agrobacterial strain C58 and its T6SS-**
824 **deficienct mutants.**

825 For each experiment, 14-20 wounded tomato seedlings were grown in soil containing
826 one of the strains tested (i.e., C58 WT, Δ*tssL*, and Δ*tssB*) and collected at 60 days post
827 inoculation (dpi). (A) Disease incidence plotted by strain and color-coded according
828 to the month of inoculation (light yellow, June; orange, July; light blue, October; dark
829 blue, November). Lines and error bars indicate mean ± SD. Statistical significance
830 was tested using two-way ANOVA followed by Tukey's multiple comparisons; *p*-
831 value = 0.025 and 0.027 when comparing the wild type to Δ*tssL* and Δ*tssB*,
832 respectively. (B) Weight distribution of the galls collected (See details in Table S7).
833 No significant difference among the three strains (*p*-value= 0.15, Kruskai-Wallis test).
834 (C) Correlation plot of disease incidence and temperature of daily average. (D) Crown
835 galls generated through soil inoculation, example from inoculation on 11/15/2018. N
836 indicated no gall formation in some of inoculated plants.

837

838 **Figure 2. Optimizing 16S rRNA gene amplification by using PCR blockers.**

839 Three crown gall samples (named as 21W, 22W and 25W) induced by C58 wild type
840 were used for analysis. V1-3, V3-4, V5-7 and V6-8 represent different primer pairs
841 targeting different variable regions on 16S rRNA gene. (A) The OTU counts of 16
842 rRNA gene amplicons after filtering non-bacterial OTUs. (ns, not statistically
843 significant; *, *p-value* < 0.05, student's t-test). The bar indicated mean of OTUs ± SD.
844 (B) Alpha rarefaction curves of the observed bacterial OTUs based on datasets from
845 amplicon sequencing run II. Sample size in X-axis indicates different sub-sampling
846 depth of each dataset.

847

848 **Figure 3. Gallobiome composition when using different primers amplified with**
849 **or without blockers.** (A) Different primer sets (V1-3, V3-4, V5-7 and V6-8**)** for
850 analysis of three crown gall samples (21W, 22W and 25W, see details in Table S8)
851 induced by C58 wild type were indicated. Top 10 observed families were listed. "-":
852 amplification without blockers; "+": amplification with blockers. Principle coordinate
853 analysis (PCoA) plots of the bacterial composition of the datasets were generated
854 based on the weighted Unifrac matrix, and color coded by (B) blocker usage (*p*-value
855 = 0.068, R^2 = 0.026, ADONIS), (C) different primer sets (*p*-value = 0.001, R^2 = 0.524,

856 ADONIS) and (D) different crown gall samples (*p*-value = 0.001, R^2 = 0.238,
857 ADONIS).
858
859 **Figure 4. Principle coordinate analysis (PCoA) of bacterial composition in 53**
860 **crown galls induced by C58 WT and T6SS mutants.**
861 The plots were drawn based on weighted Unifrac matrix of the bacterial communities.
862 Crown galls induced by different strains (C58 WT, Δ*tssB,* Δ*tssL*) (A) or inoculated in
863 different months (B) were labeled with different colors in each panel. The datasets
864 were further split based on the month of inoculation, and the PCoA plots of
865 gallobiomes from (C) July, (D) October and (E) November were indicated above.
866 Statistical differences in clustering were evaluated via ADONIS permutation test, and
867 the corresponding *p*-values and R^2 are indicated.
868
869 **Figure 5. Alpha diversity and composition of crown galls generated in different**
870 **months by C58 WT and T6SS mutants.**
871 (A) Observed OTU, (B) Shannon index, (C) evenness, and (D) Faith's phylogenic
872 diversity of each sample generated in different months was indicated. Lines and error
873 bars indicate mean ± SD. Two-way ANOVA followed by Tukey's multiple
874 comparisons test were performed, and the asterisks indicated the statistically
875 significant difference between two strains (*p*-value < 0.05). (E) Bacterial composition
876 of 53 crown galls. For each strain, the samples are grouped by the month of
877 inoculation. Top-ten most abundant families were listed and colored differently, and
878 the remaining ones are combined into "Others". (F) Relative abundance of
879 Rhizobiaceae in galls inoculated in different months and strains. (G) Scatter plot of
880 relative abundance of agrobacteria OTU in gallobiome - weight of the crown galls. A
881 positive correlation between weight of crown gall and relative abundance of
882 *Agrobacterium* C58 was observed (Spearman r = 0.333, *p*-value = 0.01).
883
884 **Figure 6. Analysis of composition of microbiomes (ANCOM) for gallobiomes in**
885 **July.**
886 The ANCOM volcano plots were drawn based on the centred-log-ratio-transformed
887 abundance of (A) OTUs or bacterial families. The taxa that exhibit differential
888 abundance (DA) between gallobiomes induced by C58 WT and T6SS mutants are
889 indicated by red arrows. The two DA OTUs belonging to *Sphingomonadaceae* were

890 named SphinOTU1 and SphinOTU2, respectively. The relative abundance of focal
891 taxa was shown in panels (B) and (C). Lines and error bars indicate mean ± SD.
892
893 **Fig. 7 Competition between C58 and *Sphingomonas* sp. R1 *in vitro* and on**
894 **wounded site of tomato stems.**
895 Each of the three *Agrobacterium* strains (i.e., C58 WT, Δ*tssL*, and Δ*tssB*) was
896 individually tested for competition against *Sphingomonas* sp. R1 isolated from
897 rhizosphere of tomato. (A) *Agrobacterium* -only or co-infected with *Sphingomonas*
898 sp. R1 at 1:1 ratio in soil were recovered from the surface of wounded stem segments
899 at 10 dpi, the CFU of *Agrobacterium* and *Sphingomonas* sp. R1 were plotted. T-test
900 indicated the CFU differences of *Sphingomonas* sp. R1 inoculation alone or co-
901 infection with T6SS mutants as compared to WT with indicated *p* values. Data are
902 mean ± SD of three independent experiments, each with five seedlings inoculated for
903 each strain. ANOVA test indicated no significant difference among means (*p*-value =
904 0.0945 and 0.0551 for *Agrobacterium* and *Sphingomonas* sp. R1 CFUs, respectively).
905 (B) The *in vitro* competition results based on *Agrobacterium* :*Sphingomonas* sp. R1 at
906 1:1 and 10:1 ratio, and the survival of *Sphingomonas* sp. R1 was plotted; lines and
907 error bars indicate mean ± SD of four biological replicates from two independent
908 experiments. Based on ANOVA followed by Tukey's multiple comparison test, C58
909 WT significantly reduced *Sphingomonas* sp. R1 CFUs when mixed in 10:1 ratio (*p*-
910 value = 0.0153) but not in 1:1 ratio (*p*-value = 0.2753).
911

912 **Figure S1. DNA sequence alignment of 16S rRNA genes of selected bacterial**
913 **strains and tomato KnowYou 301 chloroplast and mitochondria.**
914 The 16S rRNA gene sequences of chloroplasts and mitochondria in *Solanum*
915 *lycopersicum* (tomato) cultivar Known-You 301 were obtained from this study. All of
916 the bacterial 16S rRNA gene sequences were accessed from Reference Sequence
917 (RefSeq) database in National Center of Biotechnology Information (NCBI). The
918 species, strain name, and accession number of 16S rRNA gene were listed on the left
919 of each sequence. The alignment of sequences mentioned above were aligned in
920 MEGAX via ClustalW multiple alignment with gap opening penalty 15.00 and gap
921 extension penalty 6.66 (default). The levels of shaded blue color reflect the degree of
922 identity. The regions of each primer and blocker were underlined. The sequences

923 highlighted by red, black, yellow and green are the annealing region of primer sets for
924 V1-3, V3-4, V5-7 and V6-8 in 16S rRNA gene, respectively. And the gray framed
925 sequences in tomato chloroplast and mitochondrial 16S rRNA genes are the blockers
926 for cognate primers.

927

928 **Figure S2. Alpha rarefaction curves of the observed bacterial OTUs based on**
929 **amplicon sequencing of 53 gall samples in sequencing run III.**
930 The rarefaction curves were plotted after clustering DADA2-output ASVs into 99%
931 OTUs. Sample size in X-axis indicates different sub-sampling depth of each dataset;
932 Y-axis indicates the number of observed OTUs under certain sub-sampling
933 depth. Label of each curves indicates the sample ID in metadata.
934

935 **Figure S3. Competition of *Agrobacterium* and *Sphingomonas* sp. R1 in tomato**
936 **gall on stem at 28 dpi.**
937 Each of the three *Agrobacterium* strains (i.e., C58 WT, Δ*tssL*, and Δ*tssB*) and R1 were
938 mixed at 1:1 ratio then inoculated on wounded tomato stem. The galls were harvested
939 and homogenized for plating at 28 dpi. *Agrobacterium* strains and *Sphingomonas* sp.
940 R1 were recovered on 523 medium plates containing proper antibiotics. CFU data for
941 agrobacteria are mean ± SD of three independent experiments/batches, each with five
942 seedlings inoculated for each strain. R1 could only be recovered from galls in batch 3.
943 The *p*-value of ANOVA against CFU numbers of C58 for each batch is 0.786, 0.0023
944 and 0.230; the *p*-value of ANOVA against CFU numbers of R1 was 0.6413. The
945 significance of CFU numbers of C58 in second batch is due to the low CFU counts of
946 Δ*tssL* single inoculation group, which could not be replicated in other two batches.
947

948 **Figure S4. Agrobacteria colonization on wounded stem segments.**
949 The colonies of *Agrobacterium* strains C58 WT, Δ*tssL*, and Δ*tssB* recovered from the
950 surface of wounded stem segments following the soil inoculation procedure were
951 plotted. Different symbols indicated the outcome from different batches of
952 colonization assay. *F*-test indicated the variance of Δ*tssL* and Δ*tssB* were significantly
953 different when compared to C58 WT ($p(F \leq f) = 0.00028$ and 1.77826E-05,
954 respectively). Brown-Forsythe and Welch ANOVA test indicated no significant
955 difference among means (*p*-value= 0.4847). Line indicates median.

956 **Table 1**. Incidences of crown gall disease on wounded tomato stems by soil

957 inoculation

Batch	Date of inoculation	Disease incidence (diseased/total inoculated seedlings)		
		C58 WT[a]	Δ*tssL*[b]	Δ*tssB*[b]
1	2018/11/15	0.86 (12/14)	0.86 (12/14)	N.D.*
2	2018/12/20	0.30 (6/20)	0.45 (9/20)	N.D.
3	2019/06/10	0.10 (2/20)	0.05 (1/20)	0.05 (1/20)
4	2019/07/04	0.35 (7/20)	0.35 (7/20)	0.26 (5/19)
5	2019/07/22	0.30 (6/20)	0.00 (0/20)	0.10 (2/20)
6	2019/07/29	0.27 (4/15)	0.27 (4/15)	0.14 (2/14)
7	2019/10/05	0.35 (7/20)	0.25 (5/20)	0.30 (6/20)
8	2019/10/30	0.50 (8/16)	0.25 (4/16)	0.38 (6/16)
9	2020/11/11	0.94 (15/16)	0.56 (9/16)	0.50 (8/16)

[a, b]: Two-way ANOVA followed by Tukey's multiple comparisons test on disease incidences of different strains indicated the disease incidence of Δ*tssL* and Δ*tssB* was significantly lower than *Agrobacterium* C58 WT (*p*-value are 0.0225 and 0.0227 respectively). *: Not Determined.

958

959

960 **Table 2**. Sum of read counts in six tumor samples before and after filtering non-
961 bacterial reads from amplicon sequencing Run I for protocol test

16S rRNA regions	Before filtering	Bacterial reads	Percentage of non-bacterial DNA
V3-4	140,830	1,230	99.1%
V5-7	601,299	38,320	93.6%

962
963

964 **Table 3** The read count before and after filtering non-bacterial ASVs from the three
965 crown gall DNA samples (named as W21, W22, and W25) induced by *Agrobacterium*
966 C58 wild type

Sample-id	Dataset-id	Read before filtering	Reads after filtering chloroplast and mitochondrial DNA	Percentage of non-host reads
1115-21W	T21-v1-3-B	176,544	9,914	5.6%
1115-22W	T22-v1-3-B	183,475	5,608	3.1%
1115-25W	T25-v1-3-B	152,387	2,404	1.6%
1115-21W	T21-v1-3-A	185,107	853	0.5%
1115-22W	T22-v1-3-A	247,240	379	0.2%
1115-25W	T25-v1-3-A	178,980	220	0.1%
1115-21W	T21-v3-4-B	239,243	38,462	16.1%
1115-22W	T22-v3-4-B	246,063	19,218	7.8%
1115-25W	T25-v3-4-B	209,991	11,185	5.3%
1115-21W	T21-v3-4-A	200,185	979	0.5%
1115-22W	T22-v3-4-A	205,966	796	0.4%
1115-25W	T25-v3-4-A	193,350	426	0.2%
1115-21W	T21-v5-7-B	129,422	53,715	41.5%
1115-22W	T22-v5-7-B	140,012	29,078	20.8%
1115-25W	T25-v5-7-B	135,692	18,052	13.3%
1115-21W	T21-v5-7-A	150,015	2,792	1.9%
1115-22W	T22-v5-7-A	139,974	1,507	1.1%
1115-25W	T25-v5-7-A	133,035	786	0.6%
1115-21W	T21-v6-8-B	92,167	3,691	4.0%
1115-22W	T22-v6-8-B	92,053	3,197	3.5%
1115-25W	T25-v6-8-B	82,361	2,077	2.5%
1115-21W	T21-v6-8-A	121,145	1,604	1.3%
1115-22W	T22-v6-8-A	138,186	1,273	0.9%
1115-25W	T25-v6-8-A	121,820	708	0.6%
	NC-v3-4-B	1,012	979	96.7%

967

968

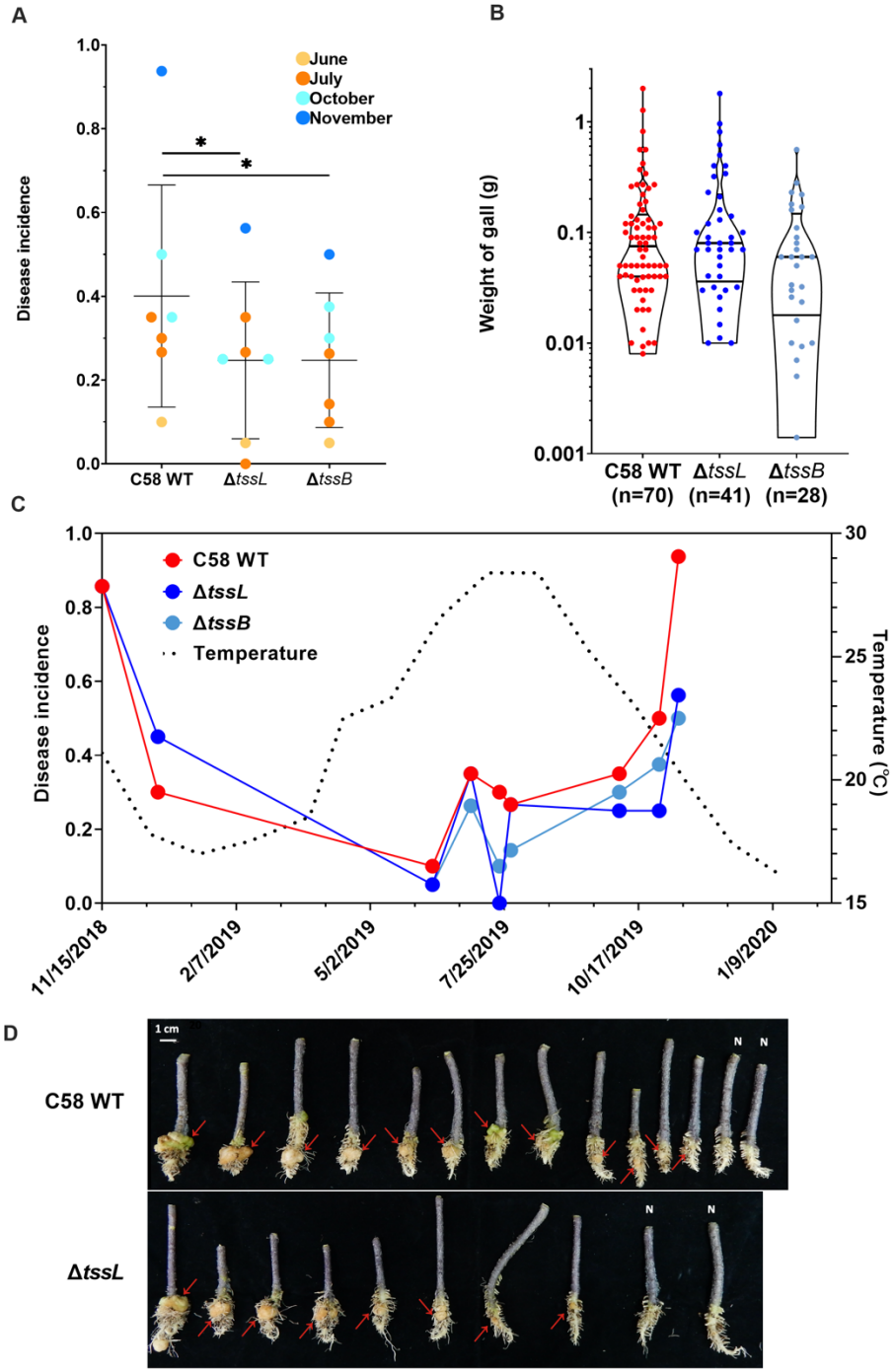

Figure 1. Tumorigenesis assay of agrobacterial strain C58 and its T6SS-deficienct mutants.

For each experiment, 14-20 wounded tomato seedlings were grown in soil containing one of the strains tested (i.e., C58 WT, Δ*tssL*, and Δ*tssB*) and collected at 60 days post inoculation (dpi). (A) Disease incidence plotted by strain and color-coded according to the month of inoculation (light yellow, June; orange, July; light blue, October; dark blue, November). Lines and error bars indicate mean ± SD. Statistical significance was tested using two-way ANOVA followed by Tukey's multiple comparisons; *p*-value = 0.025 and 0.027 when comparing the wild type to Δ*tssL* and Δ*tssB*, respectively. (B) Weight distribution of the galls collected (See details in Table S7). No significant difference among the three strains (*p*-value= 0.15, Kruskai-Wallis test). (C) Correlation plot of disease incidence and temperature of daily average. (D) Crown galls generated through soil inoculation, example from inoculation on 11/15/2018. N indicated no gall formation in some of inoculated plants.

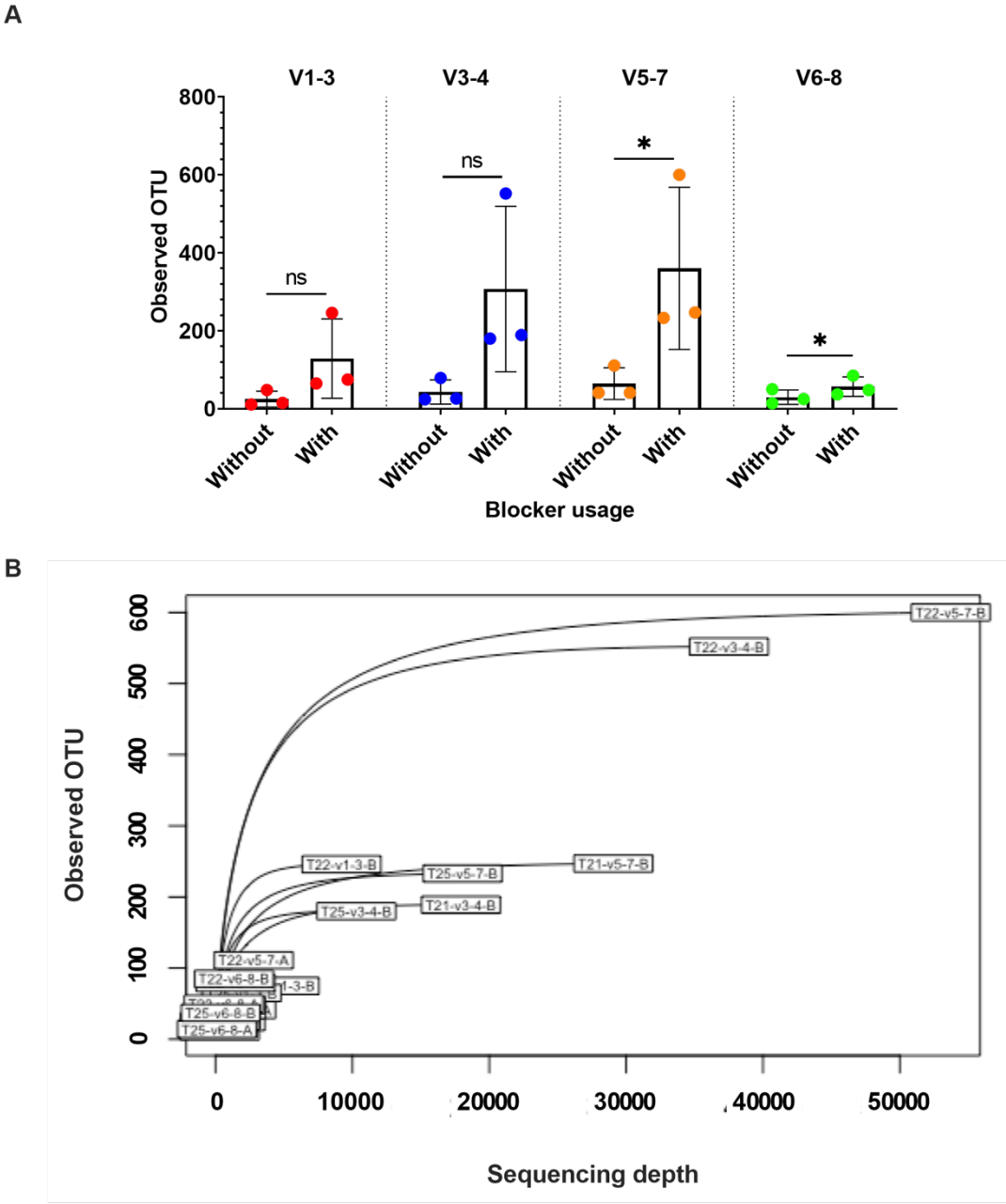

Figure 2. Optimizing 16S rRNA gene amplification by using PCR blockers.

Three crown gall samples (named as 21W, 22W and 25W) induced by C58 wild type were used for analysis. V1-3, V3-4, V5-7 and V6-8 represent different primer pairs targeting different variable regions on 16S rRNA gene. (A) The OTU counts of 16 rRNA gene amplicons after filtering non-bacterial OTUs. (ns, not statistically significant; *, *p-value* < 0.05, student's t-test). The bar indicated mean of OTUs ± SD. (B) Alpha rarefaction curves of the observed bacterial OTUs based on datasets from amplicon sequencing run II. Sample size in X-axis indicates different sub-sampling depth of each dataset.

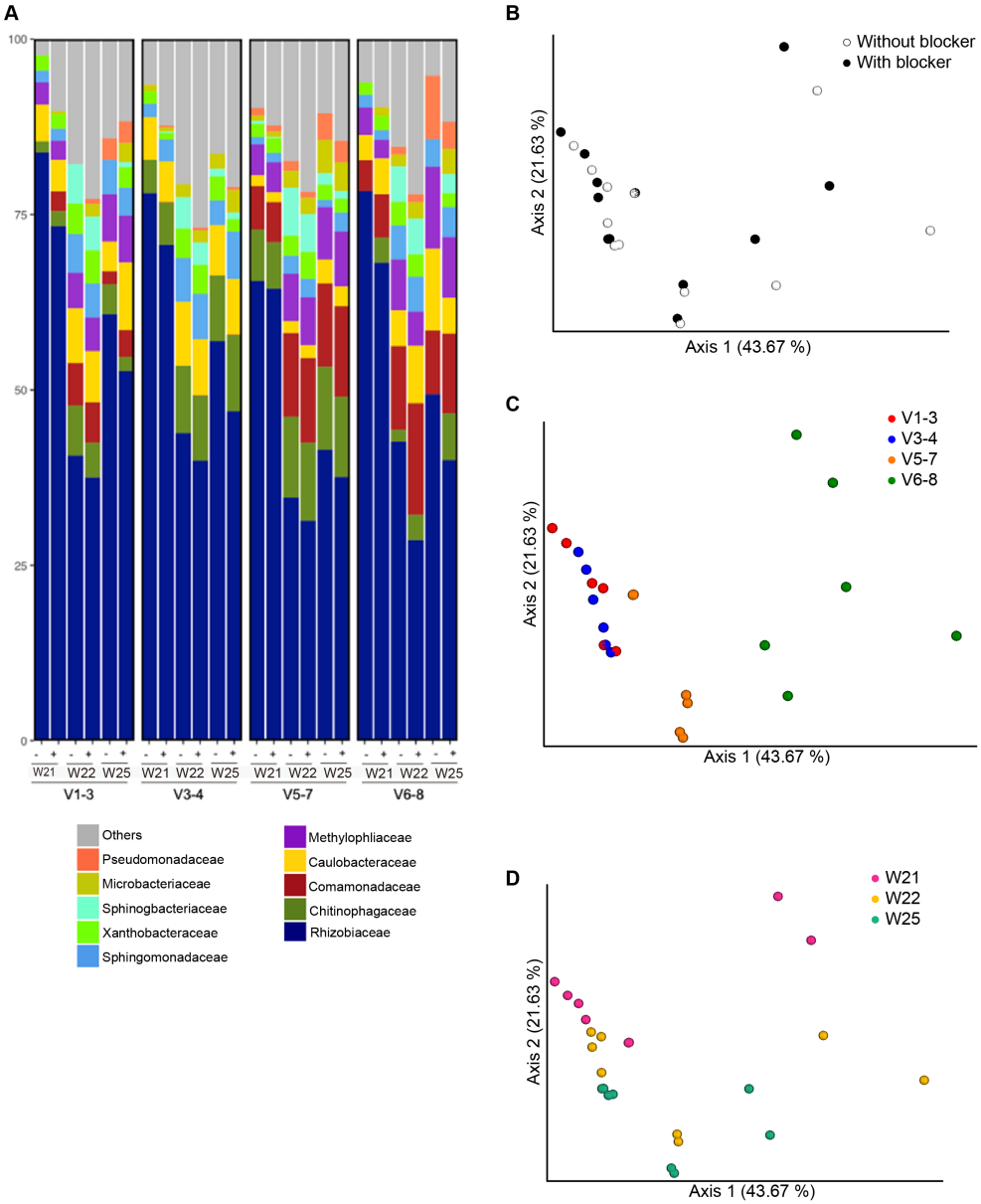

Figure 3. Gallobiome composition when using different primers amplified with or without blockers. (A) Different primer sets (V1-3, V3-4, V5-7 and V6-8**)** for analysis of three crown gall samples (21W, 22W and 25W, see details in Table S8) induced by C58 wild type were indicated. Top 10 observed families were listed. "-": amplification without blockers; "+": amplification with blockers. Principle coordinate analysis (PCoA) plots of the bacterial composition of the datasets were generated based on the weighted Unifrac matrix, and color coded by (B) blocker usage (p-value = 0.068, R^2 = 0.026, ADONIS), (C) different primer sets (p-value = 0.001, R^2 = 0.524, ADONIS) and (D) different crown gall samples (p-value = 0.001, R^2 = 0.238, ADONIS).

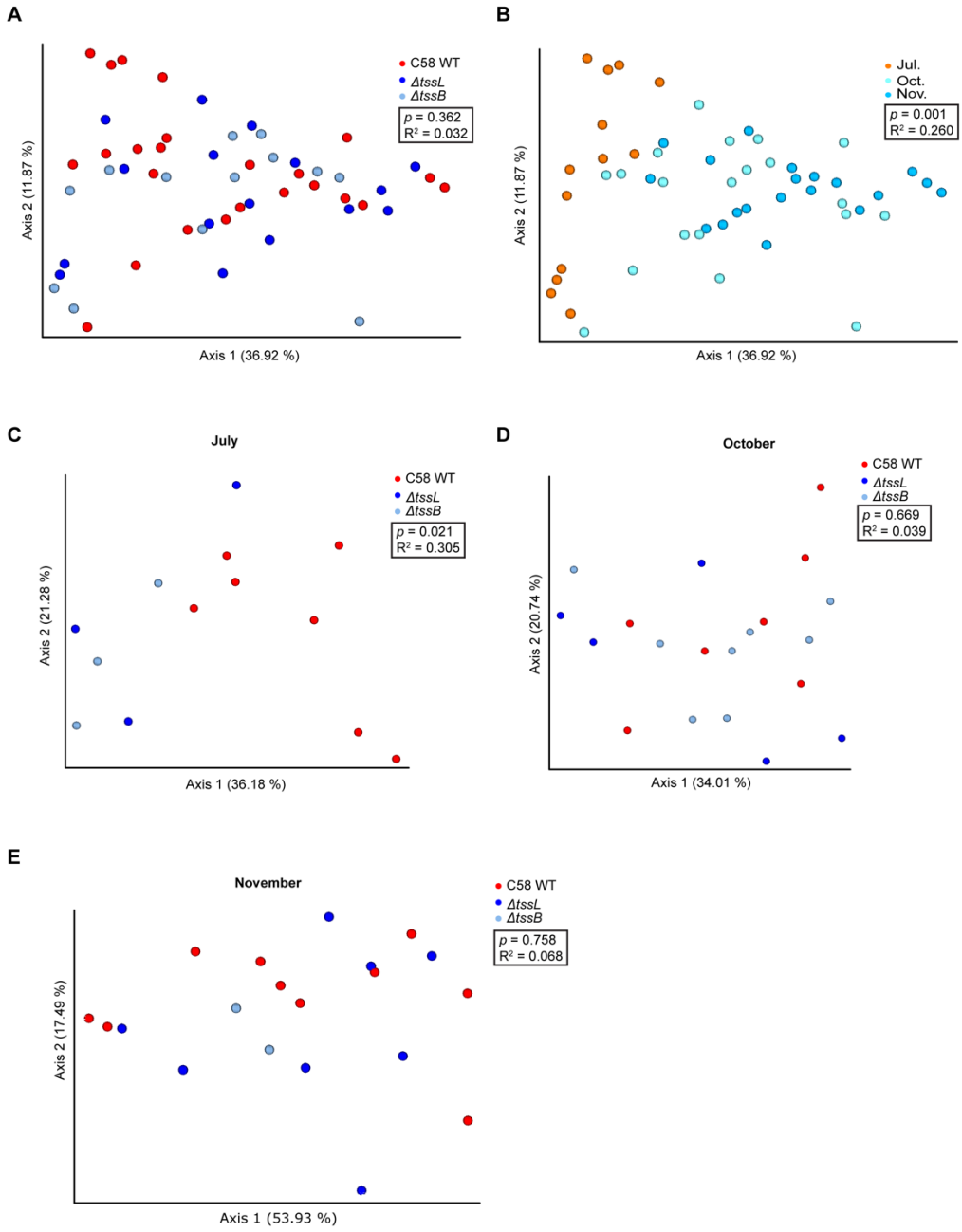

Figure 4. Principle coordinate analysis (PCoA) of bacterial composition in 53 crown galls induced by C58 WT and T6SS mutants.

The plots were drawn based on weighted Unifrac matrix of the bacterial communities. Crown galls induced by different strains (C58 WT, Δ*tssB,* Δ*tssL*) (A) or inoculated in different months (B) were labeled with different colors in each panel. The datasets were further split based on the month of inoculation, and the PCoA plots of gallobiomes from (C) July, (D) October and (E) November were indicated above. Statistical differences in clustering were evaluated via ADONIS permutation test, and the corresponding *p*-values and R^2 are indicated.

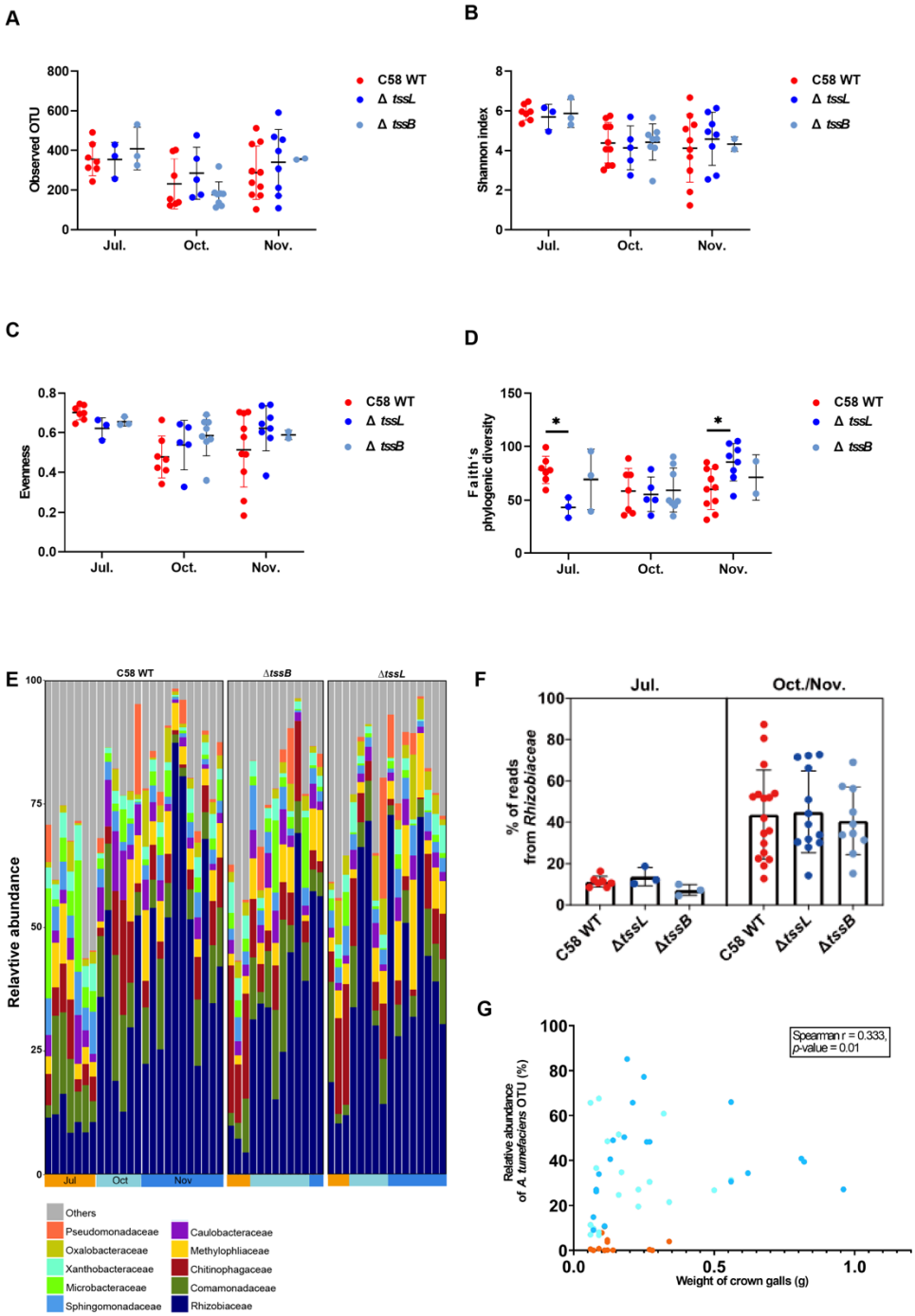

Figure 5. Alpha diversity and composition of crown galls generated in different months by C58 WT and T6SS mutants. (A) Observed OTU, (B) Shannon index, (C) evenness, and (D) Faith's phylogenic diversity of each sample generated in different months was indicated. Lines and error bars indicate mean ± SD. Two-way ANOVA followed by Tukey's multiple comparisons test were performed, and the asterisks indicated the statistically significant difference between two strains (p-value < 0.05). (E) Bacterial composition of 53 crown galls. For each strain, the samples are grouped by the month of inoculation. Top-ten most abundant families were listed and colored differently, and the remaining ones are combined into "Others". (F) Relative abundance of Rhizobiaceae in galls inoculated in different months and strains. (G) Scatter plot of relative abundance of agrobacteria OTU in gallobiome - weight of the crown galls. A positive correlation between weight of crown gall and relative abundance of *Agrobacterium* C58 was observed (Spearman r = 0.333, p-value = 0.01).

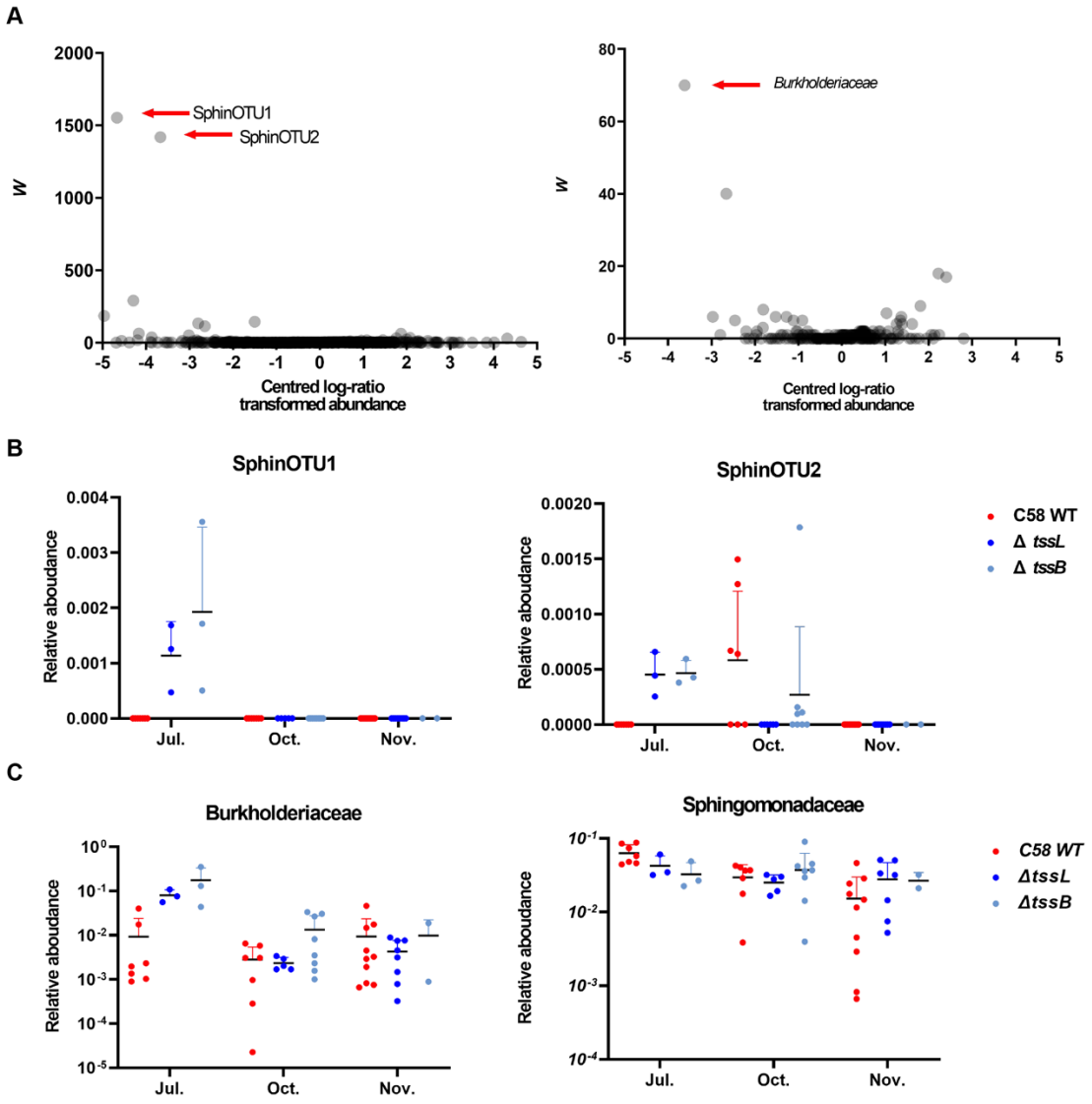

Figure 6. Analysis of composition of microbiomes (ANCOM) for gallobiomes in July.

The ANCOM volcano plots were drawn based on the centred-log-ratio-transformed abundance of (A) OTUs or bacterial families. The taxa that exhibit differential abundance (DA) between gallobiomes induced by C58 WT and T6SS mutants are indicated by red arrows. The two DA OTUs belonging to *Sphingomonadaceae* were named SphinOTU1 and SphinOTU2, respectively. The relative abundance of focal taxa was shown in panels (B) and (C). Lines and error bars indicate mean ± SD.

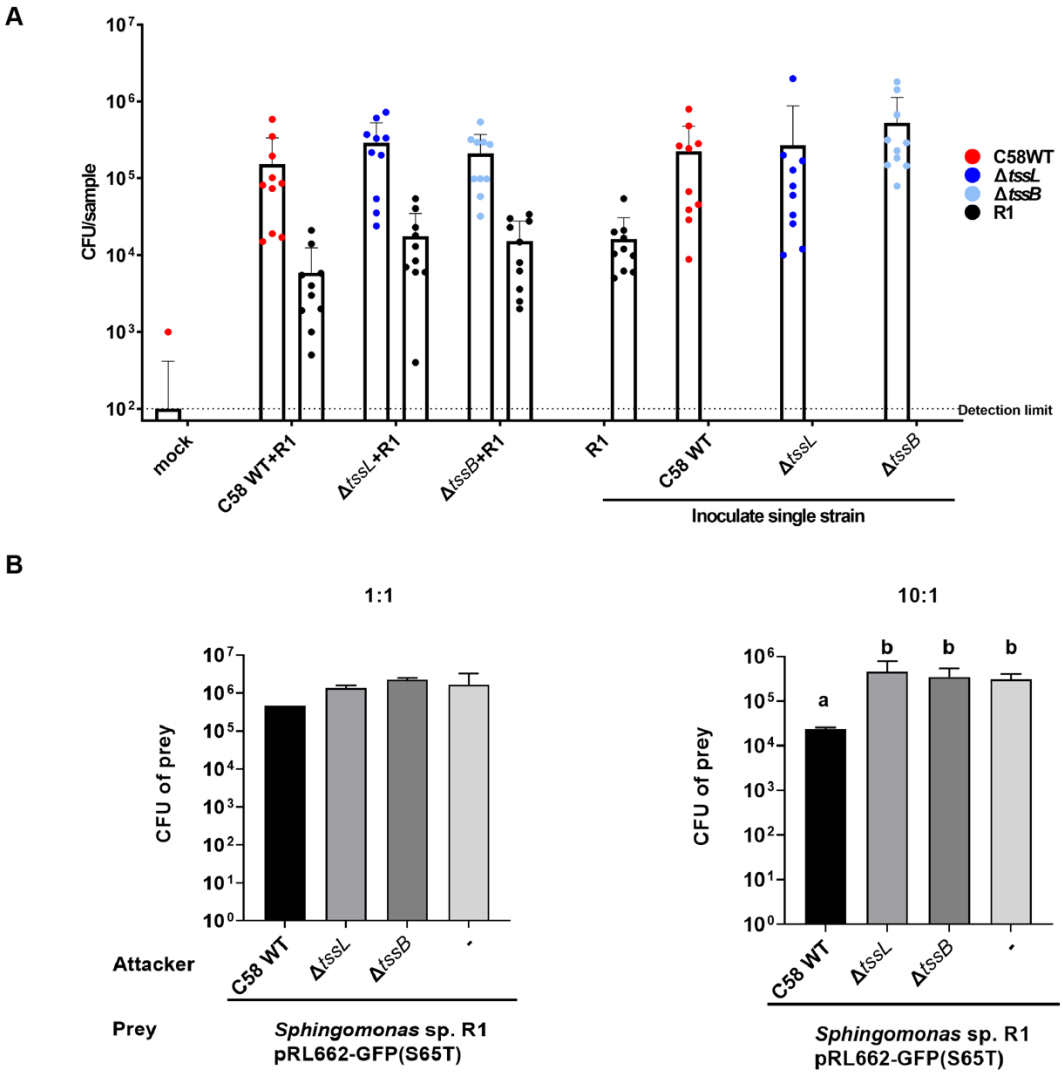

Fig. 7 Competition between C58 and *Sphingomonas* sp. R1 *in vitro* and on wounded site of tomato stems.

Each of the three *Agrobacterium* strains (i.e., C58 WT, Δ*tssL*, and Δ*tssB*) was individually tested for competition against *Sphingomonas* sp. R1 isolated from rhizosphere of tomato. (A) *Agrobacterium* -only or co-infected with *Sphingomonas* sp. R1 at 1:1 ratio in soil were recovered from the surface of wounded stem segments at 10 dpi, the CFU of *Agrobacterium* and *Sphingomonas* sp. R1 were plotted. T-test indicated the CFU differences of *Sphingomonas* sp. R1 inoculation alone or co-infection with T6SS mutants as compared to WT with indicated *p* values. Data are mean ± SD of three independent experiments, each with five seedlings inoculated for each strain. ANOVA test indicated no significant difference among means (*p*-value = 0.0945 and 0.0551 for *Agrobacterium* and *Sphingomonas* sp. R1 CFUs, respectively). (B) The *in vitro* competition results based on *Agrobacterium* :*Sphingomonas* sp. R1 at 1:1 and 10:1 ratio, and the survival of *Sphingomonas* sp. R1 was plotted; lines and error bars indicate mean ± SD of four biological replicates from two independent experiments. Based on ANOVA followed by Tukey's multiple comparison test, C58 WT significantly reduced *Sphingomonas* sp. R1 CFUs when mixed in 10:1 ratio (*p*-value = 0.0153) but not in 1:1 ratio (*p*-value = 0.2753).