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Authors

Li, Qian

Guo, Rongjun

Li, Yujia

et al.

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1Insight into the Bacterial Endophytic Communities of Peach

2Cultivars Related to Crown Gall Disease Resistance

3

4Qian Li,^{a,b,c} Rongjun Guo,^b Yujia Li,^b Wyatt H. Hartman,^c Shifang Li,^b

5Zhixiang Zhang,^b Susannah G. Tringe,^{c,#} Hongqing Wang^{a,#}

6

7^aDepartment of Fruit Science, College of Horticulture, China Agricultural

8University, Beijing, China

9^bState Key Laboratory for Biology of Plant Diseases and Insect Pests,

10Institute of Plant Protection, Chinese Academy of Agricultural Sciences,

11Beijing, China

12^cDepartment of Energy Joint Genome Institute, Walnut Creek, California,

13USA

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15Running Head: Peach endophytes and crown gall resistance

16

17[#]Address correspondence to Susannah G. Tringe, sgtringe@lbl.gov, or

18Hongqing Wang, wanghq@cau.edu.cn.

19ABSTRACT Crown gall disease caused by *Agrobacterium tumefaciens*
 20severely impacts the production of peach and other fruit trees. Several
 21peach cultivars are partially resistant to *A. tumefaciens*, but little is known
 22about the roles of endophytic microbiota in disease resistance. In the
 23present study, the endophytic bacterial communities of resistant and
 24susceptible peach cultivars ‘Honggengansutao’ and ‘Okinawa’ were
 25analyzed using universal 16S rRNA gene amplicon sequencing in parallel
 26with cultivation and characterization of bacterial isolates. A total of
 271,357,088 high-quality sequences representing 3,160 distinct OTUs
 28(Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes) and 1,200
 29isolates of 20 genera and 305 distinct ribotypes were collected in peach
 30roots and twigs. It was found that factors including plant developmental
 31stage, cultivar, and *A. tumefaciens* invasion strongly influenced the peach
 32endophytic communities. The community diversity of endophytic bacteria
 33and the abundance of culturable bacteria were both higher in the roots of
 34resistant cultivar, particularly after inoculation. Strikingly, the pathogen
 35antagonists *Streptomyces* and *Pseudomonas* in roots and *Rhizobium* in
 36twigs were most frequently detected in resistant plants. Our results
 37suggest that the higher abundance and diversity of endophytic bacteria
 38and increased proportions of antagonistic bacteria might contribute to the
 39natural defense of resistant cultivar against *A. tumefaciens*. This work
 40reveals the relationships between endophytic bacteria and disease
 41resistance in peach **plants**, and provides important information for
 42microbiome-based biocontrol of crown gall disease in fruit trees.

43IMPORTANCE *Agrobacterium tumefaciens* as the causal agent of peach

44crown gall disease can be controlled by planting resistant cultivars. This
45study profiles the endophytic bacteria in susceptible and resistant peach
46cultivars, advancing our understandings of the relationships between
47endophytic bacterial communities and peach crown gall disease, with
48potential implications for other complex microbiome-plant-pathogen
49interactions. The resistant cultivar may defend itself by increasing the
50diversity and abundance of beneficial endophytic bacteria. The
51antagonists identified among the genera *Streptomyces*, *Pseudomonas*,
52and *Rhizobium* may have application potential for biocontrol of crown gall
53disease in fruit trees.

54**KEYWORDS** peach, endophytic bacteria, high throughput sequencing,
55crown gall disease, resistance, *Agrobacterium tumefaciens*

56INTRODUCTION

57*Agrobacterium tumefaciens*, the causal agent of crown gall disease,
 58infects dicotyledonous plants of approximately one hundred botanical
 59families (1). Based on comparative 16S rRNA analyses, *A. tumefaciens* has
 60been formally reclassified into *Rhizobium radiobacter* (2), which
 61encompasses both pathogenic and non-pathogenic strains. In the current
 62study, we still treated the pathogenic strains as *A. tumefaciens* to
 63distinguish it from non-pathogenic *R. radiobacter*. The pathogen can
 64survive in soil or plant debris, and infects host plants through fresh
 65wounds using chemotactic sensing and motility. By injecting the transfer
 66DNA (T-DNA) derived from a tumor-inducing (Ti) plasmid into the plant
 67genome, *A. tumefaciens* causes overgrowths of the host, appearing as
 68galls on root collars, roots and twigs (3). Small, soft, and white lumps first
 69appear a few days after infection, which harden to form woody galls; as a
 70result, the water and nutrient transport by vascular tissues is limited,
 71ultimately stunting the plant growth and causing the yield loss of fruit (4).

72 Crown gall disease accounts for significant economic losses of peach
 73production in China (5). There are two effective measures to control this
 74disease in orchards, i.e. planting resistant cultivars and introduction of
 75biological antagonists. Although peach cultivars ‘Mr.S.2/5’ (6), ‘Cadaman’
 76(7), ‘St. Julien 655/2’ (8), and ‘Honggengansutao’ and ‘Xibei13-1’ (9) have
 77shown resistance to crown gall disease, the resistance does not appear
 78consistent across geographic locations. The antagonistic bacterium *R.*
 79*radiobacter* K84 and its genetically modified strain K1026 can suppress *A.*
 80*tumefaciens* through agrocin-84 production (10) and niche competition

13

81(11, 12), and have been successfully developed as biocontrol agents.

82However, universal biocontrol of crown gall disease by these antagonists

83is challenged by the resistance of many *A. tumefaciens* strains to K84 (13,
8414).

85 Microorganisms that spend at least part of their life cycle inside plants

86are called endophytes (15), and their communities may represent the

87extended phenotype of a host (16). Endophytic microbiota are shaped by

88both the host plant and environmental stimuli, and in turn may enhance

89the biotic and abiotic tolerance of their host plants as a multispecies

90functional unit (17). The abundance and diversity of endophytic microbial

91communities vary a lot in resistant and susceptible cultivars of some

92plants (18–21), and the community composition may also be altered by

93pathogen infection (22–25). Previous studies indicate that endophytic

94communities can inhibit pathogen invasion and prevent or reduce disease

95development by outcompeting phytopathogens, producing antimicrobial

96compounds, or inducing plant resistance (26). The colonization of specific

97endophytes has been demonstrated to successfully reduce disease

98incidence and severity in several fruit trees including citrus (18),

99grapevine (22), banana (27), and apple (28). In peach roots, five

100endophytic bacteria (*Brevundimonas diminuta*, *Leifsonia shinshuensis*,

101*Sphingomonas parapaucimobilis*, *B. vesicularis*, and *R. radiobacter*)

102isolated from *in vitro* cultures were found to produce indole-3-acetic acid

103(IAA, a plant hormone), fix nitrogen, and solubilize phosphate (29).

104Moreover, endogenous *Enterobacter*, *Pantoea* and *Rhizobium* isolated

105from the resistant peach cultivar 'Xibei 13-1' demonstrate antagonism to

14

15

106A. *tumefaciens* *in vitro* and in greenhouse trials (30). Therefore,
107endophytes with resistance-promoting capabilities are of great scientific
108and economic importance for fruit trees.

109 Endophytic bacteria can be characterized by using culture-dependent
110approaches, which are conducive to physiological and functional analysis
111(32, 33), or can be analyzed by DNA sequencing, which provides insight
112into the structure and diversity of endophyte community (34–37). The
113combination of isolation, phenotypic testing, and massively parallel
114sequencing enables more precise dissection of the whole bacterial
115community (43). Thus, the present study employed both culture-
116dependent and -independent methods to determine the bacterial
117endophyte communities of two peach cultivars, resistant
118‘Honggengansutao’ and susceptible ‘Okinawa’, and focused on the
119endophyte responses to *A. tumefaciens* invasion. We aim to provide a
120better understanding of complex microbiota-plant-pathogen interactions,
121and reveal which endophytic microbiota may contribute to plant
122resistance to root diseases.

123

124RESULTS

125 **Susceptibility of peach cultivars to crown gall disease.** The
126susceptibility of different peach cultivars to crown gall disease was tested
127on peach roots in the greenhouse and newly grown twigs in the field.
128Disease onset occurred in both roots and twigs 10 days post-inoculation
129(D10), and crown gall tumors developed rapidly thereafter until D60 (Figs.
1301A and 1B). In root collars, crown gall disease was severe in susceptible

cultivar 'Okinawa', as evidenced by larger galls (2.1 vs. 0.6 gall/stem diameter ratio) and higher incidence rates (84.6% vs. 48.7%) and disease index (74.8 vs. 28.2) than the resistant 'Honggengansutao' ($P \leq 0.01$ in all cases; Fig. 1C). Similar results were observed in twigs, with average gall/twig diameter ratios of 2.2 vs. 0.8, incidence rates of 92.7% vs. 75.2%, and disease index of 71.6 vs. 28.1 in cultivars 'Okinawa' and 'Honggengansutao' ($P \leq 0.05$ in all cases; Fig. 1D), respectively. No symptoms were observed in uninoculated plants. The results indicated that the resistant cultivar 'Honggengansutao' was highly effective in deterring gall development in peach roots and twigs.

141

142 **Endophytic bacterial communities in peach roots and twigs.**

Tissues from cultivars 'Okinawa' and 'Honggengansutao' with and without *A. tumefaciens* inoculation were collected in triplicate from both roots (of greenhouse-grown trees) and twigs (of field-grown trees) at D0, D10 and D60, resulting in 60 samples (Fig. S1). The V5–V7 region of the bacterial 16S rRNA gene, approximately 400 bp in length, was amplified using PCR and sequenced using the Illumina Miseq platform, generating a total of 1,357,088 high-quality sequences (9,484–46,736 sequences per sample; Table S1). After clustering using >97% sequence similarity and removing OTUs of less than 5 counts, 1,842 and 1,318 distinct OTUs were observed in roots and twigs, respectively (Table S1). To describe the endophytic bacterial communities of the root and twig microbiota, a representative sequence of each OTU was assigned to a taxonomic classification by comparison with the Silva database. **Negative controls had no specific**

156product amplified.

157 Differences were observed in the community compositions of
 158endophytic bacteria in peach roots and twigs. Overall, endophytic
 159assemblages were dominated by Proteobacteria, Actinobacteria,
 160Bacteroidetes and Firmicutes at the phylum level, accounting for 49.8–
 16199.0% of the total bacterial community regardless of compartment,
 162cultivar, treatment or time of sampling (Fig. S2). At the genus level,
 163*Streptomyces* (average abundance of 23.2%) in roots and *Rhizobium*
 164(average abundance of 24.7%, including *A. tumefaciens*) in twigs were
 165dominant (Fig. 2). The other top genera largely different between roots
 166and twigs (Fig. 2); only *Pseudomonas* and *Rhizobium* were abundant in
 167both. In comparison to the relatively stable distribution of root
 168endophytes, the community composition of twig endophytes varied
 169notably with time and pathogen inoculation.

170

171 **Factors affecting the community composition of bacterial**

172**endophytes.** Nonmetric multidimensional scaling (NMDS) ordination of
 173the root and twig community data (Figs. 3A and 3B **respectively**) and
 174multiple regression tree analysis (Fig. S3) indicated that endophyte
 175communities were first structured by sampling time, followed by cultivar
 176and pathogen inoculation. These effects were validated by permutational
 177multivariate analysis of variance (PERMANOVA, Table S2), randomForest
 178classification (Table S3) and one-way analysis of similarity (ANOSIM, Fig.
 179S4). The susceptible and resistant cultivars also showed different
 180responses to pathogen invasion, displaying similar bacterial communities

181at D10, but divergent ones at D60 (Figs 3A, 3B and S3). It's interesting
 182that the differences in endophytic communities between the two peach
 183varieties were larger in mock inoculated plants compared to those in the
 184infected plants, especially at D10 when the endophytes in mock
 185inoculated plants (Figs. S3 and S5) were more divergent in relation to
 186cultivars. Measures of Shannon diversity also indicated that peach
 187endophytic microbiota changed across sampling time, cultivar, and
 188pathogen inoculation. In roots, of the two cultivars, resistant
 189'Honggengansutao' exhibited significantly higher diversity than 'Okinawa',
 190particularly in the inoculated samples ($P \leq 0.05$, Fig. 3C). In contrast, the
 191endophyte diversity declined in twigs after inoculation; both cultivars
 192showed similar response to the pathogen inoculation, exhibiting a sharp
 193drop at D10 in the inoculated samples, and partial recovery at D60 (Fig.
 1943D). This indicates that *A. tumefaciens* infection has effects on the
 195structure and dynamics of endophyte communities in peach roots and
 196twigs, which differ in susceptible and resistant cultivars. In combination
 197with the bacterial abundance analysis, the population fluctuations of
 198inoculated pathogen might contribute to the changes of community
 199diversity.

200

201 **Differentially abundant endophytic bacteria in peach cultivars.**

202A total of 57 and 34 OTUs were significantly enriched in the roots of
 203'Honggengansutao' and 'Okinawa' (Kruskal-Wallis test, $P \leq 0.05$; Fig. S6A),
 204respectively, at one or more time point(s), but only 5 and 1 were
 205consistently elevated in each cultivar. *Pseudomonas* sp. (OTU_18r) was

206 found to be closely associated with resistant ‘Honggengansutao’ (Fig. 4A).
 207 According to the similarity percentage analysis (SIMPER), OTU_18r
 208 contributed 19.8%, 8.9% and 7.9% to the dissimilarities of root endophytic
 209 communities at D0, D10 and D60 (Table S4), respectively. Another major
 210 root endophyte of ‘Honggengansutao’, *Streptomyces* sp. (OTU_1r), was
 211 more abundant in inoculated roots at D10 (30.2% vs. 13.4% of mock-
 212 inoculated roots, $P \leq 0.05$), and was the major differential component of
 213 the ‘Honggengansutao’ bacterial community at D60 (30.2% vs. 11.6% of
 214 ‘Okinawa’ roots, $P \leq 0.05$; Fig. 4A and Table S4). Candidate division OD1
 215 (9/10 OTUs), Planctomycetes (9/9 OTUs) and Chloroflexi (4/7 OTUs) were
 216 also abundant in ‘Honggengansutao’ at D10 (Fig. S6A).

217 In agreement with the cultivar comparison in roots, more OTUs were
 218 enriched in the twigs of the resistant cultivar (65 vs. 40; Kruskal-Wallis
 219 test, $P \leq 0.05$; Fig. S6B), but the differential OTUs were different from
 220 those in roots. More differentially abundant endophytic bacteria emerged
 221 with the plant growth, as identified in both Kruskal-Wallis test and SIMPER
 222 analysis (Table S5). Genera *Rhizobium*/*Agrobacterium* (OTU_2t),
 223 *Pseudomonas* (OTU_6t), *Pantoea* (OTU_11t), *Curtobacterium* (OTU_12t),
 224 and *Massilia* (OTU_22t) were enriched in resistant ‘Honggengansutao’ at
 225 D60 (Fig. 4B and Table S5). The abundance of some bacterial endophytes
 226 in twigs also responded to *A. tumefaciens* inoculation (Fig. 4B). Most
 227 notably, OTU_2t, which matched the inoculated *A. tumefaciens* as well as
 228 *Rhizobium* sp., accounted for 70.1% of the total Bray-Curtis dissimilarity,
 229 and made up 64.7% and 31.4% sequences of the inoculated and mock-
 230 inoculated twigs at D10, respectively (Table S5). However, the

proportional frequencies and the difference of genus *Rhizobium* between the mock inoculated and inoculated plants disappeared by D60. Although *Bacillus*, the well-known antagonist, represented a low proportion of amplicon sequences, it still had higher abundance in the resistant cultivar ($P \leq 0.05$; Fig. S7).

236

Cultivation of peach endophytic bacteria. Endophytic bacteria from both roots and twigs were enumerated, isolated and identified. More colonies were obtained from the resistant cultivar ‘Honggengansutao’ than susceptible ‘Okinawa’ per gram of tissue, especially in roots (3.3×10^4 vs. 2.0×10^3 ; Fig. S8). Sixty bacterial isolates of each subset (cultivar/sampling time/treatment/peach compartment; the total of 1200) were then selected for further studies.

Based on full-length 16S rRNA sequences, 600 isolates from roots were assigned to 10 genera and 143 unique 16S sequences (ribotypes) (Fig. S9A and Table S6). *Pseudomonas* (32.8%) and *Rhizobium* (18.7%, including *A. tumefaciens*) were the most frequently cultivated genera, followed by *Paenibacillus* (15%), *Bacillus* (13.7%) and *Streptomyces* (8.7%). The 600 isolates from peach twigs were assigned to 15 genera and 162 ribotypes, including *Rhizobium* (36.2%, including *A. tumefaciens*), *Pantoea* (11.7%), *Staphylococcus* (8.9%), *Pseudomonas* (5.8%), *Bacillus* (4.2%), and *Enterobacter* (3.3%), etc (Fig. S9B and Table S7). *Rhizobium* (encompassing the inoculated *A. tumefaciens*) was strikingly enriched in twigs at D10, accounting for 85.8% and 36.7% of culturable isolates in the inoculated and mock-inoculated plants, respectively.

Phylogenetic analysis indicated that the 305 distinct ribotypes (143 from roots and 162 from twigs) were clustered into five branches (α -, β -, and γ -Proteobacteria, Actinobacteria, and Firmicutes) and 20 genera (Fig. 5A). Almost all of the *Rhizobium* isolates (84/85) were closely related to *R. radiobacter* (Fig. 5B, including *A. tumefaciens*). In contrast, *Pseudomonas* isolates were more diverse, belonging to ten species with most closely related to *P. putida* and *P. poae* (Fig. 5C).

263

264 **Antagonistic/pathogenic characterization of bacterial isolates.**

The antagonistic activities of 305 endophytic isolates against *A. tumefaciens* were tested *in vitro* (Fig. 5A, Tables S6 and S7). Fifty-four strains, mainly belonging to *Rhizobium*, *Pseudomonas*, *Bacillus*, and *Pantoea* showed significant antagonism. These antagonists were mostly isolated from resistant ‘Honggengansutao’ (14/18 in roots and 25/36 in twigs, respectively; $P \leq 0.05$; Table S8). Approximately 50% of the antagonists from ‘Honggengansutao’ were isolated from the mock-inoculated samples, which was higher than that in ‘Okinawa’ ($P \leq 0.05$; Table S8). It suggested that resistant cultivar ‘Honggengansutao’ may possess inherently antagonistic endophytes even in the absence of *A. tumefaciens*.

Further analysis of the pathogenicity-related *ipt* gene by PCR and re-inoculation tests in sunflowers indicated that none of the *Rhizobium* strains from roots were pathogenic, while 12 of the 56 *Rhizobium* isolates from twigs harbored the pathogenic gene *ipt*. Among the 12 *Rhizobium*

280 strains, 8 of them derived from the inoculated susceptible ‘Okinawa’, and
 281 4 from the inoculated ‘Honggengansutao’ (Fig. 5B).

282

283 **DISCUSSION**

284 The crown gall disease caused by *A. tumefaciens* is one of the most
 285 important diseases in peach. Continuous plantation leads to the
 286 accumulation of *A. tumefaciens* in soil (5), and makes the disease more
 287 serious. **Until now**, only one biocontrol agent, K84, was commercialized;
 288 however, its application is limited due to the sole efficacy on nopaline
 289 strains of *A. tumefaciens* (38) and inconsistent effects in different
 290 environments (39). Other strategies to control crown gall disease are
 291 thereby urgently needed. Previous studies indicated that plant endophytes
 292 can make up a “second genome” of their host and fulfill important host
 293 functions (15, 40). However, few studies on endophytic bacteria have
 294 been conducted on peach, and their roles in disease resistance are
 295 unknown. In the present study, we focused on the ecological responses of
 296 the bacterial endophyte community to *A. tumefaciens* invasion, and
 297 characterized the relationships among endophytic microbiota,
 298 antagonistic endophytes, and plant resistance to *A. tumefaciens*. The
 299 results not only reveal the composition of microbiota in susceptible and
 300 resistant cultivars, but also facilitate the development of beneficial
 301 endophytes for biocontrol purposes.

302 High throughput 16S rRNA gene sequencing gives a detailed picture of
 303 microbiota in terms of diversity and composition, and may provide clues
 304 to microbial functions when coupled with bioinformatic tools. Another

305solution relies on partnering culture-independent studies with culture-
 306dependent ones, i.e. community analysis and characterization of isolates,
 307where dominant or differential bacteria can be selectively isolated for
 308function verification *in vitro* (43–45). Some culturable strains of
 309*Rhizobium*, *Pseudomonas*, *Bacillus* and *Pantoea* are successful biocontrol
 310agents (46–47) or have high biological control potential against crown gall
 311disease (30, 48–55). In this study, similar genera were found to be
 312strongly associated with pathogen invasion in resistant peach cultivar, and
 313some strains showed antagonistic activity via *in vitro* test (Fig. S10).
 314*Streptomyces*, a well-known biocontrol agent and the dominant member
 315(23.2%) of the peach root community, had no antagonistic activity in the
 316pair culturing test (Fig. S11). It could contribute to disease suppression
 317through indirect mechanisms, such as systemic acquired resistance and
 318production of volatile organic compounds (56). However, some important
 319bacteria in the resistant cultivar are relatively unculturable, including
 320prevalent bacterial groups like *Actinoplanes* and *Massilia* as well as
 321seldom characterized and less abundant organisms like Candidate phylum
 322OD1, Planctomycetes, and Chloroflexi (Fig. S2). To verify their functions,
 323new cultivation and screening strategies, like optimization of the culture
 324medium (57) and conditions (58) or multiple *in vitro* tests involved in
 325different suppressive mechanisms, should be considered.

326 The microbiota associated with healthy or crown gall diseased trees
 327has been studied previously by Ji *et al.* (59) using the PCR-DGGE
 328technique, with results indicating that the severity of crown gall disease
 329had no effect on the community structure of rhizosphere bacteria.

330 Similarly, Faist *et al.* (60) reported that the presence/absence of crown gall
 331 disease has no effect on the microbial community compositions of
 332 rhizosphere soil and grapevine roots and canes. However, our results
 333 indicated that the endophytic bacterial community of resistant
 334 'Honggengansutao' is higher in density and diversity in roots, contains
 335 more antagonists against *A. tumefaciens*, and has distinct responses to
 336 pathogen invasion. These findings endorsed the hypothesis that the
 337 endophytic community is not made up of random guests in the plant
 338 habitat (17, 61). Instead, during community assembly, selective pressure
 339 enables the endophytic community to adapt and specialize to host plants;
 340 this coevolution and interactions between plants and beneficial microbes
 341 make endophytes essential to their hosts (62). For example, the resistant
 342 cultivar 'Honggengansutao' hosts a sufficient diversity of 'protective'
 343 endophytes, including *Rhizobium*, *Streptomyces*, *Pseudomonas*, *Pantoea*
 344 and *Bacillus*, which can be provoked by pathogen attack and convey
 345 protective antagonism against phytopathogens (17). And fewer
 346 pathogenic *A. tumefaciens* harboring *ipt* gene were present in the
 347 inoculated 'Honggengansutao' than the inoculated 'Okinawa' (Fig. 5B).
 348 The efficient inhibition of *A. tumefaciens* by 'protective' endophytes might
 349 maintain the pathogen population below the threshold required for
 350 quorum sensing, restrict the T-DNA transfer from *A. tumefaciens* to peach,
 351 and cause smaller galls (63). Furthermore, it has been reported that
 352 salicylic acid (SA)-induced systematic acquired resistance was activated in
 353 'Honggengasutao' by pathogen infection (9). For biocontrol application, a
 354 threshold population level of 10^5 CFU/g root is required for a significant

355 suppression of pathogens (Raaijmakers et al., 1995; Kawaguchi et al.,
 356 2012). However, the peach endophyte populations are within $10\text{--}10^5$ CFU/
 357 g fresh tissue, which is low to directly suppress pathogens. Considering
 358 the diverse and balanced microbial system would be more conducive to
 359 disease resistance (van Elsas et al. 2012), this probiotic consortia may
 360 enhance disease suppression efficacy via intensified resource competition
 361 and interference with the pathogen. For example, the consortia of
 362 *Pseudomonas* spp. with high complexity made better protection to tomato
 363 plants against the root pathogen *Ralstonia solanacearum* than that with
 364 low-complexity (Hu et al., 2016). Although endophytic bacteria are low in
 365 abundance, they might be essential to prevent pathogen establishment
 366 and stimulate host immunity (Jousset et al., 2017). These endophytic
 367 microbes may also involve in other indirect mechanisms, such as plant
 368 growth promotion, systemic resistance induction, better plant interior
 369 niche adaptation, etc, also could make them contribute to plant health
 370 (15, 26).

371 Pathogen invasion brought changes to the cultivar-inherent
 372 endophyte communities, enriching the endophytic *Streptomyces* and
 373 *Rhizobium* in roots and twigs at D10, respectively. Similarly, drought
 374 weakens host selection of grass root microbiota, with a significant
 375 enrichment of Actinobacteria within the host roots (Naylor, 2017). It has
 376 been reported that stress alters internal plant responses, which may have
 377 cascading effects on the structure and function of endophyte community
 378 and provoke enrichment of some specific bacterial taxa (15).

379 In the present study, the peach endophytic bacteria mainly belonged

380to phyla Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes,
381which were dominant during all developmental stages as previously
382reported (66). Although both peach roots and twigs had *Pseudomonas* and
383non-pathogenic *Rhizobium* as the dominant genera, similar to sorghum
384roots and shoots (67), they also harbored tissue-specific endophyte
385representatives (Fig. 2), i.e. *Streptomyces* in roots and *Rhizobium* in twigs.
386Because 16S rRNA sequencing technique cannot distinguish the
387inoculated pathogenic *Agrobacterium* from other Rhizobia, it is necessary
388to decide whether the enrichment of Rhizobia was ascribed to the
389inoculated *A. tumefaciens*. Molecular detection of virulent *ipt* gene
390indicated that no pathogenic *A. tumefaciens* was present in uninfected
391roots and twigs. In infected twigs, approximately half of *A. tumefaciens*
392harbored the *ipt* gene, suggesting that the enrichment of *Rhizobium* in
393inoculated twigs partly resulted from the pathogen infestation. It's in line
394with the study of New and Kerr (46) that non-pathogenic *Rhizobium* was
395present in healthy trees, while both non-pathogenic and pathogenic
396*Rhizobium* were detected in the roots of infected trees. The dominant
397bacterial assemblages of *Rhizobium* at D10 in twigs possibly ascribed to
398the incisions during inoculation that leads to the specific chemotactic
399movement of *Rhizobium* toward wound exudates (Currier and Strobel,
4001976; Aguilar et al., 1988), or the stress resistance of *Rhizobium*
401(Gopalakrishnan et al., 2014). Along with the plant growth, their
402proportion largely dropped, which might be related to the different
403nutrient supply by plants (Yuan et al., 2015) or be buffered by other
404endophytic bacteria (Bulgarelli et al., 2013). However, the proportion of

antagonistic *Rhizobium* was always higher in the resistant cultivar, suggesting that *Rhizobium* might be responsible for the peach resistance to *A. tumefaciens*. *Rhizobium* sp. K84 (46) and PAR (Li et al., 2018) have been confirmed having high efficacy in inhibiting the gall formation. However, whether *Rhizobium* plays a role in peach plant resistance requires further confirmation by inoculation assay on sterile seedlings.

To decipher the plant-microbe interactions and apply specific endophyte to control crown gall disease in fruit trees, several strategies should be employed. One is to combine genomics, transcriptomics, metabolomics, and molecular biocontrol mechanism analysis. By using this comprehensive practice, Carrier et al. (44) identified specific members of the Burkholderiaceae family that contribute to soil suppressiveness via the production of sulfurous volatile compounds. The other strategy is to inoculate host plants with putative antagonists (single or multiple) or plant-microbiota extract and determine their impartment in suppressing disease. Similar to the control of damping-off disease by supplementing suppressive soils (73), it is possible to develop plant resistance by promoting specific microbial consortia prior to planting or even develop customized biocontrol agents for field use. However, without sterile peach seedlings, it's impossible to determine which bacterial strains are associated with peach resistance against *A. tumefaciens*.

426

427 MATERIALS AND METHODS

428 **Plant materials and *A. tumefaciens* inoculation.** Peach cultivars
429 'Honggengansutao' and 'Okinawa' have been grown in the National Peach

430 Germplasm Repository of China (NPGRC, Zhengzhou, China) for 20 years,
 431 and their seeds were collected in 2012 and 2015 for field and greenhouse
 432 trials, respectively. All seeds were washed thoroughly, surface sterilized in
 433 0.5% NaClO for 5 min, a rinsed 3× in sterile deionized water before
 434 stratifying at 4°C for 3 months. After germination in autoclaved
 435 vermiculite for 1 week at 28°C, seedlings were grown in homogenized
 436 soils (0–20 cm depth) collected from the field of origin. For field study,
 437 seedlings were grown in the same experimental field next to NPGRC, and
 438 treated with the same agronomic practice (no fertilizer or pesticide
 439 applied) for two years. For greenhouse trial, seedlings were planted
 440 individually in 90 mm plastic pots and grown for two months in a
 441 greenhouse. Peach trees and seedlings were then subjected to
 442 experimental treatments as described below.

443 *A. tumefaciens* strain TA-AT-2 (biovar 2), isolated from a peach tree in
 444 Taian, China, was cultured in yeast extract and beef extract broth (YEB; 9)
 445 on a rotary shaker (200 rpm) at 28°C for 20 h, and aliquots used for
 446 inoculation were adjusted to a cell density of 10⁹ CFU/ml.

447 A total of 180 peach plants were grown in a greenhouse experiment
 448 (90 per cultivar), including 45 plants of each cultivar inoculated with *A.*
 449 *tumefaciens* on root collars (I) and 45 uninoculated plants used as controls
 450 (M). The pathogen inoculation was performed as described by Hao *et al.*
 451 (9). Cuts of 1 cm in length were made into the cambium at the root collar,
 452 and either 20 µl of bacterial inoculum (10⁹ CFU/ml) or sterile deionized
 453 water was applied to the incision, which was then covered by autoclaved
 454 vermiculite. Five trees of each cultivar were selected for the twig

455inoculation assay. Six newly-grown twigs of each tree were randomly
 456selected, and each twig was inoculated at five sites (with 5 cm interval
 457between inoculation sites) with *A. tumefaciens* suspension. Similar twigs
 458inoculated with sterile deionized water were taken as mock control. At the
 459end of the incubation period (60 days), the gall incidence, maximum
 460diameter of each tumor, and diameters of stems and twigs of each plant
 461(13 plants × 3 replicates per treatment for roots, 5 trees × 6 twigs × 5
 462sites per treatment for twigs) were measured, and used for the calculation
 463of disease index (5). The data were statistically analyzed using Student's
 464t-Test in R V3.4.3.

465

466 **Sample collection.** Peach roots and twigs were collected from the
 467two cultivars with or without inoculation at three time points (D0, D10 and
 468D60) as shown in Fig. S1. Peach roots were collected from three randomly
 469selected peach samples planted in the greenhouse. Roots were surface
 470sterilized using a phosphate buffer wash followed by sonication (30 s at
 471150–60 Hz for 3 times; 37) and homogenized. The roots were dried on
 472sterile filter paper and imprinted on the agar plates of tryptic soy medium
 473(TSA; 30). No colonies appeared after incubating the plates at 28°C for 5
 474days, confirming the effectiveness of the surface sterilization procedure.
 475An aliquot was snap-frozen and stored at –80°C for DNA extraction, and
 476the remainders were stored at 4°C for bacterial isolation.

477 Similarly, peach twigs were collected from trees in orchards at D0, D10
 478and D60. Twigs from each cultivar were randomly selected at D0, while at
 479D10 and D60, three inoculated or uninoculated twigs of different

480orientations were collected from each tree by sterile pruning shears. The
 481leaves were removed, and the twigs were washed 3× with sterile
 482deionized water, followed by sterilization with 70% ethanol for 30 s and
 4831% NaOCl for 3 min, and 5× sterile deionized water washes. Duplicates of
 484the last rinse (100 µl) were placed on TSA plates at 28°C for 5 days to
 485confirm complete sterilization. Three twigs from each tree were
 486homogenized after discarding segments near inoculation sites ($\pm$ 0.5 cm).
 487A tissue aliquot was snap-frozen for DNA extraction and storage as
 488described above.

489

490 **DNA extraction and amplicon sequencing.** One gram of frozen
 491root or twig tissue from each sample was ground in liquid nitrogen into
 492powders, and genomic DNA was extracted using the FastDNA SPIN kit for
 493soil (MP Biomedicals, USA) according to the manufacturer's instructions.
 494The quality of extracted DNA was checked by 1% agarose gel
 495electrophoresis and spectrophotometry (OD 260/280 nm). DNA samples
 496were stored at -20°C for subsequent analyses.

497 Using the DNA extracts as templates, the V5–V7 region of the bacteria
 49816S rRNA gene spanning ~400 bp was amplified with the universal
 499primers 799F (5'-AACMGGATTAGATACCCKG-3'; 76) and 1193R (5'-
 500ACGTCATCCCCACCTTCC-3'; 77). These primers contained a set of 8-
 501nucleotide barcode sequences unique to each sample. The PCR program
 502was as follows: 95°C for 5 min, 25 cycles of 95°C for 30 s, 56°C for 30 s,
 503and 72°C for 40 s, and a final extension of 72°C for 10 min. PCR reactions
 504were performed in triplicate, and the 25 µl mixture system contained 2.5

64

505 μ l of 10 $\times$ Pyrobest buffer, 2 μ l of 2.5 mM dNTPs, 1 μ l of each primer (10
506 μ M), 0.4 U of Pyrobest DNA polymerase (TaKaRa, Japan), and 15 ng of
507 template DNA. Sterile RNase-free water was used as negative control in
508 each PCR run.

509 Amplicons with bacterial products of approximately 400 bp were
510 extracted from 2% agarose gels and purified using the AxyPrep DNA Gel
511 Extraction Kit (Axygen Biosciences, USA) according to the manufacturer's
512 instructions and quantified using QuantiFluor™ -ST (Promega, USA).
513 Purified amplicons were pooled in equimolar ratios and subjected to
514 paired-end sequencing (2 $\times$ 300) by Allwegene (Beijing, China) using the
515 Miseq PE300 sequencing platform (Illumina, USA).

516

517 **Processing of sequencing data.** Sequencing data were processed
518 by the personalized pipeline developed by Allwegene (Beijing, China). Raw
519 DNA sequences were filtered based on sequence length and quality, and
520 primer and tag sequences were removed using the QIIME software v1.2.1
521 (78). Sequences that overlapped more than 10 bp were assembled using
522 FLASH v1.2.7 (79), while read pairs which could not be assembled were
523 discarded. Paired-end sequences were clustered into operational
524 taxonomic units (OTUs) at 97% sequence similarity using UCLUST (80),
525 and chimeric sequences were removed using USEARCH v8.0.1623 (81).
526 Taxonomy of these OTUs was assigned by UCLUST using the Silva 119 16S
527 rRNA database (82, 83) as a reference, with assignments made using a
528 confidence threshold of 90%. OTUs identified as plastids (0.003–0.03%
529 reads in roots and 6.52–33.68% reads in twigs) or mitochondria (0.19–

65
66

530 1.37% reads in roots and 1.18–13.54% reads in twigs) were removed.

531

532 **Amplicon sequencing data analysis.** OTU tables derived from 16S
 533 amplicon sequencing data analyses were analyzed in R v3.4.3 using the
 534 phyloSeq (84), Vegan (85), ggplot2 (86), randomForest (87) and mvpart
 535 (88) packages. Nonmetric multidimensional scaling (NMDS) ordinations
 536 were generated using the metaMD function in ‘Vegan’. Multiple regression
 537 tree (MRT) analysis and permutational multivariate analysis of variance
 538 (PERMANOVA; 89) were used to compare the effects of time, cultivar, and
 539 inoculation on the whole bacterial community. The Shannon diversity
 540 index (90) was used to account for both the abundance and evenness of
 541 present OTUs in each treatment, computed with the ‘phyloSeq’ package
 542 plot_richness function. One-way analysis of similarities (ANOSIM) was used
 543 to detect the difference in endophyte assemblages among different time
 544 points using anosim in ‘Vegan’, while ANOVA was used to test other
 545 significant differences among groups. The relative strength of each
 546 experimental factor contributing to the patterns in microbial community
 547 composition across samples was tested using the function randomForest
 548 in the ‘randomForest’ package in R. Differentially abundant OTUs were
 549 identified with similarity percentage (SIMPER) analyses and a Kruskal-
 550 Wallis test. Phylogenetic trees of the 16S rRNA sequences (OTU
 551 abundance >0.5%) and alignments between OTUs and isolates were
 552 generated by Geneious 11.0.5 (Biomatters, New Zealand), and visualized
 553 using the Interactive Tree of Life (iTOL) v4.1.1 (91).

554

555 **Isolation and identification of bacteria from roots and twigs.**

556 One gram sample of root or twig tissues was ground in 9 ml of phosphate
 557 buffer, pH 7.2 with sterile quartz sand using a sterile mortar and pestle.
 558 Serial dilutions were subsequently prepared in sterile deionized water. An
 559 aliquot of 100 µl of the suspension was plated on TSA and incubated at
 560 28°C. The colony numbers and morphologies were counted after 24–48 h
 561 growth, and logarithm numbers of colony-forming units per gram
 562 ($\log_{10}$ CFU/g) were calculated. Sixty isolates of each subset
 563 (time/cultivar/treatment, Fig. S1) were randomly selected from both peach
 564 roots and twigs, confirming that all morphologies were represented, to
 565 give the total of 1200 single colonies for antagonistic assay *in vitro*.

566 Individual colonies were cultured separately in tryptic soy broth (TSB;
 567 30) on a rotary shaker (200 rpm) at 28°C overnight. Bacterial suspensions
 568 of selected colonies (2 ml) were used for DNA extraction using the
 569 genomic DNA extraction kit (TIANGEN, China). Universal primers 27f/1492r
 570 were employed for the 16S rRNA gene amplification (92), and
 571 amplification was confirmed using a 1.2% agarose gel prior to Sanger
 572 sequencing by Sango, China. Sequences were evaluated and assembled
 573 using DNASTAR Lasergene v7.1 (DNASTAR, USA). And top hits (all >97%
 574 sequence identity) of BLAST search (<http://blast.ncbi.nlm.nih.gov>) were
 575 used to identify the highest possible taxonomic resolution of isolates to
 576 genus or species level.

577

578 **Antagonistic assay.** One strain of each ribotype (a group of isolates
 579 with identical 16S rRNA sequences) was selected for the antagonistic test.

Antagonistic assays were conducted by using the pair culturing method (93). Briefly, 1 ml of the *A. tumefaciens* cell suspension mixture (10^8 CFU/ml) of strains ATCC 23308^T (biovar 1) and TA-AT-2 (biovar 2) was combined with 20 ml of YEB medium and plated on Petri dishes. Peach endophyte isolate cultures were then inoculated on these plates on three corners of Petri dishes. After 2 days incubation at 28°C, the diameter of each inhibition zone was measured. Antagonistic assays were performed in three biological replicates. Non-inoculated plates served as controls.

588

PCR screening for pathogenic genes in *Rhizobium* isolates and inoculation tests. Each endophyte isolate which was identified as *Rhizobium* by sequencing was subjected to further pathogenic analysis. PCR-based screening for pathogenic *Rhizobium* was performed using ipt 3R primers and corresponding PCR amplification protocol, which targeted a conserved portion of T-DNA affecting the strain's pathogenicity (Akiyoshi et al., 1984; Buchmann et al., 1985, 5). PCR products were visualized on a 1.2% agarose gel, and specific amplicons of pathogenic *Rhizobium* of 247 bp in length were identified. The pathogenicity of the *Rhizobium* isolates was also confirmed by inoculating the sunflower stems with bacterial suspension and inducing the formation of galls (Loper and Kado 1979).

601

Accession numbers. The 16S rRNA gene amplicon sequences were deposited in the NCBI Sequence Read Archive (SRA) database under accession numbers SRR6801696–SRR6801755. The 16S rRNA nucleotide

605 sequences of bacterial isolates were deposited at GenBank under
606 accession numbers MG835926–MG836230.

607

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617

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963FIGURE LEGENDS

964**FIG 1** Disease occurrence on the peach root collars and twigs of resistant

965cultivar ‘Honggengansutao’ and susceptible cultivar ‘Okinawa’ 60 days

966after inoculation with *A. tumefaciens*. (A and B) Symptomatic

967development on peach root collars and twigs, respectively. (C and D)

968Disease indices of peach root collars and twigs, respectively. Statistical

969comparisons between groups were conducted by **Student’s t-Test**. *

970indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, and *** indicates $P \leq 0.001$. M,

971mock; I, inoculated with *A. tumefaciens*; H, ‘Honggengansutao’; O,

972‘Okinawa’.

973

974**FIG 2** Distribution of endophytic bacteria from roots (A) and twigs (B)

975across sampling time, cultivar, and treatment. Unidentified genera and

976genera with a proportion of less than 0.5% are combined into the group

977“Others”. Genus *Rhizobium* contains the former genus *Agrobacterium*. H,

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978'Honggengansutao'; O, 'Okinawa'; R, root; T, twig; M, mock; I, inoculated
979with *A. tumefaciens*.

980

981**FIG 3** Distribution of the endophyte microbiota in peach roots (A) and
982twigs (B) within a nonmetric multidimensional scaling (NMDS) ordination,
983and Shannon diversity index of the microbiota of peach roots (C) and
984twigs (D) based on 16S rRNA sequences. The analysis was conducted
985based on the Bray-Curtis **dissimilarity** at OTU level. Statistical comparisons
986between groups were conducted by one-way ANOVA test. * indicates $P \leq$
9870.05, ** indicates $P \leq 0.01$, and *** indicates $P \leq 0.001$. **U, uninoculated;**
988M, mock; I, inoculated with *A. tumefaciens*; H, 'Honggengansutao'; O,
989'Okinawa'.

990

991**FIG 4** Phylogenetic distribution and heatmaps of the **most abundant OTUs**
992(**with abundance >0.5%**) in the **endophytic** microbiota of peach roots (A)
993and twigs (B) under different sampling time, cultivars and treatment. The
994phylogenetic trees were constructed with 1000 boot-strapresamplings and
995annotated using iTOL. Branch lengths are ignored. **The lowest taxonomic**
996**resolution of OTUs was labeled**. Heatmaps show the relative abundances
997of OTUs across sample replicates. P values are calculated according to the
998Kruskal-Wallis analysis, and significant differences ($P \leq 0.05$) are indicated
999with asterisks. **U, uninoculated;** M, mock; I, inoculated with *A.*

1000*tumefaciens*; H, 'Honggengansutao'; O, 'Okinawa'.

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1002**FIG 5** Phylogenetic analysis of all endophytic isolates (A), *Rhizobium*
1003isolates (B), and *Pseudomonas* isolates (C) based on the 16S rRNA
1004sequences (1350 bp). The phylogenetic trees were constructed with 1000
1005boot-strap resamplings and annotated using iTOL. Pathogenetic
1006*Rhizobium* was determined based on PCR amplification of virulent *ipt* gene
1007and inoculation assay in sunflower; antagonistic strains were determined
1008by pair culturing method; and other strains were defined as commensal.
1009The branches in panel A are colored according to different genera shown
1010in Fig. S9. H, 'Honggengansutao'; O, 'Okinawa'; R, root; T, twig; M, mock;
1011I, inoculated with *A. tumefaciens*.

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