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Authors

Li, Qian

Guo, Rongjun

Li, Yujia

et al.

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Insight into the Bacterial Endophytic Communities of Peach Cultivars Related to Crown Gall Disease Resistance

Qian Li,[a,b,c] Rongjun Guo,[b] Yujia Li,[b] Wyatt H. Hartman,[c] Shifang Li,[b] Zhixiang Zhang,[b] Susannah G. Tringe,[c,#] Hongqing Wang[a,#]

[a]Department of Fruit Science, College of Horticulture, China Agricultural University, Beijing, China

[b]State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China

[c]Department of Energy Joint Genome Institute, Walnut Creek, California, USA

[#]Address correspondence to Susannah G. Tringe, sgtringe@lbl.gov, or Hongqing Wang, wanghq@cau.edu.cn.

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

IMPORTANCE *Agrobacterium tumefaciens* as the causal agent of peach

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

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

INTRODUCTION

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

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

(11, 12), and have been successfully developed as biocontrol agents. However, universal biocontrol of crown gall disease by these antagonists is challenged by the resistance of many *A. tumefaciens* strains to K84 (13, 14).

Microorganisms that spend at least part of their life cycle inside plants are called endophytes (15), and their communities may represent the extended phenotype of a host (16). Endophytic microbiota are shaped by both the host plant and environmental stimuli, and in turn may enhance the biotic and abiotic tolerance of their host plants as a multispecies functional unit (17). The abundance and diversity of endophytic microbial communities vary a lot in resistant and susceptible cultivars of some plants (18–21), and the community composition may also be altered by pathogen infection (22–25). Previous studies indicate that endophytic communities can inhibit pathogen invasion and prevent or reduce disease development by outcompeting phytopathogens, producing antimicrobial compounds, or inducing plant resistance (26). The colonization of specific endophytes has been demonstrated to successfully reduce disease incidence and severity in several fruit trees including citrus (18), grapevine (22), banana (27), and apple (28). In peach roots, five endophytic bacteria (*Brevundimonas diminuta*, *Leifsonia shinshuensis*, *Sphingomonas parapaucimobilis*, *B. vesicularis*, and *R. radiobacter*) isolated from *in vitro* cultures were found to produce indole-3-acetic acid (IAA, a plant hormone), fix nitrogen, and solubilize phosphate (29). Moreover, endogenous *Enterobacter*, *Pantoea* and *Rhizobium* isolated from the resistant peach cultivar ‘Xibei 13-1’ demonstrate antagonism to

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

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

RESULTS

Susceptibility of peach cultivars to crown gall disease. The susceptibility of different peach cultivars to crown gall disease was tested on peach roots in the greenhouse and newly grown twigs in the field. Disease onset occurred in both roots and twigs 10 days post-inoculation (D10), and crown gall tumors developed rapidly thereafter until D60 (Figs. 1A 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.

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

product amplified.

Differences were observed in the community compositions of endophytic bacteria in peach roots and twigs. Overall, endophytic assemblages were dominated by Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes at the phylum level, accounting for 49.8–99.0% of the total bacterial community regardless of compartment, cultivar, treatment or time of sampling (Fig. S2). At the genus level, *Streptomyces* (average abundance of 23.2%) in roots and *Rhizobium* (average abundance of 24.7%, including *A. tumefaciens*) in twigs were dominant (Fig. 2). The other top genera largely different between roots and twigs (Fig. 2); only *Pseudomonas* and *Rhizobium* were abundant in both. In comparison to the relatively stable distribution of root endophytes, the community composition of twig endophytes varied notably with time and pathogen inoculation.

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

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

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

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

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

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).

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*

strains, 8 of them derived from the inoculated susceptible ‘Okinawa’, and 14 from the inoculated ‘Honggengansutao’ (Fig. 5B).

DISCUSSION

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

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

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

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

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

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

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

In the present study, the peach endophytic bacteria mainly belonged

to phyla Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes, which were dominant during all developmental stages as previously reported (66). Although both peach roots and twigs had *Pseudomonas* and non-pathogenic *Rhizobium* as the dominant genera, similar to sorghum roots and shoots (67), they also harbored tissue-specific endophyte representatives (Fig. 2), i.e. *Streptomyces* in roots and *Rhizobium* in twigs. Because 16S rRNA sequencing technique cannot distinguish the inoculated pathogenic *Agrobacterium* from other Rhizobia, it is necessary to decide whether the enrichment of Rhizobia was ascribed to the inoculated *A. tumefaciens*. Molecular detection of virulent *ipt* gene indicated that no pathogenic *A. tumefaciens* was present in uninfected roots and twigs. In infected twigs, approximately half of *A. tumefaciens* harbored the *ipt* gene, suggesting that the enrichment of *Rhizobium* in inoculated twigs partly resulted from the pathogen infestation. It's in line with the study of New and Kerr (46) that non-pathogenic *Rhizobium* was present in healthy trees, while both non-pathogenic and pathogenic *Rhizobium* were detected in the roots of infected trees. The dominant bacterial assemblages of *Rhizobium* at D10 in twigs possibly ascribed to the incisions during inoculation that leads to the specific chemotactic movement of *Rhizobium* toward wound exudates (Currier and Strobel, 1976; Aguilar et al., 1988), or the stress resistance of *Rhizobium* (Gopalakrishnan et al., 2014). Along with the plant growth, their proportion largely dropped, which might be related to the different nutrient supply by plants (Yuan et al., 2015) or be buffered by other endophytic 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, Carrior 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*.

MATERIALS AND METHODS

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

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

A. tumefaciens strain TA-AT-2 (biovar 2), isolated from a peach tree in Taian, China, was cultured in yeast extract and beef extract broth (YEB; 9) on a rotary shaker (200 rpm) at 28ºC for 20 h, and aliquots used for inoculation were adjusted to a cell density of 10^9 CFU/ml.

A total of 180 peach plants were grown in a greenhouse experiment (90 per cultivar), including 45 plants of each cultivar inoculated with *A. tumefaciens* on root collars (I) and 45 uninoculated plants used as controls (M). The pathogen inoculation was performed as described by Hao *et al.* (9). Cuts of 1 cm in length were made into the cambium at the root collar, and either 20 µl of bacterial inoculum (10^9 CFU/ml) or sterile deionized water was applied to the incision, which was then covered by autoclaved vermiculite. Five trees of each cultivar were selected for the twig

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

Sample collection. Peach roots and twigs were collected from the two cultivars with or without inoculation at three time points (D0, D10 and D60) as shown in Fig. S1. Peach roots were collected from three randomly selected peach samples planted in the greenhouse. Roots were surface sterilized using a phosphate buffer wash followed by sonication (30 s at 150–60 Hz for 3 times; 37) and homogenized. The roots were dried on sterile filter paper and imprinted on the agar plates of tryptic soy medium (TSA; 30). No colonies appeared after incubating the plates at 28°C for 5 days, confirming the effectiveness of the surface sterilization procedure. An aliquot was snap-frozen and stored at −80ºC for DNA extraction, and the remainders were stored at 4°C for bacterial isolation.

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

orientations were collected from each tree by sterile pruning shears. The leaves were removed, and the twigs were washed 3× with sterile deionized water, followed by sterilization with 70% ethanol for 30 s and 1% NaOCl for 3 min, and 5× sterile deionized water washes. Duplicates of the last rinse (100 µl) were placed on TSA plates at 28°C for 5 days to confirm complete sterilization. Three twigs from each tree were homogenized after discarding segments near inoculation sites (± 0.5 cm). A tissue aliquot was snap-frozen for DNA extraction and storage as described above.

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

Using the DNA extracts as templates, the V5–V7 region of the bacteria 16S rRNA gene spanning ~400 bp was amplified with the universal primers 799F (5´-AACMGGATTAGATACCCKG-3´; 76) and 1193R (5´-ACGTCATCCCCACCTTCC-3´; 77). These primers contained a set of 8-nucleotide barcode sequences unique to each sample. The PCR program was as follows: 95°C for 5 min, 25 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 40 s, and a final extension of 72°C for 10 min. PCR reactions were performed in triplicate, and the 25 µl mixture system contained 2.5

μl of 10× Pyrobest buffer, 2 μl of 2.5 mM dNTPs, 1 μl of each primer (10 μM), 0.4 U of Pyrobest DNA polymerase (TaKaRa, Japan), and 15 ng of template DNA. Sterile RNase-free water was used as negative control in each PCR run.

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

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

67
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

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

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

Antagonistic assay. One strain of each ribotype (a group of isolates 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.

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 3F/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).

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

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

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FIGURE LEGENDS

FIG 1 Disease occurrence on the peach root collars and twigs of resistant cultivar ‘Honggengansutao’ and susceptible cultivar ‘Okinawa’ 60 days after inoculation with *A. tumefaciens*. (A and B) Symptomatic development on peach root collars and twigs, respectively. (C and D) Disease indices of peach root collars and twigs, respectively. Statistical comparisons between groups were conducted by Student’s t-Test. * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, and *** indicates $P \leq 0.001$. M, mock; I, inoculated with *A. tumefaciens*; H, ‘Honggengansutao’; O, ‘Okinawa’.

FIG 2 Distribution of endophytic bacteria from roots (A) and twigs (B) across sampling time, cultivar, and treatment. Unidentified genera and genera with a proportion of less than 0.5% are combined into the group “Others”. Genus *Rhizobium* contains the former genus *Agrobacterium*. H,

‘Honggengansutao’; O, ‘Okinawa’; R, root; T, twig; M, mock; I, inoculated with *A. tumefaciens*.

FIG 3 Distribution of the endophyte microbiota in peach roots (A) and twigs (B) within a nonmetric multidimensional scaling (NMDS) ordination, and Shannon diversity index of the microbiota of peach roots (C) and twigs (D) based on 16S rRNA sequences. The analysis was conducted based on the Bray-Curtis dissimilarity at OTU level. Statistical comparisons between groups were conducted by one-way ANOVA test. * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, and *** indicates $P \leq 0.001$. U, uninoculated; M, mock; I, inoculated with *A. tumefaciens*; H, ‘Honggengansutao’; O, ‘Okinawa’.

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

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