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RESEARCH ARTICLE

Isolation and characterization of diverse tumorigenic *Rhizobiaceae* strains associated with crown gall on flowering cherry (*Prunus serrulata* var. *lannesiana*) in Ningbo, China

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ABSTRACT

Crown gall disease threatens flowering cherry (*Prunus serrulata* Lindl. var. *lannesiana* (Carrière) Makino) cultivation in Ningbo, China. Six tumorigenic bacterial strains were isolated from gall tissues of diseased cherry roots. Based on biochemical phenotypic assays and partial 16S rRNA gene sequences, strain CA-6 was preliminarily assigned to the *Agrobacterium tumefaciens* species complex (syn. *Rhizobium radiobacter*), and isolates CA3-1 to CA3-5 shared high sequence similarity with *Rhizobium calliandrae* and are therefore referred to as *R. calliandrae*-like strains. The polymerase chain reaction (PCR) screening verified that all six isolates harbored three key virulence genes (*virD2*, *tms2*, and *ipt*) borne on the tumor-inducing (Ti) plasmids. Pathogenicity assays fulfilling Koch's postulates demonstrated that all strains could induce crown galls on tomato, sunflower, and *Prunus* seedlings, and all six isolates were confirmed to be pathogenic on the three test hosts. This work offers preliminary data regarding the virulence traits of local cherry crown gall pathogens to support follow-up integrated disease management research.

Key words: *Agrobacterium tumefaciens* species complex, crown gall, pathogenicity, *Rhizobium calliandrae*-like strains, tumorigenic genes.

INTRODUCTION

Prunus serrulata var. *lannesiana* (Carrière) Makino, a cultivated variety within the genus *Prunus*, is predominantly distributed in temperate and subtropical regions of the Northern Hemisphere, with notably high diversity in East Asia (Zhang et al., 2017). Cherry blossoms, which are representative ornamental plants within this variety, hold significant aesthetic and cultural value in China (Chin et al., 2014). This taxon is widely utilized for enhancing green spaces in urban environments, including parks, residential communities, and along roadways. Moreover, the market demand for flowering cherry blossom saplings continues to increase, demonstrating promising commercial prospects (Song et al., 2024). As an important ornamental tree in China, cherry blossoms (*P. serrulata* var. *lannesiana*) are widely cultivated in Ningbo, Zhejiang Province, where the climatic conditions are highly suitable for their growth (Yan et al., 2017). Notably, in areas such as Yuyao, Fenghua, and Yinzhou district within the Siming Mountain region of Ningbo, the production scale, annual output value, and market share of cherry blossom saplings rank among the highest nationwide, making it a truly renowned "Hometown of the cherry blossom" (Wang et al., 2014; Fu et al., 2020). The production and sale of cherry blossom saplings have become the primary economic source for local farmers in the Siming Mountain area (Cheng et al., 2021; Chen et al., 2024). This reliance also renders the industry vulnerable to diseases such

as crown gall. This is because flowering cherry trees are commercially propagated via grafting, where crown gall susceptibility is largely determined by the rootstock rather than the scion cultivar. The disease severely impairs the growth rate and vigor of flowering cherry saplings, diminishing their ornamental quality and commercial value (Zhang et al., 2025).

Crown gall is a globally prevalent bacterial plant disease caused by the *Agrobacterium tumefaciens* with a wide distribution worldwide (Escobar and Dandekar, 2003). Its host range encompasses 643 plant species from 331 genera and 93 families, primarily targeting dicotyledonous plants, though certain monocotyledonous species are also susceptible. Among fruit trees, common hosts include grape, peach, cherry, plum, apricot, apple, pear, jujube, chestnut, and walnut. Infected plants exhibit yellowing, weakness, reduced fruit yield, and diminished ornamental value, which may eventually lead to plant death (Kawaguchi, 2022; Bouri and Rhouma, 2025). The disease is widespread throughout Europe, North America, Africa, and parts of Asia. In China, it is primarily distributed in Fujian, Hebei, Henan, Jilin, Liaoning, Shandong, and Zhejiang provinces, among other regions, with nursery incidence rates ranging from 20% to 100% (Yan et al., 2020). In Ningbo, preliminary surveys indicate that the average incidence of crown gall in major seedling production bases exceeds 30%, with severely affected areas reaching infection rates as high as 92%.

Crown gall is caused by pathogenic *Rhizobiaceae* strains (*Agrobacterium*), including multiple species from the genera *Rhizobium*, *Allorhizobium*, and *Agrobacterium*. The case of *Agrobacterium tumefaciens* is somewhat more complex. Genomic analyses based on DNA-DNA hybridization have revealed that compared with that of the other two species, this species exhibits greater heterogeneity. It is not a single uniform species but rather a species complex; hence, it is more appropriate to refer to it as the "*Agrobacterium tumefaciens* species complex" (Xiao et al., 2023). This nomenclature has gained official recognition. Currently, this complex comprises 15 genomic species: G1-G9, G13, G14, G15, G19, G20, and G21. Among these, only six have formally validated Latin binomials*: A. fabrum* (G8), *A. pusense* (G2), *A. radiobacter* (G4), *A. nepotum* (G14), *A. salinitolerans* (G9), and *A. deltaense* (G7) (Holmes et al., 2023; Xiao et al., 2023; Vargas Ribera et al., 2024). Single-locus 16S rRNA gene sequencing cannot reliably resolve cryptic taxa belonging to the family *Rhizobiaceae*. Multilocus sequence analysis (MLSA) and whole-genome phylogenomics, by comparison, serve as the benchmark methodologies for unambiguous species-level delineation of agrobacteria and rhizobial strains (Ma et al., 2023; Vargas Ribera et al., 2024). To maintain consistency, the pathogen is referred to as *A. tumefaciens* throughout this study. The pathogen can survive in soil for extended periods and can infect plants through wounds. The pathogen primarily enters through wounds in plant tissues and induces the formation of gall tumors of various sizes on root crown, lateral roots, or stem nodes. As these galls enlarge, they severely disrupt the phloem, impairing the transport of nutrients, leading to a progressive decline in plant vigor, significantly reduced yield and quality, and ultimately, the death of the plant. Transmission occurs mainly via contaminated soil and infected seedlings during transplantation (Kado, 2014). During the process of pathogen infection, tumorigenic strains harbor large tumor-inducing plasmids (pTis) carrying genes such as *virD2*, *ipt*, and *tms2* (Haas et al., 1995; Tan et al., 2003). The expression of these genes is crucial for the development of crown gall disease in plants, and they serve as marker genes for the identification of *A. tumefaciens*, making them valuable tools for the isolation of the pathogen responsible for crown gall disease (Tan et al., 2003).

The objectives of this study were to isolate the wild tumorigenic bacteria responsible for crown gall disease in flowering cherry trees in Ningbo, China, and to characterize its taxonomic classification and pathogenicity.

MATERIALS AND METHODS

Samplings and bacterial isolation

From 2023 to 2024, we obtained 30 flowering cherry trees (*Prunus serrulata* Lindl. var. *lannesiana* (Carrière) Makino) with crown gall disease from 20 nurseries located in Ningbo city, Zhejiang Province, China. The sampling was conducted during the spring and summer seasons (March to September) when disease symptoms were most apparent. The 20 nurseries were located in various districts of Ningbo, including both urban and suburban areas, reflecting the regional distribution of cherry cultivation in this area. The tumor fragments were excised from the plant roots and rinsed with sterile distilled water. Following drying with sterile filter paper, the fragments were disinfected with 75% ethanol for 1 min, crushed in a sterile mortar and ground with a small quantity of normal saline solution. The mixture was transferred to a 2 mL sterile centrifuge tube and left to sit

for 1 h. Subsequently, 100 µL supernatant were applied onto plates containing selective Moore-Warren (MW) medium (per liter: 10 g Mannitol, 2 g $NaNO_3$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.5 g KH_2PO_4, 0.2 g K_2HPO_4, 0.3 g yeast extract, 15 g agar, pH 7.0) for the isolation of *Agrobacterium tumefaciens*. The colonies were selected from the MW medium and transferred onto fresh yeast extract mannitol (YEM) agar plates (per liter: 10 g Mannitol, 0.4 g yeast extract, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 15 g agar, pH 7.0) and pure single colonies were obtained by continuous streaking. Single colonies were incubated in YEM liquid medium at 28 °C for 1 d, after which sterile glycerol was added to reach a final concentration of 20% glycerol, and the solution was mixed and stored at-80 °C for further analysis (Wang et al., 2000).

Molecular biological identification

Total genomic DNA of each purified isolate was extracted using a commercial bacterial genomic DNA extraction kit (Sangon Biotech, Shanghai, China) following the manufacturer's standard protocol. The 16S rRNA gene sequences of the various isolates were amplified with the universal primers 27F and 1492R (Table 1). The polymerase chain reaction (PCR) conditions were as follows: Initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min; and an additional extension at 72 °C for 5 min. The PCR amplification products were sent to Sangon Biotech for sequencing. The 16S rRNA gene sequence analysis of the isolates for the closest related type strains was conducted online using Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information [NCBI], Bethesda, Maryland, USA). The 16S rRNA gene sequences of the strains and other related species were subsequently aligned using the CLUSTAL W method (Edgar and Batzoglou, 2006). A phylogenetic tree was constructed using MEGA 12.0 software with neighbor-joining methods (Kumar et al., 2024).

Table 1. Primer pairs used in this study. Ti: Tumor-inducing; Ri: root-inducing.

Target	Primer name	5′-3′ sequence	Size of amplicon (bp)	Location of the target sequence
16S rRNA	8F	AGAGTTTGATCCTGGCTCAG	~ 1500	Chromosomes
	1492R	GGCTACCTTGTTACGACTT		
*tms*2(iaaH)	tms2A	CGCCACACAGGGCTGGGGGTAGGC	220	Ti plasmid
	tms2B	GGAGCAGTGCCGGGTGCCTCGGGA		
ipt(tmr)	CYT	GATCG(G/C)GTCCAATG(C/T)TGT	427	Ti plasmid
	CYT	GAT ATCCATCGATC(T/C)CTT		
*vir*D2	VirD2A	ATGCCCGATCGAGCTCAAGT	224	Ti/Ri plasmid
	VirD2C'	TCGTCTGGCTGACTTTCGTCATAA		

Determination of pathogenicity associated genes

The PCR was used to amplify pathogenicity-associated genes located on the tumorigenic (Ti) or rhizogenic (Ri) plasmids (Table 1). Two primer pairs (tms2A-tms2B and CYT-CYT') complementary to the *tms2* and *ipt* genes of the T-DNA region located on the Ti plasmid were used. Another primer pair (virD2A-virD2C') complementary to the *virD2* gene of the vir region from the Ti/Ri plasmids was also used. The PCR cycles were as follows: (1) For the tms2A-tms2B primers, denaturation was set for 2 min at 94 °C, followed by a cycle profile of 94 °C for 10 s, 67 °C for 15 s, and 72 °C for 10 s. After 40 cycles, a final elongation was performed at 72 °C for 5 min. (2) For the CYT-CYT' primers, denaturation was set for 1 min at 94 °C, followed by 40 cycles at 94, 43, and 72 °C for 1 min at each temperature. Furthermore, the final elongation was set at 72 °C for 5 min. (3) For the virD2A-virD2C' primers, the initial denaturation was set at 94 °C for 1 min, followed by a cycle profile of 94, 55, and 72 °C for 1 min at each temperature. After 40 cycles, the final extension was performed at 72 °C for 5 min. All PCR products were separated on 1.2% agarose gel (1×TAE, 120 V, 25 min), stained with GoldView and visualized under UV light.

Pathogenicity assays

Three host plants were used to test the pathogenicity of all isolates: Tomato (*Solanum lycopersicum* L.), sunflower (*Helianthus annuus* L.), and a locally selected flowering cherry cultivar FH101 (*P. serrulata* var.

lannesiana ‘FH101’). Cultivar FH101 is locally bred, characterized by vigorous growth, strong adaptability, and early spring flowering, with a tree habit typically reaching 2-6 m in height. This cultivar was selected for pathogenicity tests owing to its prevalence in the Ningbo region, its frequent use as a local rootstock for cherry grafting, and its known susceptibility to the target pathogen. Yeast extract beef medium (YEB) was used for bacterial suspension preparation, containing 5 g L^{-1} tryptone, 5 g L^{-1} beef extract, 1 g L^{-1} yeast extract, 5 g L^{-1} sucrose, 2 mM $MgSO_4 \cdot 7H_2O$, pH 7.2. All bacterial suspensions were cultured in YEB for 48 h and adjusted to a concentration of 10^8 cfu mL^{-1}. Two separate inoculation methods were applied to different hosts. Needle-prick inoculation was carried out on the young stems of tomato and sunflower seedlings. For ‘FH101’ cherry seedlings, roots were thoroughly cleaned; fresh wounds were cut with sterilized gardening scissors, and the wounded roots were soaked in bacterial suspension for 30 min. Sterile blank YEB medium served as the negative control for all three hosts. Each treatment contained four replicate plants, and the whole experiment was repeated twice. All inoculated plants were cultivated in growth chambers at 24 ± 3 °C and watered every 2 d. The development of gall symptoms was inspected weekly. To satisfy Koch’s postulates, strains were re-isolated from gall tissues of diseased plants, and their identity was verified using 8F/1492R and tms2A/tms2B primer pairs.

RESULTS

Strains isolated from cherry crown galls

The crown gall on the root of an infected flowering cherry tree is shown in Figures 1a and 1b. Furthermore, isolation from diseased flowering cherry tree tissues yielded bacterial colonies that were white, circular, domed and glistening (Figure 1c). Disease symptoms appeared on tomato (d), sunflower (e), and *Prunus* (f) following artificial inoculation with strain CA-6, which was isolated from *Prunus* crown galls.

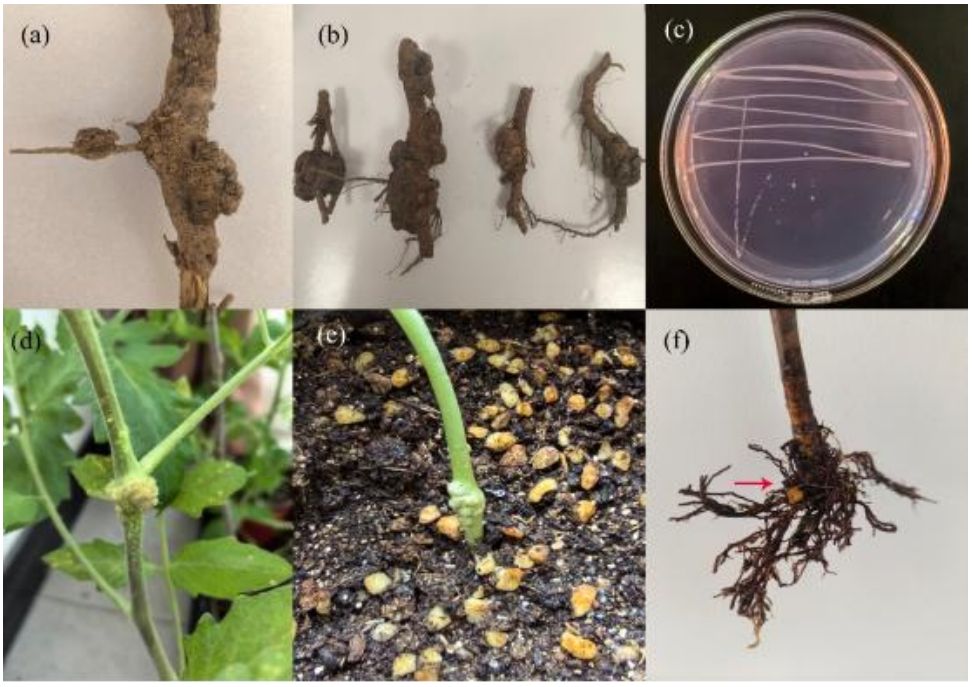

Figure 1. (a) Natural tumors of crown gall on flowering cherry cv. FH101. (b) Sample with crown gall for laboratory analysis. (c) Colony morphology of strains on selective MW plates. (d)-(f) Disease symptoms on plants artificially inoculated with strain CA-6 isolated from crown galls. (d) Tomato. (e) Sunflower. (f) FH101.

Identification of the bacterial strains

The identity of the strains was confirmed by sequence analysis of the 16S rRNA gene. In the 16S rRNA gene sequence analysis, almost the full length (approximately 1380 bp) of the 16S rRNA gene sequences of the isolates were determined. Moreover, the 16S rRNA gene sequence of the isolated strain CA-6 was 99.85% similar to that of the type strain of *A. radiobacter* strain IAM 12048^{T}, suggesting its tentative membership within the *A. tumefaciens* species complex.

The phylogenetic tree constructed with the 16S rRNA sequences of isolates identified at the species level was clustered. Five isolates, CA3-1, CA3-2, CA3-3, CA3-4 and CA3-5, were clustered into a clade and exhibited 16S rRNA gene sequence similarities of 99.85%, 99.78%, 99.83%, 99.78% and 99.56%, respectively, to the type strain of *Rhizobium calliandrae* strain CCGE524^{T}, indicating a close phylogenetic relationship with these species (Figure 2).

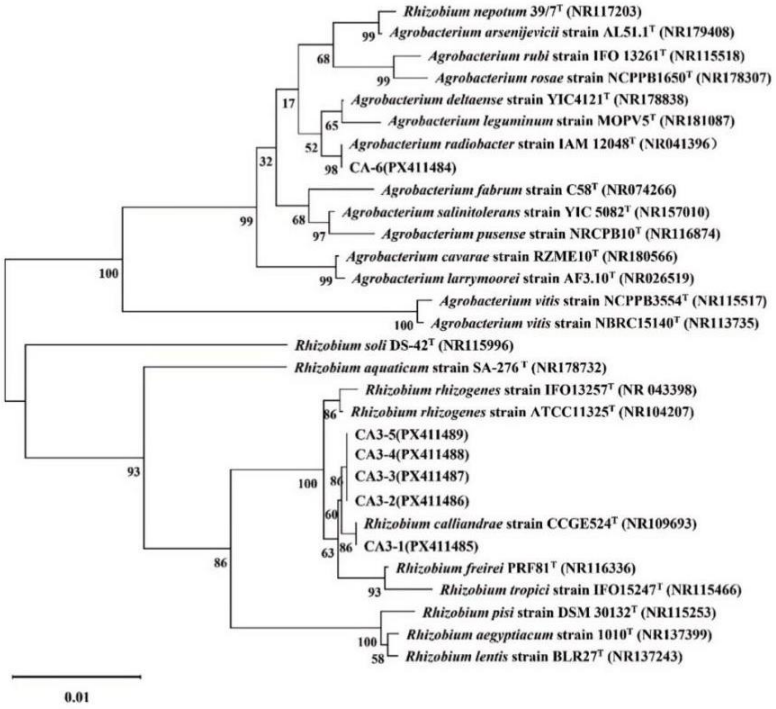

Figure 2. Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships among strains CA-6, CA3-1, CA3-2, CA3-3, CA3-4 and CA3-5. Bootstrap values calculated for 1000 replications are indicated. GenBank accession numbers are given in parentheses. The scale bar equals 0.01 changes per nucleotide position.

As shown in Table 2, the isolate CA-6 survived in 2% NaCl and growth at 35 °C, along with positive results for 3-ketolactose production, citrate utilization, and pigment formation on ferric ammonium citrate medium. It produced acid from ethanol and sodium malonate, while generating alkali from erythritol and in litmus milk. These bacteriological characteristics of the CA-6 coincided perfectly with those of the reference strain *R. radiobacter* CFCC1369^{T}. By contrast, isolates CA3-1 and CA3-5 all exhibited consistent physiological profiles: They grew in 2% NaCl but not at 35 °C, and were negative for 3-ketolactose production, citrate utilization, and pigment formation. They uniformly produced acid in litmus milk and from erythritol, and alkali from ethanol and sodium malonate. All tested isolates were pathogenic to tomato, sunflower, and 'FH101'. In addition, a strain of *A. radiobacter* (CA-6) was included in the phylogenetic analysis, and it is a member of the *A. tumefaciens* species complex (Figure 2). The other five isolates (CA3-1 to CA3-5) belong to *Rhizobium* sp. These strains, which cause crown gall disease, were previously classified as *A. tumefaciens* (crown gall bacterium).

Table 2. Phenotypic characterization of pathogenic strains used in this study. (+): Positive; (-): negative: NT: not tested.

Strains	3-Ketolactose production	Grow at 35 °C	Grow in 2% NaCl	Reaction in litmus milk	Reaction in erythritol	Reaction in ethanol	Reaction in in sodium malonate	Pigmentation in ferric ammonium citrate broth	Citrate utilization	Pathogenicity		
										Tomato	Sunflower	FH101
Rhizobium radiobacter CFCC1369^T	+	+	+	Alkaline	Alkaline	Acid	Acid	+	+	NT	NT	NT
CA-6	+	+	+	Alkaline	Alkaline	Acid	Acid	+	+	+	+	+
CA3-1, CA3-5	−	−	+	Acid	Acid	Alkaline	Alkaline	−	−	+	+	+
CA3-2, CA3-3, CA3-4	−	−	−	Acid	Acid	Alkaline	Alkaline	−	−	+	+	+

Identification and analysis of pathogenicity-associated genes

A PCR assay was used to detect the *tms2*, *virD2*, and *ipt* genes located on the plasmid DNA. The PCR with the tms2A-tms2B and CYT-CYT' primer pairs yielded the expected 220 and 427 bp specific bands, respectively, for all the tested isolates (Figure 3). Moreover, the targeted sequences were successfully amplified by PCR with the virD2A-virD2C' primer pair, yielding a band with a length of 224 bp in the case of all the isolates (Figure 3). Sequence alignment of the amplified products with reference sequences in GenBank confirmed their identities, with the 220 bp *tms2* amplicon sharing 98% nucleotide identity (GenBank accession no. AH003431.2), the 224 bp *virD2* amplicon displaying 99% homology (GenBank accession no. J03320.1), and the 427 bp *ipt* fragment exhibiting 85.75% similarity (GenBank accession no. NC_003065.3). These results indicated successful amplification of all three virulence genes. In the pathogenicity assays, symptoms associated with crown gall disease developed on inoculated tomatoes within 1 mo, on sunflower within 3 wk and on 'FH101' after 2 mo (Figures 1c, 1d). The experiments were repeated three times, and the reisolated strains had the same colony morphology as those inoculated. Moreover, the 16S rRNA gene sequence of the reisolated strains was 100% similar to that of the inoculated strains (CA-6, CA3-1, CA3-2, CA3-3, CA3-4 and CA3-5), and the targeted sequences were successfully amplified by PCR with the *tms*2A-*tms*2B primer pair in the case of all reisolates. No symptoms were observed on the plants that were inoculated with YEB medium. Taken together, the results confirmed that the isolated strains are pathogenic.

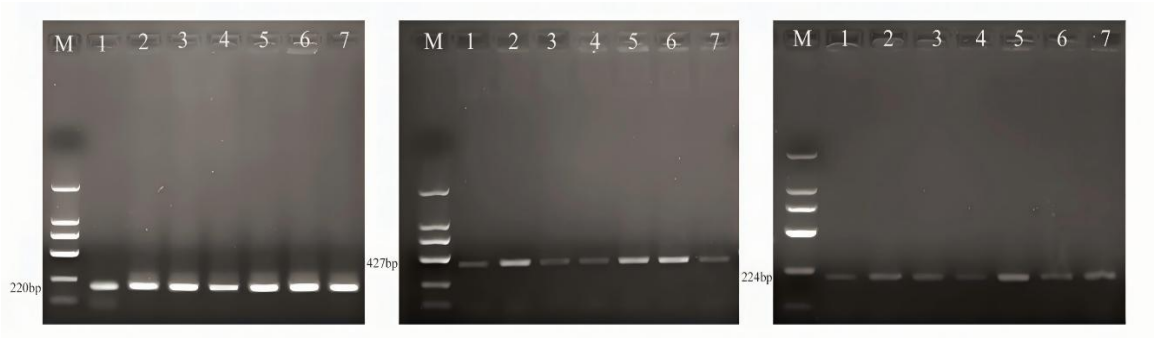

Figure 3. Agarose gel (1.2%) electrophoresis of the T-DNA and virulence region on the tumorigenic plasmids (*tms*2A-B, CYT-CYT' and *vir*D2A-C') of the isolates revealed PCR products of 220, 427 and 224 bp, respectively. Lane M: DM 2000 DNA Marker (from top to bottom: 2000, 1000, 750, 500, 250, and 100 bp); Lane 1: *Rhizobium radiobacter* CFCC1369^T control strain; Lanes 2-7: CA-6, CA3-1, CA3-2, CA3-3, CA3-4, and CA3-5, respectively.

DISCUSSION

Crown gall, caused by Gram-negative pathogenic agrobacteria, is a typical disease in dicot plants (Pulawska, 2010). In Ningbo, China, flowering cherry (*Prunus serrulata* var. *lannesiana*) is a key ornamental species and a notable agricultural industry, with a substantial planting area. However, recent local monitoring data indicate a yearly increase in the incidence of crown gall disease, caused by tumorigenic agrobacteria, within nursery bases, posing a threat to the healthy development of this industry (Escobar and Dandekar, 2003). However, specialized research reports on crown gall disease of flowering cherry remain relatively limited, and the pathogen can be transmitted over long distances through the transfer of seedlings, further complicating prevention and control efforts. Currently, an integrated management strategy focusing on quarantine prevention is primarily adopted to control crown gall disease of flowering cherry. This approach involves strict pathogen detection during seedling introduction to minimize input risks, combined with physical measures such as pruning tool sterilization to reduce wound infection (Chand et al., 2017). Chemical interventions using K84 biocontrol agents or Cu-based compounds are promptly applied during early disease stages, while long-term sustainable control is achieved through agricultural practices, including the selection of disease-resistant rootstocks, the implementation of crop rotation systems, and scientific nutrient and water management (Farri and Khezri, 2021). In addition, considerable research has been conducted on the biological control of crown gall disease in recent years (Ahmed et al., 2022; Liu et al., 2024). Therefore, the efficient detection of tumorigenic agrobacteria in flowering cherry seedlings is a key step in the propagation of plant materials as well as in preventing the further spread of this disease.

In this study, selective MW medium was used to isolate bacteria from tumor tissue, with meticulous attention given to maintaining sterility. Species identification of all the isolated strains was achieved using biochemical tests and 16S rRNA sequence genetic analysis. Consistent with the well-documented limitations of single-gene 16S rRNA barcoding, this single molecular marker fails to fully distinguish closely related cryptic lineages in *Rhizobiaceae*. For the tumorigenic isolates recovered from diseased flowering cherry in this work, only supplementary multilocus sequence analysis (MLSA) or whole-genome phylogenomic datasets can deliver definitive species-level identification (Ma et al., 2023; Vargas Ribera et al., 2024). In the phylogenetic tree constructed via the neighbor-joining method with the 16S rRNA sequence, the isolated strain CA-6 was grouped into one clade. Strains CA3-1, CA3-2, CA3-3, CA3-4, and CA3-5 were clustered in another clade. The *Rhizobium radiobacter* type strain CFCC-1369 was in a separate clade. This suggests that the isolated strains belong to two different putative species.

A series of biochemical tests was subsequently conducted in accordance with Bergey's Manual of Determinative Bacteriology (Bai et al., 2022). The results indicated that the isolated strain CA-6 belongs to the *Agrobacterium tumefaciens* species complex, as determined by comparison with the type strain of *A. radiobacter* (synonym: *R. radiobacter*), IAM 12048^{T}, which was originally isolated from The Netherlands (Bautista-Zapanta et al., 2009). Consequently, strain CA-6 was identified as a member of the *A. tumefaciens* species complex (biovar 1). Additionally, the isolated strains CA3-1, CA3-2, CA3-3, CA3-4, and CA3-5 showed close taxonomic affiliation to the type strain of *R. calliandrae* CCGE524^{T}, which was previously isolated from root nodules of the medicinal legume *Calliandra grandiflora* (Rincon-Rosales et al., 2013). Notably, our strains carry tumor-inducing (Ti) virulence genes and induce galls, which conflicts with the typical symbiotic feature of standard *R. calliandrae.* They are likely an unreported tumorigenic subgroup closely related to this species rather than true *R. calliandrae*. Further MLSA or comparative whole-genome data are required to clarify the precise taxonomic identity of these gall-inducing isolates. This is the first record of such rhizobial pathogens causing cherry crown gall in local nurseries.

Typically, crown gall disease is a bacterial disorder that affects most dicotyledonous plants and some monocots. It is caused by organisms belonging to the *Agrobacterium*, *Rhizobium*, and *Allorhizobium* genera. The key characteristic of its pathogenesis involves the presence of a Ti plasmid (pTi). Most of the genes responsible for pathogenicity are located on this plasmid. Researchers have employed universal polymerase chain reaction (PCR) primers to detect phytopathogenic *Agrobacterium* strains. The Ti plasmids carry genes such as *tms2*, *virD2*, and *ipt*. The expression of these genes is essential for the occurrence of crown gall disease (Tan et al., 2003; Bai et al., 2022). In this study, relevant gene fragments could be detected via PCR in the strains isolated from the flowering cherry tree roots. The PCR analysis of pathogenicity-associated genes confirmed

the presence of Ti plasmids in the genomes of the isolated strains, and these results are consistent with the findings of numerous studies. However, recovering tumorigenic strains linked to crown gall of flowering cherry using selective MW medium posed obvious technical obstacles in the present study. The MW is a semi-selective medium originally designed for *Agrobacterium* isolation; it does not exert inhibitory effects on closely related *Rhizobium* species within *Rhizobiaceae*, due to their overlapping nutritional requirements, which accounts for the frequent co-isolation of *Agrobacterium* and *Rhizobium* strains. The main interference originated from *Pseudomonas* sp. and *Rhizobium* sp. (Holmes et al., 2023). To reduce such interference in future studies, several strategies can be considered: optimize the MW medium (e.g., adjust C/N ratios or add selective inhibitors) to suppress non-target bacteria, and apply species-specific PCR primers for rapid pre-screening of candidate colonies to improve isolation efficiency. Notably, although sampling was performed from March to September, crown gall-affected flowering cherry samples are most suitable for bacterial isolation during March to April, corresponding to the bud-swelling and early-flowering stages. At this time, metabolically active gall tissues harbor higher pathogen loads, favoring successful isolation. We hypothesize that this observation is linked to the incidence dynamics of crown gall disease on flowering cherry and conditions conducive to *A. tumefaciens* infection (Gohlke and Deeken, 2014; Barton et al., 2018).

The pathogenicity of bacterial isolates was assessed by needle inoculation into the young stems of tomato and sunflower plants (Xiao et al., 2023). Infection occurred within 1 mo, with tomato plants demonstrating greater susceptibility than sunflower plants did, suggesting host-specific differences. Moreover, an identical inoculation of flowering cherry roots resulted in distinct crown gall symptoms after 2 mo. Macroscopic observation suggested that strain CA-6 tended to form larger galls than the other isolates across all three hosts, but quantitative measurements were not performed in this study; therefore, conclusions regarding differential virulence are preliminary (Figure 1). Prior research indicates variable virulence among root-isolated rhizobia may be shaped by environmental temperature, seedling age, or intrinsic bacterial genetics (Bai et al., 2022). Uniform gall induction on all tested hosts verifies that all six isolates are tumorigenic.

Given the high incidence of crown gall disease in cherry nurseries in Ningbo, these findings underscore the importance of routine monitoring and quarantine measures to prevent the spread of highly virulent strains. Moreover, the observed variability in virulence among isolates points to the potential for breeding cherry rootstocks with enhanced resistance or for developing targeted biological control strategies against the most aggressive strains, thereby contributing to sustainable disease management in local production.

CONCLUSIONS

In this study, by integrating morphological, biochemical and 16S rRNA molecular identification, six tumorigenic isolates including strain CA-6 received preliminary taxonomic classification. Strain CA-6 was tentatively grouped into the *Agrobacterium tumefaciens* species complex (biovar 1), whereas isolates CA3-1 to CA3-5 shared high 16S rRNA homology with *Rhizobium calliandrae* and are therefore referred to as *R. calliandrae*-like strains. Single-locus 16S rRNA sequencing lacks sufficient resolution to differentiate cryptic taxa in *Rhizobiaceae*; complementary multilocus sequence analysis (MLSA) or genome-wide comparative analysis is required to clarify their accurate taxonomic placement. Visible variation in gall size was observed across inoculation treatments, and more evidence is required to confirm inter-strain virulence differences. This study preliminarily identifies the pathogenic bacteria associated with flowering cherry crown gall in Ningbo and establishes a basis for further research on local agrobacterial distribution and the development of integrated management approaches.

Author contribution

Conceptualization: Z.W. Methodology: K.F. Software: L.W. Validation: Q.G., L.L., F.Y. Formal analysis: Q.Y. Investigation: W.L., F.L., T.F. Resources: Z.W. Data curation: L.L. Writing-original draft preparation: L.L. Writing-review and editing: L.L. All co-authors reviewed the final version and approved the manuscript before submission.

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