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Phylogenetic relationships and pathogenicity of *Agrobacterium* in *Cinnamomum camphora*

Relaciones filogenéticas y patogenicidad de *Agrobacterium* en *Cinnamomum camphora*

Toshiyuki Kawamura[1,*], Yui Naito[1], Yosyun Onishi[1], Hayato Kato[1], Rajesree A/P Sivakumaran[1], Kentaro Kameyama[2], Yuichi Takaku[3], Osamu Haraguchi[4], Madoka Hirobe[5], Yuichi Kataoka[5] & Eiki Matsui[1]

[1]Department of Chemistry and Biology, National Institute of Technology, Fukui College, Geshi-cho, Sabae City, Fukui, 916-8507 Japan.
[2]Department of Mechanical Engineering, National Institute of Technology, Fukui College, Geshi-cho, Sabae City, Fukui, 916-8507 Japan.
[3]Department of Electrical and Electronics Engineering, National Institute of Technology, Fukui College, Geshi-cho, Sabae City, Fukui, 916-8507 Japan.
[4]Department of General Education, National Institute of Technology, Fukui College, Geshi-cho, Sabae City, Fukui, 916-8507 Japan.
[5]Educational Research Support Center, National Institute of Technology, Fukui College, Geshi-cho, Sabae City, Fukui, 916-8507 Japan.
*Corresponding author: kawamura@fukui-nct.ac.jp

ABSTRACT

Camphor trees (*Cinnamomum camphora*) are renowned for their natural resistance to bacterial diseases resulting from their production of camphor, a bioactive compound with antimicrobial properties. Despite extensive research on *Agrobacterium* as a soil-borne plant pathogen, its association with camphor trees has not been previously documented. To address this research gap, we investigated the potential interactions between *Agrobacterium* and camphor trees by isolating *Agrobacterium* strains using a copper-containing medium screening method. A molecular phylogenetic analysis of 16S rDNA sequences was employed to identify and characterize the isolates. The *Agrobacterium* strains formed a distinct phylogenetic cluster closely related to *Agrobacterium cavarae* derived from maize (*Zea mays* L.) roots and *Agrobacterium larrymoorei* derived from *Ficus benjamina*. These findings provide the first evidence of *Agrobacterium* in association with camphor trees, thereby expanding our understanding of plant-microbe interactions and highlighting a potentially unexplored aspect of *Agrobacterium* ecology. This work underscores the importance of investigating plant-associated microbial communities, particularly in species with unique antimicrobial traits.

Keywords: *Agrobacterium*, *Cinnamomum camphora*, Copper-containing medium.

RESUMEN

Los alcanforeros (*Cinnamomum camphora*) son famosos por su resistencia natural a las enfermedades bacterianas, derivada de su producción de alcanfor, un compuesto bioactivo con propiedades antimicrobianas. A pesar de la amplia investigación sobre *Agrobacterium* como patógeno de plantas transmitido por el suelo, no se ha documentado previamente su asociación con los alcanforeros. Para abordar esta laguna en la investigación, investigamos las posibles interacciones entre *Agrobacterium* y los alcanforeros aislando cepas de *Agrobacterium* mediante un método de cribado en un medio que contiene cobre. Se empleó un análisis filogenético molecular de secuencias 16S rDNA para identificar y caracterizar las cepas aisladas. Las cepas de *Agrobacterium* formaban un grupo filogenético distinto estrechamente relacionado con *Agrobacterium cavarae* derivado de raíces de maíz (*Zea mays* L.) y *Agrobacterium larrymoorei* derivado de *Ficus benjamina*. Estos hallazgos proporcionan la primera prueba de la asociación de *Agrobacterium* con árboles de alcanfor, ampliando así nuestra comprensión de las interacciones planta-microbio y destacando un aspecto potencialmente inexplorado de la ecología de *Agrobacterium*. Este trabajo subraya la importancia de investigar las comunidades microbianas asociadas a plantas, especialmente en especies con rasgos antimicrobianos únicos.

Palabras clave: *Agrobacterium*, *Cinnamomum camphora*, medio que contiene cobre.

INTRODUCTION

Agrobacterium is a well-known genus of soil-borne plant pathogenic bacteria that cause crown gall disease in a wide range of host species by transferring part of its DNA, known as T-DNA, into the host plant's genome (Chilton *et al.* 1977). Once *Agrobacterium* infects a plant and its T-DNA is integrated, the expression of genes encoded on the T-DNA induces the biosynthesis of auxins and cytokinins (Morris 1986). The increased levels of these plant hormones promote abnormal growth and crown gall formation (Gohlke & Deeken 2014).

Camphor trees (*Cinnamomum camphora*) are native to regions such as southern China, Taiwan, southern Japan, Korea, India, and Vietnam and thus are widely distributed across Asia; moreover, they have been introduced to several other countries (Orwa *et al.* 2009, Li *et al.* 2019, Li *et al.* 2023). These trees can grow up to 20-30 m in height and are the primary source of camphor, which is extracted from the wood, stems, and leaves through steam distillation (Berchtold & Presl 1825). Camphor trees are generally regarded as resistant to pathogens and pests. The essential oil derived from camphor trees has demonstrated antibacterial activity, as tested on *Escherichia coli* (Wu *et al.* 2019), antifungal properties against *Choanephora cucurbitarum* (Pragadheesh *et al.* 2013), and resistance to pests such as *Tribolium castaneum* and *Lasioderma serricorne* (Guo *et al.* 2016).

According to the Supplement to the List of Plant Diseases in Japan, as of November 22, 2023, crown gall and hairy root diseases caused by *Agrobacterium* species have been reported in 73 different plant species in Japan. However, camphor trees, known for their relatively strong resistance to pathogens, have not yet been reported as hosts for *Agrobacterium* infection (The Phytopathological Society of Japan 2023).

Takács *et al.* (2020) reported that in Hungary, 12.2% of 531 cases of disease in old trees were caused by polypore fungi, 22.8% by *Agrobacterium*, and 29.6% by ivy infestation, with 51.2% of the cases attributed to other pests and diseases. Globally, *Agrobacterium* has reportedly infected various crops such as grapes (Kuzmanović *et al.* 2018a) and nuts (Pulawska 2010), as well as trees like poplar (Nesme *et al.* 1987), oak (Bouzar & Moore 1987, Glits & Folk 2000), cypress (Basavand *et al.* 2019), and citrus (Bozkurt *et al.* 2023). Additionally, experimental studies have shown that the host range of tumorigenic *Agrobacterium*/*Rhizobium* strains, the causative agent of crown gall disease, is extensive, affecting dicots as well as monocots and gymnosperms. Pathogenicity has been confirmed in 643 plant species across 93 families and 331 genera out of 1193 plant species tested (De Cleene & De Ley 1976).

The genus *Agrobacterium* is renowned for its role as a plant pathogen capable of causing crown gall disease and other detrimental conditions in a wide variety of plants (Smith & Townsend 1907). Its ability to integrate genetic material into host plants has represented a concern for plant health, yet has also become a cornerstone for genetic engineering research (Gelvin 2003). Despite extensive studies on the biology of *Agrobacterium* and its infection mechanisms across numerous tree species, the susceptibility of certain species, such as camphor trees (*Cinnamomum camphora*), remains underexplored. This gap is particularly notable given that camphor trees have traditionally been regarded as resistant to numerous pathogens, positioning *C. camphora* as a critical model for understanding pathogen-host interactions in robust tree species.

The purpose of this study was to obtain new knowledge about *Agrobacterium* infection in camphor trees through detailed analyses, including isolating *Agrobacterium* from camphor trees for the first time and identifying the various species through phylogenetic analysis and examining the morphology of cells and colonies.

MATERIALS AND METHODS

Isolation of bacteria from plant leaves

Leaves of camphor trees were collected and placed in plastic bags (Ziploc, Asahi Kasei), followed by an addition of sterile water and a thorough suspension. The camphor tree leaves used as samples were taken from branches of an approximately 50-year-old tree planted in Sabae City, Fukui, Japan (Blinded for Review). The supernatant was then cultured in Luria-Bertani (LB) medium supplemented with 0.06 mM copper sulfate at 25 °C for one week.

Agrobacterium possesses the cop operon responsible for copper resistance (Nawapan *et al.* 2009). Following their findings, we attempted to isolate *Agrobacterium* by adding copper ions to the medium, with the goal of leveraging this resistance mechanism. Although copper resistance is not specific to *Agrobacterium*, and many other bacterial species possess copper resistance genes, copper-supplemented medium was used as a preliminary screening step to narrow down potential candidates. More specific identification was subsequently performed using phylogenetic analysis and morphological observation under a microscope.

Culture conditions for purity and growth verification

The purity and growth of the isolates were verified by culturing them on Difco LB Broth Lennox (Becton Dickinson, USA) solidified with agar. The cultures were incubated at 25 °C for 48 h under aerobic conditions.

Morphological observations

The morphology of the cell in terms of size and shape and characteristics of the cell wall were observed using Gram staining and microscopic observations (FAVOR G, Nissui Pharmaceutical, Japan). Observations were conducted with a light microscope (BX50F4, Olympus, Japan) and stereomicroscope (SMZ800N, Nikon, Japan).

The colonies grown on agar plates were suspended in distilled water and then dropped onto natural mica plates (Crystal Base Co., Ltd., V-4 (GS)). After drying, carbon coating of the sample surface was performed using a Quick Carbon Coater FC-701C (Sanyu Denshi Co., Ltd., Japan). The samples were then observed and analyzed using a scanning electron microscope (SEM) (JEOL, Japan; JSM-6340F).

PCR amplification and sequencing of the 16s rDNA region

The 16S rDNA region was amplified and sequenced using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGCTACCTTGTTACGACTT-3') (Lane 1991, Turner *et al.* 1999). The PCR products were subjected to agarose gel electrophoresis, excised, and purified. Sequencing reactions were performed with the above primers using the BigDye Terminator v3.1 Cycle Sequencing Kit, and the products were analyzed on an Applied Biosystems (USA) 3730xl DNA Analyzer.

The determined nucleotide sequences (Accession No. PQ721985) were analyzed using ChromasPro 2.1 (Technelysium, Australia). An analysis of the 1,368 base sequence of the determined 16S rDNA was performed, which excluded the region near the primer binding region. A BLAST homology search (Altschul *et al.* 1997) was performed on December 24, 2024, using the analysis software ENKI v3.2 (TechnoSuruga Laboratory, Japan), database DB-BA17.0 (TechnoSuruga Laboratory), and international nucleotide sequence database (DDBJ/ENA/GenBank). Molecular phylogenetic analysis was performed based on multiple sequence alignment using CLUSTALW (Thompson *et al.* 1994), alignment editing was performed using BioEdit ver. 7.2 (Hall 1999), molecular phylogenetic tree estimation was performed using MEGA ver. 12 (Kumar *et al.* 2016), phylogenetic tree estimation was performed using the neighbor-joining (NJ) method (Saitou & Nei 1987), base substitution modelling was performed using the Kimura 2-parameter model (Kimura 1980), and the maximum likelihood (ML) test (Felsenstein 1981) was performed using the Tamura–Nei base substitution model (Tamura & Nei 1993). The reliability of the tree was assessed with the bootstrap method (Felsenstein 1985) (1000 iterations).

When identifying bacteria, it is important to compare them with the type strain (the naming standard for species and subspecies) that serves as the basis for classification. In this study, the taxonomic group of the samples was estimated based on the results of a comparison and analysis with the 16S rDNA base sequence of the type strain of each bacterial species and subspecies. A total of 12 *Agrobacterium*/*Rhizobium* strains were used for phylogenetic analysis via the NJ and ML methods.

Detection of Vir genes responsible for t-DNA integration into plant genomes (estimation of plant pathogenicity)

Colonies on agar plates were transferred to PCR tubes, suspended in sterile water, and heated at 95 °C for 5 min. The *VirD2* gene region was amplified using MightyAmp DNA Polymerase (Takara Bio) and the primer set described below. The PCR products were then subjected to electrophoresis on a 3% agarose gel, stained with ethidium bromide, and observed under ultraviolet light.

PCR reactions were performed using universal PCR primers designed by Haas *et al.* (1995) for detecting pathogenic *Agrobacterium*: primer A (5'-ATG CCC GAT CGA GCT CAA GT-3' [coordinates 1 to 20]), primer C' (5'-TCG TCT GGC TGA CTT

TCG TCA TAA-3' [coordinates 224 to 201]) and primer E' (5'-CCT GAC CCA AAC ATC TCG GCT GCC CA-3') [coordinates 338 to 313]). PCR using the A-C' primer set yielded a 224 bp product, and PCR using the A-E' primer set yielded a 338 bp product. As a positive control for this experiment, the *Agrobacterium tumefaciens* LBA4404 strain, which possesses the *VirD2* gene but lacks tumorigenic pathogenicity, was used. The 100 bp DNA Ladder from FastGene was used as the marker DNA.

RESULTS

Characterization of *Agrobacterium* isolates: morphological and gram staining analysis

Clone 10 exhibited colony characteristics typical of *Agrobacterium*, with a creamy, moist appearance (Fig. 1A). Furthermore, the bacterium was gram-negative and rod-shaped (Fig. 1B). Gram staining revealed that the isolate did not form spores. The cells also varied in size, ranging from short cells of 1 µm in length to cells longer than 2 µm in length (Figs. 1C, 1D), which was consistent with the typical morphology of *Agrobacterium*. These morphological features further support the identification of Clone 10 as *Agrobacterium* and are consistent with the known characteristics of this genus.

Phylogenetic analysis and genetic relationship of *Agrobacterium* strain clone 10 with closely related species

The tree revealed three major clades with varying bootstrap support levels. Clone 10 was most closely related to *A. larrymoorei* AF3.10 and *A. cavarae* RZME10. The difference between Clone 10 and *A. larrymoorei* and *A. cavarae* showed extremely strong support with a bootstrap value of 100.

Additionally, the evolutionary distance between Clone 10 and *A. skierniewicense* Ch11 as well as *A. rosae* NCPPB1650 was approximately 0.01 in both the NJ and ML methods, thus supporting the possibility that these strains form an exceedingly close cluster. The bootstrap values for this difference were 63 for the NJ method and 58 for the ML method, indicating moderate support for the branching (Figs. 2A, 2B).

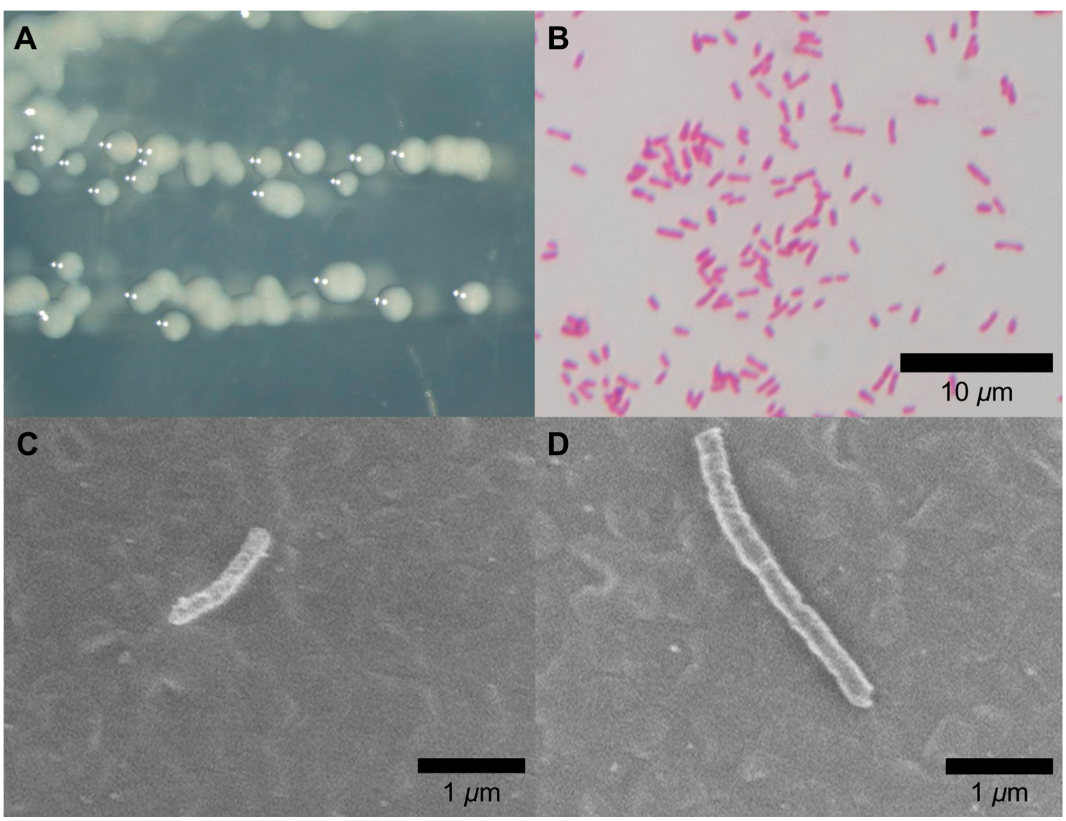

Figure 1. Morphology of the clones isolated from camphor trees. (A) Colony appearance, (B) Gram staining, (C) and (D) represent cells observed under a scanning electron microscope, where (C) is a short cell and (D) is a long cell. / Morfología de los clones aislados de los alcanforeros. Morfología de los clones aislados de alcanforeros. (A) Aspecto de la colonia, (B) Tinción de Gram, (C) y (D) representan células observadas al microscopio electrónico de barrido, donde (C) es una célula corta y (D) es una célula larga.

Indirect estimation of *Agrobacterium* pathogenicity in plants through detection of the *VirD2* gene

Amplification of the *VirD2* gene region by colony PCR revealed a signal corresponding to the expected 224 and 338 bp products in the control experiment with *A. tumefaciens* LBA4404. However, no amplification signal was detected in Clone 10, which was isolated from the *Cinnamomum* trees (Fig. 3).

The morphology of the camphor tree from which *Agrobacterium* was isolated contained several areas that look like a mass, although a bumpy mass-like appearance is common for camphor trees. Presumably, changes in pruning and branch breakage areas resemble a mass over time. We could not determine whether this mass-like morphology was due to *Agrobacterium* pathogenicity.

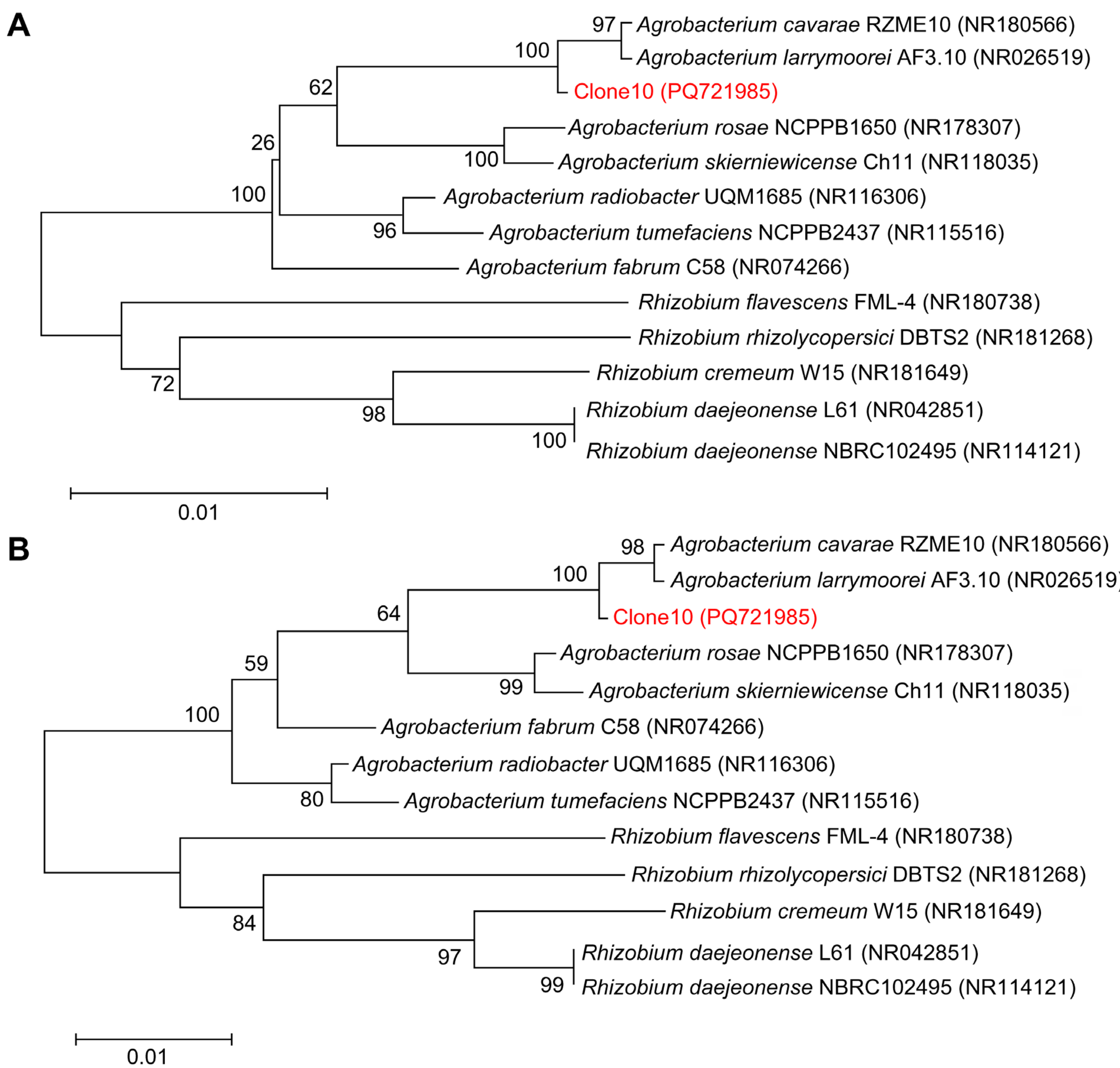

Figure 2. Molecular phylogenetic tree based on the 16S rDNA sequence of strain Clone10. (A) Neighbor-joining method. (B) Maximum likelihood method. The scale bar is located at the top left. Numbers at the branching points represent bootstrap values. / Árbol filogenético molecular basado en la secuencia de ADNr 16S de la cepa Clone10. Árbol filogenético molecular basado en la secuencia de ADNr 16S de la cepa Clone10. (A) Método de unión de vecinos. (B) Método de máxima verosimilitud. La barra de escala se encuentra en la parte superior izquierda. Los números de los puntos de ramificación representan valores bootstrap.

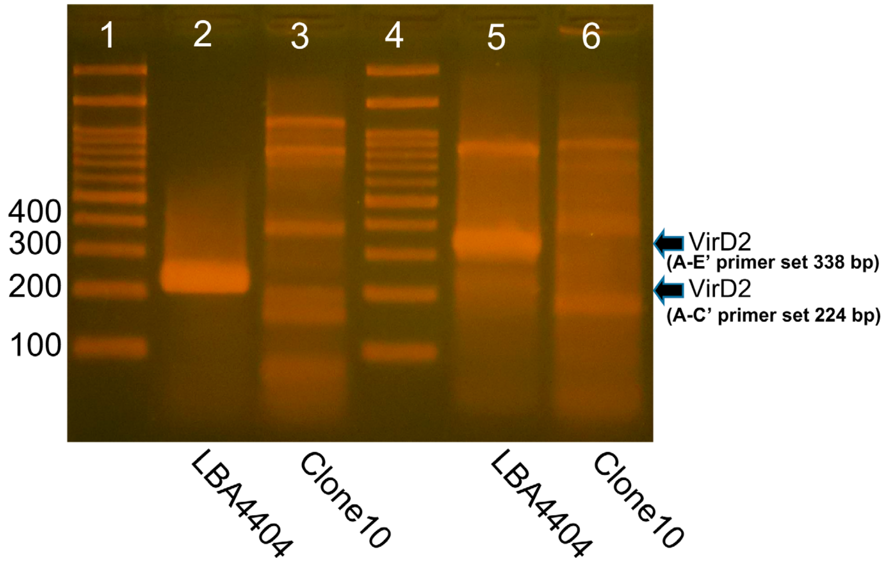

Figure 3. Detection of the *VirD2* gene by PCR (Indirect Pathogenicity Prediction). Lanes 1 and 4 show the ladder marker (numbers indicate 400 bp, 300 bp, 200 bp, and 100 bp). Lanes 2 and 5 show the PCR product from *Agrobacterium tumefaciens* LBA4404 strain used as the positive control, and Lanes 3 and 6 show the PCR product from Clone 10 isolated from the *Cinnamomum* tree. Lanes 2 and 3 show PCR products using the A-C' primer set, and lanes 5 and 6 show PCR products using the A-E' primer set. / Detección del gen VirD2 Detección del gen *VirD2* por PCR (predicción indirecta de patogenicidad). Los carriles 1 y 4 muestran el marcador ladder (los números indican 400 pb, 300 pb, 200 pb y 100 pb, respectivamente). Los carriles 2 y 5 muestran el producto de la PCR de la cepa *Agrobacterium tumefaciens* LBA4404 utilizada como control positivo, y los carriles 3 y 6 muestran el producto de la PCR del clon 10 aislado del árbol *Cinnamomum*. Los carriles 2 y 3 muestran los productos de la PCR utilizando el conjunto de cebadores A-C', y los carriles 5 y 6 muestran los productos de la PCR utilizando el conjunto de cebadores A-E'.

DISCUSSION

Properties of the isolated *Agrobacterium* as predicted via molecular phylogenetic analysis

Clone 10 isolated from camphor trees is closely related to *A. larrymoorei* and *A. cavarae* (Figs. 2A, 2B). Furthermore, the closeness of Clone 10 to *A. skierniewicense* and *A. rosae* was consistent with the results of both the NJ and ML methods. However, low to moderate bootstrap values of 62 and 64 were recorded using the NJ and ML methods, respectively, and the branch support rate was moderate (Figs. 2A, 2B). Therefore, there was insufficient variation/polymorphism in the 16S-rDNA sequence length for *Agrobacterium* species identification, and *Agrobacterium* species could not be differentiated due to low bootstrap values of the nodes. An incomplete *Agrobacterium* taxonomy is obtained from phylogenetic trees based on single marker genes such as the *16S rRNA* gene or the *recA* gene; however, genomic species can be accurately and efficiently identified by combining multilocus sequence analysis (MLSA) and average nucleotide identity (ANIb) (Vargas Ribera *et al.* 2024). The Average Nucleotide Identity (ANI) and Digital DNA-DNA Hybridization (dDDH) are critical for determining the genetic relatedness of strains. ANI values above 95-96% and dDDH values above 70% typically indicate the same species (Richter & Rosselló-Móra 2009). Phenotypic and biochemical characterization tests such as carbon source utilization, opine metabolism, and other enzymatic activities complement molecular approaches (Kersters *et al.* 2006).

By combining these methods, researchers can ensure an increasingly reliable and robust identification of *Agrobacterium* species. However, Naranjo *et al.* (2023) also suggested that *A. larrymoorei*, *A. cavarae*, *A. skierniewicense*, and *A. rosae* formed a cluster in a phylogenetic analysis. Therefore, Clone 10, which we isolated from camphor tree, may also belong to this group.

Raio *et al.* (2004) demonstrated that *A. larrymoorei* is capable of surviving and migrating within the xylem of weeping fig (*Ficus benjamina*), where it induces tumors in the aerial parts of wounded plants. This behavior is notable because most pathogenic *Agrobacterium* species do not exhibit such xylem migration (Zoina *et al.* 2002; Raio *et al.* 2004).

The bacterial strain RZME10T, isolated from maize (*Zea mays* L.), belonged to the *Agrobacterium* genus and showed the closest relationship to *A. larrymoorei* through a 16S rRNA gene sequence analysis. However, the species' similarity with other genes (*rpoB*, *recA*, *gyrB*, *atpD*, and *glnII*) was low, and genome analysis revealed clear differences. Based on chemical characteristics such as the composition of major fatty acids and phenotypic analysis, RZME10T was classified as a new species and named *A. cavarae* (Flores-Félix *et al.* 2020). Moreover, pathogenicity tests confirmed that it does not induce tumors in tomato or carrot roots. The existence of such non-pathogenic species of *Agrobacterium* is not inconsistent with the lack of detection of the *VirD2* gene, which is an indicator of pathogenicity of Clone 10 (Fig. 3).

A. skierniewicense and *A. rosae*, which belong to the cluster that includes the Clone 10 strain, *A. larrymoorei*, and *A. cavarae*, have been isolated from crown gall lesions of chrysanthemum and rose, respectively, and are plant pathogenic (Puławska *et al.* 2012; Kuzmanović *et al.* 2018b).

The bacterium *A. larrymoorei*, discovered in fig trees, and *A. cavarae*, isolated from maize roots, are genetically extremely closely related. However, the former exhibits pathogenicity toward plants (Bouzar & Jones 2001; Bouzar *et al.* 1995), whereas the latter is considered non-pathogenic (Flores-Félix *et al.* 2020). This dynamic suggests that the *Agrobacterium* strain we discovered in camphor trees that is currently considered non-pathogenic but genetically similar to pathogenic species may potentially acquire pathogenicity through evolutionary processes. *Agrobacterium* was previously thought to be incapable of infecting camphor trees; however, its successful infection of this species and close relationships with pathogenic species indicate the need for vigilance in future tree management practices. This discovery underscores a potential threat posed by *Agrobacterium* to trees, warranting proactive monitoring and preventive measures.

Perspective on the plant pathogenicity of *Agrobacterium* derived from *Cinnamomum*

Our study revealed that clone 10 forms a genetic cluster with *A. cavarae* and *A. larrymoorei*. Notably, these two species exhibited contrasting levels of phytopathogenicity, with *A. cavarae* lacking phytopathogenic traits and *A. larrymoorei* reportedly causing plant diseases under specific conditions. This discrepancy suggests that genetic similarity alone cannot reliably predict phytopathogenic potential, underscoring the intricate interplay of genetic, environmental, and host factors in determining pathogenicity. Vargas Ribera *et al.* (2024) reported that *Agrobacterium* species may or may not present pathogenicity discrepancies; thus, we suggest adopting a holistic perspective and investigating regulatory genes and host-specific interactions. Our research also documents the first isolation of *Agrobacterium* from camphor trees (*C. camphora*), which suggests that new tree infections can always arise. Phylogenetic and morphological analyses revealed that the isolated strain shared considerable similarities with pathogenic *Agrobacterium* species, extending its known host range. This finding raises concerns for tree species traditionally considered pathogen-resistant, exposing potential vulnerabilities in urban forests. Despite extensive studies on *Agrobacterium* biology and infection mechanisms across various tree species, the susceptibility of camphor trees remains underexplored. Given their historical classification as resistant to several pathogens, camphor trees present a valuable model for understanding pathogen-host interactions in robust tree species.

This discovery holds important implications, particularly for Japan's ageing urban trees, many of which were planted during its rapid economic growth period. As these trees age, their vulnerability to pathogens such as *Agrobacterium* increases, thus posing risks to urban safety and environmental sustainability. Proactive surveillance and targeted management strategies are essential to mitigate pathogen spread and preserve urban ecosystems.

These long-lived trees have sophisticated defense mechanisms, such as the production of specialized plant metabolites (SPMs) (Cui *et al.* 2024). However, as bacteria battle with the superior SPMs that long-lived trees have acquired during evolution, both may continue to produce innovative functions our study highlights the susceptibility of camphor trees, a species previously considered pathogen-resistant, thus challenging long-held assumptions about tree resilience.

Conclusion & future directions

To build on these findings, future research should leverage advanced genomic and transcriptomic approaches to uncover the molecular mechanisms underlying camphor tree susceptibility to *Agrobacterium*. Additionally, longitudinal studies examining the impact of environmental stressors—such as urban pollution and soil degradation—on pathogen-host dynamics are essential. Integrating ecological and genomic perspectives will enhance our understanding of *Agrobacterium* spread and resilience in urban forestry.

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