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Research paper

Aluminum-tolerant, growth-promoting endophytic bacteria as contributors in promoting tea plant growth and alleviating aluminum stress

Xiaolan Jiang^{1,†}, Wei-Wei Li^{1,†}, Menglin Han¹, Gao Chen¹, Jing Wu¹, Sanyan Lai¹, Zhouping Fu¹, Shuxiang Zhang², Wei-Wei Deng¹, Liping Gao^{2,3} and Tao Xia^{1,3} 

¹State Key Laboratory of Tea Plant Biology and Utilization, Anhui Agricultural University, 130 West Changjiang Rd, Hefei 230036, Anhui, China; ²School of Life Science, Anhui Agricultural University, 130 West Changjiang Rd, Hefei 230036, China; ³Corresponding authors: T.Xia (xiatao62@ahau.edu.cn); L.Gao (gaolp62@126.com)

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Unlike that of other crops, the growth of tea plants can be promoted by aluminum, but its regulation mechanism remains unclear. Some endophytes can also promote growth of plant hosts. In this paper, tea roots treated with aluminum were used to study the growth-promoting traits and aluminum tolerance of endophytes. Meta-16S rDNA analysis revealed that *Burkholderia* was enriched in tea roots after aluminum treatment, and it was the dominant strain for hydroponic tea roots and field tea roots. *Actinomycetes* constituted the dominant strains in hydroponic tea seedlings treated with aluminum. Sixteen endophytic bacteria, including 12 strains of *Firmicutes*, 2 strains of *Proteobacteria* and 2 strains of *Actinomycetes*, were isolated and identified from hydroponic tea roots treated with different aluminum concentrations. Growth-promoting activity analysis showed that the isolated endophytic bacteria all had more than one plant growth-promoting trait. Among them, B4 (*Bacillus nealsonii*), B8 (*Brevibacterium frigoritolerans*) and A2 (*Nocardia nova*) bacteria each had three growth-promoting traits. Aluminum tolerance ability analysis indicated that endophyte A1 (*Leifsonia shinshuensis*) had the strongest aluminum tolerance ability, up to 200 mg l⁻¹ aluminum. Plant–bacteria interactions showed that endophytes A1, A2 and B4 and their synthetic community all had a growth-promoting effect on the growth of wheat lateral roots. Moreover, endophytes A1 and B4 alleviated aluminum stress in wheat. Endophyte A1 also promoted the growth of tea cuttings, especially lateral roots, with/without aluminum. Taken together, aluminum enhanced the distribution of aluminum-tolerant and growth-promoting bacteria, thereby promoting the growth of tea roots. This study provides a new aspect for research on the mechanism by which aluminum promotes tea plant growth.

Keywords: aluminum toxicity, endophyte, physiological profiles, plant–bacteria interactions, tea roots.

Introduction

Aluminum is one of the main toxic metal ions that inhibits the growth and development of crops, such as wheat, rice, rye and corn in acidic soils, and it causes a significant reduction in crop yields (Watanabe and Osaki 2002, Kochian et al. 2015). However, unlike for most crops, aluminum is a beneficial element for tea plants. Many studies have shown that low concentrations of aluminum promoted the growth of tea plants, while high concentrations, such as >2 mM, inhibited their growth (Ghanati

et al. 2005, Morita et al. 2008, Li et al. 2017, Safari et al. 2018, Fu et al. 2020, Sun et al. 2020). Research on the effect of aluminum on tea plants mainly focuses on aluminum tolerance, including the complexation of organic acids (Morita et al. 2008), complexation of polyphenols (Nagata et al. 1992, Fu et al. 2020) and modification of cell walls (Li et al. 2017, Safari et al. 2018). However, the mechanism by which aluminum promotes the growth of tea plants has not been clarified.

[†]These authors contributed equally to this work.

Plant endophytic bacteria exist in the plant for a long time, and most of them are in a stable and mutually beneficial symbiotic state and play various roles in the plant host, such as direct or indirect growth promotion and disease resistance; examples include *Pseudomonas*, *Enterobacter*, *Azotobacter* and other strains (Zamioudis et al. 2013, Santoyo et al. 2016, Rho et al. 2018, Morales-Cedeno et al. 2021). Endophytes mainly promote the growth and development of host plants by producing indole-3-acetic acid (IAA) (Santoyo et al. 2016, Duran et al. 2018, Yan et al. 2018), fixing nitrogen (Ji et al. 2014, Zhang et al. 2019) and enhancing the absorption of soil nutrients by plants (Yu et al. 2011, Hiruma et al. 2016). Due to the uncontrollability of the field environment, there are still limitations in predicting the impact of endophytes on plant growth and development under field conditions, and reducing environmental changes is essential for meaningful microbiome comparison descriptions. The synthetic community (SynCom) system can decompose the interactions between the root microbiome and the host plant into experimentally controllable factors, such as microorganisms, plant genotypes, nutrients and growth conditions (Vorholt et al. 2017, Liu et al. 2019).

There are many kinds of endophytic bacteria in plants, and the composition of endophytic bacterial communities in different tissues and organs of different plants is also very different. At present, extensive research on endophytes in crops, such as rice, sorghum, cotton and corn, has been performed. (Hardoim et al. 2015, Mareque et al. 2015, Santoyo et al. 2016). However, apart from a small number of studies on tea rhizosphere bacteria and endophytes in different tea areas, there are few reports on tea plant microorganisms (Dutta et al. 2015, Dutta and Thakur 2017, Shan et al. 2018, Yan et al. 2018, Borah et al. 2019). Most of the endophytic bacteria isolated from different tissues of tea plants showed plant growth-promoting traits (PGPTs) that positively affect the growth and production of tea plants, including producing IAA, dissolving phosphorus, fixing nitrogen, producing siderophores, etc. (Dutta et al. 2015, Dutta and Thakur 2017, Shan et al. 2018). Research by Yan et al. showed that there were very large differences in the dominant bacteria of tea plants in different periods, and most of the dominant endophytes isolated during the vigorous growth period had a growth-promoting effect (Yan et al. 2018). Our previous experiments found that the growth of tea roots was very different under three aluminum concentrations (0, 0.4 and 2.0 mM) (Fu et al. 2020). Does the distribution of endophytic bacteria in these tea roots also differ significantly in aluminum-containing environments?

To understand the relationship between tea root endophytic bacteria and tea root growth and development under aluminum treatment, tea roots exposed to different aluminum concentrations were selected for meta-16S rDNA sequencing and endophytic bacteria isolation. Then, the PGPTs of the cultivable strains were screened. Wheat seedlings and tea cuttings were treated to research plant–bacterial interaction characteristics

(Figure S1 available as Supplementary data at *Tree Physiology* Online). Our research provides a new aspect for research on the mechanism by which aluminum promotes tea plant growth and lays the foundation for the selection of tea plant endophytic strains with significant root-promoting ability for use in the cultivation and breeding of tea plants.

Materials and methods

Plant culture

In this experiment, roots of Shuchazao cultivated for 1 year in the presence of 0, 0.4 and 2 mM aluminum ($\text{Al}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$) were selected for high-throughput sequencing and isolation of endophytic bacteria. The cultivation conditions were described in our previous research (Fu et al. 2020). For tea garden aluminum treatment, 5-year-old Shuchazao plants from the Experimental Tea Garden, Anhui Agricultural University (Hefei, Anhui, China, 31.52°N 117.14°E) were treated with 2 mM aluminum for half a year from July to December 2020. The treatment frequency was once every 2 weeks, with 2 l each time. The soil pH of the Experimental Tea Garden was 5.09, and the exchangeable aluminum concentration was 66.10 mg kg⁻¹ before treatment.

Diversity analysis of endophytic bacteria

The hydroponic roots of each Al treatment (0, 0.4 and 2 mM) and roots from a tea garden (control and 2 mM Al treatment) were collected and then aliquoted into tubes. Part of the material was immediately frozen in liquid nitrogen, stored with solid carbon dioxide (dry ice) and then sent to The Beijing Genomics Institute (Wuhan, China) for meta-16S rDNA analysis. Three biological replicates of each analysis were carried out. The hydroponic roots under Al treatment were further used for endophytic bacteria separation experiments.

Bacterial strain isolation and growth conditions

Endophytic bacteria were isolated from the roots of Shuchazao cultivated for 1 year at concentrations of 0, 0.4 and 2 mM as follows: the material was first rinsed under running water for 30 min to wash off surface dirt, immersed in 75% ethanol for 30 s and rinsed for three to four times with sterilized water. Second, the cells were disinfected by soaking in 0.1% HgCl₂ for 8 min and were washed with sterilized water for three to four times. After sterilization, the roots were fully ground with an autoclaved abrasive tool containing 5 ml sterilized water. Next, 500 µl of homogenate was added into 50 ml Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract and 10 g NaCl) and was shaken overnight at 28 °C. Then, the cells were inoculated on agar-solidified LB agar plates at 28 °C for 48–72 h. The final rinse sterilized water was spread over the LB medium as a sterility check. Bacterial colonies

were selected based on their morphological characteristics for purification and inoculated on the same medium repeatedly. All aseptic operations were performed on a clean bench.

RNA extraction, polymerase chain reaction amplification and 16S rRNA gene analysis

Endophytes were first evaluated by their morphological and biochemical characteristics and were then identified by 16S rRNA analysis. Genomic DNA was extracted through high-temperature crushing at 98 °C for 15 min. The RNA amplification used 16S rRNA universal primers (27F: 5'-AGAGTTTGATCCTGGCTCA-3'; 1492R: 5'-GGTTACCTGTACGACTT-3') according to the following polymerase chain reaction (PCR) procedure: pre-denaturation at 32 cycles of 98 °C for 30 s, annealing at 56 °C for 30 s and 72 °C for 1 min, elongation at 72 °C for 10 min and finally maintaining at 4 °C. The unpurified PCR products were sent to GENERAL BIOSYSTEMS Inc. and sequenced. Partial 16S rRNA sequences obtained were compared with the GenBank database of NCBI (<https://www.ncbi.nlm.nih.gov/>) and EzBioCloud (<https://www.ezbiocloud.net/>).

PGP activity assays

IAA production assay Indole-3-acetic acid production was examined for the identified bacteria using the method of Yan et al. (2018). Tryptophan was formulated into a 2.5 mg ml⁻¹ solution, filtered through a 0.22- μ m Millipore filter for sterilization and added to nitrogen-containing liquid medium to a final tryptophan concentration of 0.5 mg ml⁻¹. The bacteria were inoculated into the above solution according to 1% of the inoculum and were shaken at 28 °C for 48–72 h. Centrifugation was performed for 15 min, and the supernatant was added to a C18 extraction column to extract IAA. Afterwards, IAA was eluted with 100% methanol, and the final 0.5 ml extract was obtained as the sample. Chromatographic separation was performed on a reverse-phase column (Phenomenex Kinetex[®] XB-C18 2.6 μ m 4.6 \times 100 mm) in a liquid chromatography system (Agilent 1,260 UPLC) equipped with an ultraviolet detector monitoring the wavelength of 261 nm. The injection volume was 5 μ l at a flow rate of 0.4 ml min⁻¹ with a mobile phase composed of solvent A (5 mM ammonium formate in 0.1% formic acid) and solvent B (100% methanol) for separation in gradient mode.

Assays of other PGP activities Solidified Pikovskayas agar was used to identify phosphate (P⁻) solubilization activity (Yan et al. 2018). The inoculated Pikovskayas agar was cultured in a 28 °C incubator in an inverted manner. Each agar plate had three bacterial spots; the presence or absence of a transparent halo around each plaque was observed. The ability to produce siderophores was verified by using an improved version of CAS medium (Wang et al. 2014). To activate the isolated strains, MKB iron-free liquid agar was used at 28 °C for 3 days. Then, the cells were inoculated onto solidified MKB iron-free agar and

cultured at 28 °C for 2 days, and a positive strain was indicated by an orange halo around the bacterial colony. The ability to use 1-amino-1-cyclopropanecarboxylic acid (ACC) was determined by ADF agar (DF agar replacing (NH₄)₂SO₄ with ACC). The isolated bacteria were inoculated onto ADF agar and were cultured at 28 °C for 7 days, and the observed growth was subcultured for three times, which indicated ACC deaminase activity.

Aluminum tolerance ability of the endophytic bacteria The aluminum tolerance ability of the selected endophytic bacteria was investigated under different aluminum treatments. The bacterial samples stored in the refrigerator at -80 °C were incubated in LB broth at 28 °C and 180 r.p.m. for 12 h. The overnight activated bacterial solution was inoculated (inoculum amount of 1%) onto LB solid medium containing different Al³⁺ concentrations (0, 20, 40, 60, 100 and 200 mg l⁻¹) and cultured at 30 °C for 12–24 h. The size of the plaque was observed and measured. Additionally, the overnight activated bacterial solution was inoculated (inoculum amount of 1%) into LB liquid medium containing different Al³⁺ concentrations (0, 20, 40, 60, 100 and 200 mg l⁻¹) and was incubated at 30 °C and 180 r.p.m. for 12–24 h, followed by measuring the OD₆₀₀ value with a spectrophotometer. At the same time, the OD₆₀₀ value of bacterial growth in LB liquid medium without Al was determined as a control.

Plant–bacteria interactions The bacterial concentration gradient treatment experimental method was referenced from the Gabriel Castrillo root microbial flora-driven phosphate stress experiment (Castrillo et al. 2017). Wheat seeds (Annonong 0711) were sterilized with 75% alcohol and then rinsed with sterile water for three to four times. Next, the sterilized seeds were soaked in a specific bacterial suspension (10⁵ c.f.u. ml⁻¹ to 10⁷ c.f.u. ml⁻¹ or 10⁶ c.f.u. ml⁻¹) for 12 h and were rinsed with sterile water for three to four times. Afterward, the wheat seeds were cultured at 25 °C for 2 days until they took root. The germination rate of wheat seeds was counted. Finally, wheat seedlings were cultivated with Hoagland's nutrient solution (KNO₃ (506 mg l⁻¹), Ca(NO₃)₂·4H₂O (945 mg l⁻¹), MgSO₄·7H₂O (493 mg l⁻¹), NH₄NO₃ (80 mg l⁻¹), KH₂PO₄ (136 mg l⁻¹), FeSO₄·7H₂O (13.9 mg l⁻¹), EDTA-Na (18.7 mg l⁻¹), KCl (0.83 mg l⁻¹), H₃BO₃ (6.2 mg l⁻¹), ZnSO₄·7H₂O (8.6 mg l⁻¹), CuSO₄·5H₂O (0.025 mg l⁻¹), H₂MoO₄ (0.25 mg l⁻¹) and CoCl₂·6H₂O (0.025 mg l⁻¹)) containing specific endophytic bacteria at 10⁶ c.f.u. ml⁻¹ and then cultured at 25 °C for 1 week. To study the influence of endophytic bacteria on the growth of wheat seedlings under aluminum treatment, 0.4 mM aluminum was added to the nutrient solution alone or at the same time as the bacteria and cultured as described above. The control was wheat seedlings cultivated in Hoagland's nutrient solution that did not contain bacteria. The

growth indicators of the roots were observed and measured, including root length and the number and length of lateral roots. The aluminum content was detected according to our previous method (Fu et al. 2020). Three biological replicates of the wheat–bacteria interaction experiment were carried out, and 40 wheat seedlings were used for each repeat.

Tea cuttings were treated with bacteria alone, aluminum alone and both. For bacterial treatment alone, the fresh tea cuttings were soaked in a specific bacterial suspension (10^6 c.f.u. ml⁻¹) for 15 min, inserted into sterilized soil (all vermiculite) and watered with 5 ml bacterial suspension every 2 weeks. To determine the effect of these endophytic bacteria on the growth of tea cuttings with aluminum, 5 ml of 0.4 mM aluminum were added to the sterilized soil alone or at the same time with A1 bacteria every 2 weeks and cultured as described above. The control was tea cuttings cultivated in sterilized soil and watered with just water. After the tea cuttings were cultivated for 6 months, the growth indicators were measured, including the weight and length of shoots and roots and the number of roots and leaves. Three biological replicates of the tea cuttings–bacteria interaction experiment were carried out, and 40 tea cuttings were used for each repeat.

Statistical analysis

The root and shoot biomass values among the wheat seedling treatments were analyzed statistically by one-way ANOVA using the software IBM SPSS Statistics V21.0, and the means were compared for significant differences by Duncan analysis; a *P*-value ≤ 0.05 was considered to be significant.

Results

Diversity analysis of endophytic bacteria in tea roots under different aluminum concentrations from hydroponic culture

Under different aluminum treatments (0, 0.4 or 2 mM), the growth of tea roots varied greatly (Figure 1a). To study the relationship between tea root growth and endophytic bacteria under different aluminum treatments, the endophytic bacteria of these roots were analyzed by meta-16S rDNA. Principal component analysis of OTU abundance showed that there were significant differences in the clustering of endophytic bacterial structures among the three groups of high aluminum (2 mM), low aluminum (0.4 mM) and no aluminum (0 mM) (Figure 1b). This finding was further verified from analysis of the bacterial phylum and genus abundance for the three groups of aluminum concentrations (Figure 1c and d).

There was a clear difference in the diversity and abundance of endophytic bacteria (from phylum to genus) in tea roots under different aluminum treatments (Figure 1c and d). The diversity and abundance of endophytic bacteria in tea roots cultured without aluminum ions were lower than those in the

high aluminum and low aluminum groups. In addition, Proteobacteria and Acidobacteria were found to be more abundant in the high and low aluminum groups with better root development.

The cladogram by LEfSe analysis indicated that in the high aluminum group (2 mM), three types of bacteria were significantly enriched, namely, Actinobacteria (from phylum to order classification level), Burkholderiales (from order to family classification level) and Opitutae (from phylum to order classification level) (Figure S2a available as Supplementary data at *Tree Physiology Online*). The result of endophytic bacteria with LDA scores >2 also confirmed that in the high aluminum group, Actinobacteria and Burkholderiales were significantly enriched (Figure S2b available as Supplementary data at *Tree Physiology Online*). It was concluded that there were significant differences in the diversity and abundance of endophytic bacterial communities in the three groups of tea roots under different aluminum treatments. Moreover, Actinobacteria and Proteobacteria may be closely related to the growth and development of tea plant roots.

Diversity analysis of endophytic bacteria in tea roots under aluminum treatment in a tea garden

To further investigate whether the composition and abundance of endophytic bacteria in the roots from a tea garden also exhibit the above differences, this study carried out aluminum treatment (2 mmol l⁻¹) on tea plants for half a year, and then, the same meta-16S rDNA analysis was performed on the aluminum-treated tea roots and untreated tea roots. From the phenotype of tea roots, it could be seen that the growth status of tea plant roots after aluminum treatment was significantly better than that of tea plants without aluminum treatment (Figure 2a and b).

The diversity and abundance of endophytic bacteria varied in tea roots under aluminum treatment in the tea garden, similar to those in hydroponic culture (Figure 2c and d). Analysis of the composition and abundance of endophytes under the phylum classification found that the relative abundances of Actinobacteria, Cyanobacteria and Proteobacteria increased after aluminum treatment. At the genus level, the relative abundances of *Actinospica* and *Burkholderia* increased (Figure 2c and d). Comparing the composition and abundance of endophytic bacteria in the phylum and genus classification of field and hydroponic seedlings, it was found that the relative abundances of Proteobacteria and its subordinate *Burkholderia* in two types after aluminum treatment were all increased, and their proportion in the root system of tea plants was relatively high; by contrast, the relative abundance of Acidobacteria was reduced after aluminum treatment (Figures 1 and 2).

The LEfSe analysis of the endophytic bacteria from the tea garden showed that in the roots of tea plants treated with aluminum, three types of bacteria were significantly enriched,

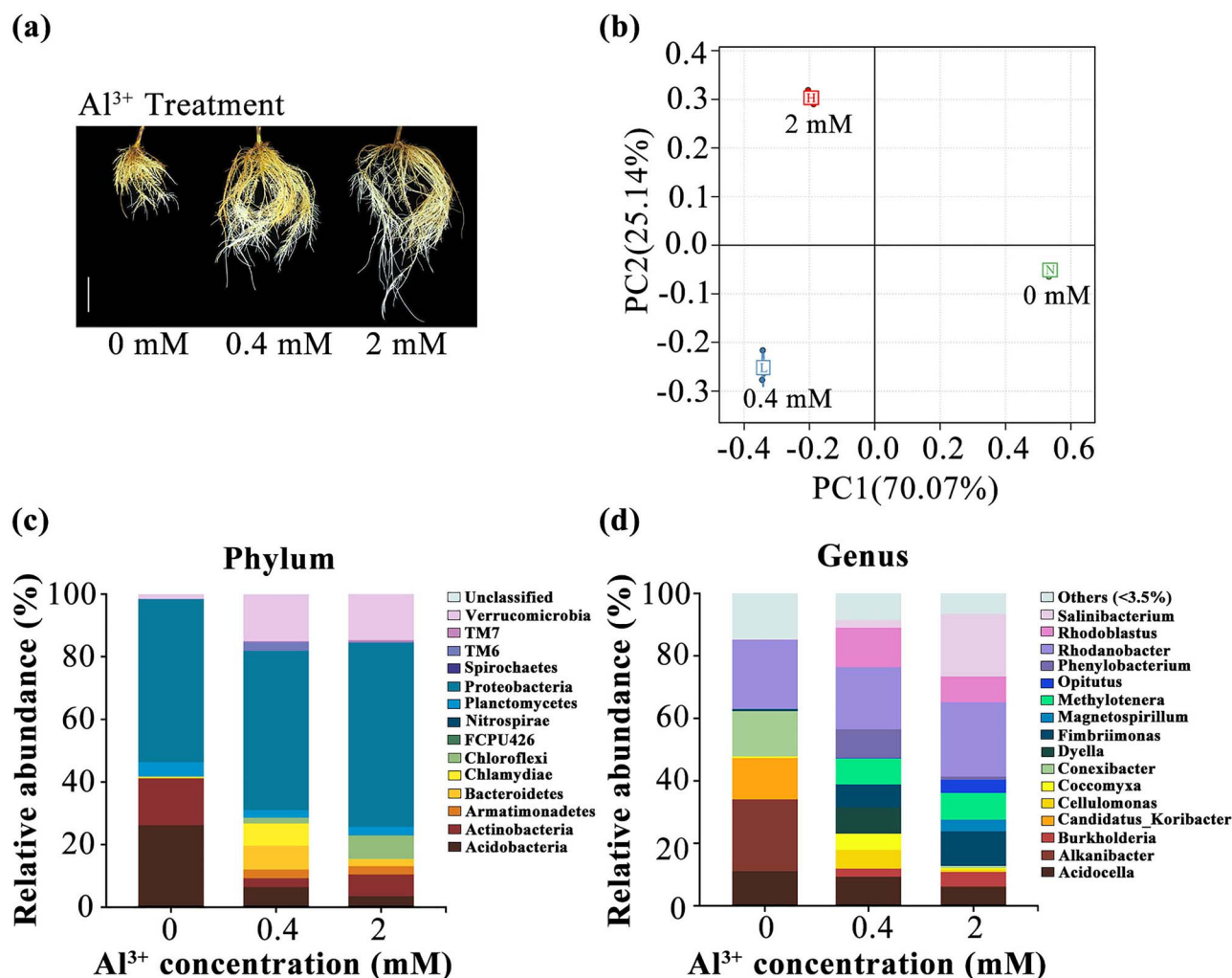


Figure 1. Classification and abundance analysis of endophytic differences in tea seedling roots under different aluminum concentrations from hydroponic culture. (a) Tea roots after treatment with 0, 0.4 and 2 mM aluminum for 1 year. (b) Principal coordinate analysis of endophytic bacteria. The values of axes 1 and 2 are the percentages that can be explained by the corresponding axis. The dots in the figure represent each sample. Different colors indicate that the samples belong to different groups. (c and d) OTU species classification and area and histogram profiles of each sample at the two classification levels of phylum and genus by comparison with the database. The relative abundance was plotted for endophytic bacteria of each sample. The phylum-level histogram of all species is presented. At the genus level, all species whose species abundance was <0.5% in all samples were merged into others.

namely, thermophilic bacteria, *Burkholderia* and acidophilic bacteria (from order to genus) (Figure S3a available as Supplementary data at *Tree Physiology* Online). The LDA histogram results showed that among the aluminum-treated materials, *Burkholderia* had the most significant advantage, and thermophilic bacteria and acidophilic bacteria were significantly enriched (Figure S3b available as Supplementary data at *Tree Physiology* Online). Integrating the results of hydroponic tea plants and field tea trees, *Burkholderia* was a dominant strain, indicating that this genus played a very important role in the growth and development of tea roots after aluminum treatment (Figures S2 and S3 available as Supplementary data at *Tree Physiology* Online). Actinomycetes represented the dominant bacteria in hydroponic tea seedlings treated with aluminum, and the relative abundance

of actinomycetes in the roots from tea gardens increased greatly after aluminum treatment, indicating that actinomycetes also affect the growth of tea plants.

Isolation and identification of endophytic bacterial strains

The three tea roots under different concentrations of aluminum treatments were further used for isolation of endophytic bacterial strains. Thirty-five endophytes were isolated and identified from the three groups. After deduplicating the sequences obtained by 16S RNA sequencing, they were compared on the NCBI and EzBioCloud websites, and 16 different endophytes were finally obtained. The 16 endophytes included 12 Firmicutes strains (B1-B12), two Proteobacteria strains (PB1

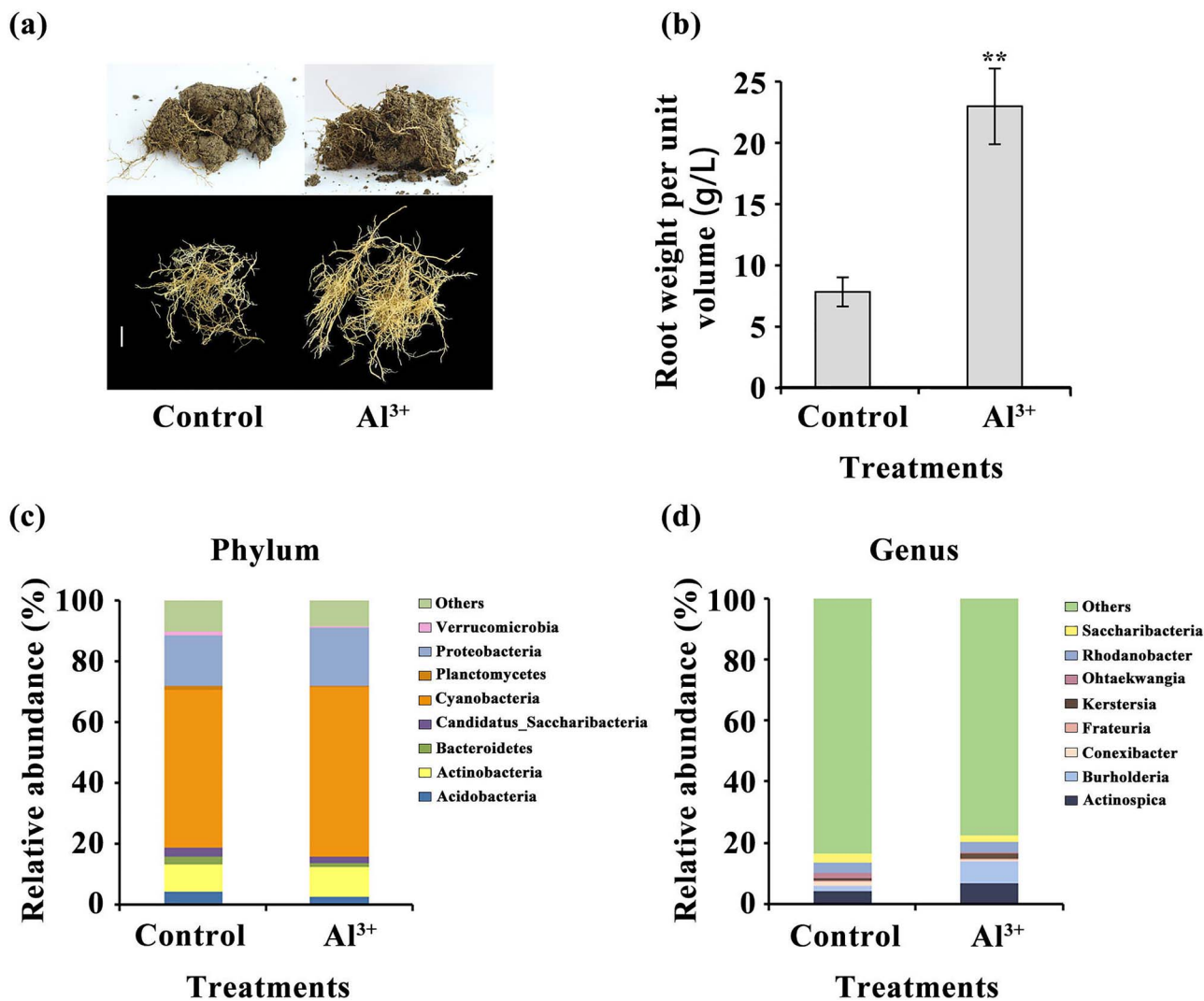


Figure 2. Classification and abundance analysis of endophytic differences in tea roots under aluminum treatment in a tea garden. (a) Tea roots after treatment with 2 mM aluminum for half a year. (b) The root weight per unit volume of soil block. (c and d) OTU species classification and area and histogram profiles of each sample at the two classification levels of phylum and genus by comparison with the database.

and PB2) and 2 Actinobacteria strains (A1 and A2) (Table 1). Most of the endophyte information obtained through sequence comparison on the NCBI website only contains sequence information, and no accurate function has been reported. Therefore, it is necessary to perform functional analysis on the isolated endophytes through further experiments.

PGPTs of identified endophytic bacterial strains

To understand the effect of the isolated endophytes on the growth of tea roots, PGP analysis was performed on the 16 isolated endophytes. The analysis indicators were phosphate solubilization ability, siderophore production, ACC utilization ability and IAA production ability, which were all beneficial indicators for plant growth.

The results showed that these 16 endophytes were all growth-promoting endophytes with different growth-promoting abilities (Table 2). Three *Bacillus* strains, B6, B7 and B8, had the ability to solubilize phosphate. Nine strains had the capacity to produce siderophores, including Firmicutes strains B1, B4, B5, B7, B8, B9, B11 and B12 and Actinobacteria strains A2, especially strains B5 and A2, which had strong siderophore production capacity. Seven strains had the ability to utilize ACC, for example, Firmicutes strains B4, B5, B8, B9 and B12, Proteobacteria strain PB1 and Actinobacteria strains A1 and A2 (Figure 3a and Table 2).

The ability of endophytes to produce IAA was qualitatively and quantitatively determined by salmon colorimetry and UPLC. The results of the experiment found that 10 kinds of endophytes could produce IAA, which was beneficial to plant growth.

Table 1. NCBI alignment of purified bacteria 16S sequence.

Strain	Species classification		16S rRNA			
	Phylum	Genus	Closest known relative	Similarity (%)		
B1	Firmicutes	<i>Bacillus</i>	<i>Bacillus aryabhatai</i>	99.5		
B2			<i>Bacillus paranthracis</i>	99.31		
B3			<i>Bacillus cereus</i>	99.1		
B4			<i>Bacillus flexus</i>	99.93		
B5			<i>Bacillus kochii</i>	100		
B6			<i>Paenibacillus</i>	<i>Paenibacillus tylopili</i>	99.72	
B7				<i>Solibacillus isronensis</i>	99.31	
B8				<i>Bacillus</i>	<i>Brevibacterium frigoritolerans</i>	99.65
B9				<i>Bacillus siamensis</i>	99.79	
B10				<i>Bacillus nealsonii</i>	99.09	
B11				<i>Lysinibacillus</i>	<i>Lysinibacillus fusiformis</i>	99.72
B12				<i>Bacillus subtilis</i>	<i>Bacillus subtilis subsp. stercoris</i>	99.29
PB1	Proteobacteria	<i>Trinickia</i>	<i>Trinickia diaoshuihuensis</i>	99.29		
PB2			<i>Limnobacter</i>	<i>Limnobacter thiooxidans</i>	99.86	
A1	Actinobacteria	<i>Leifsonia</i>	<i>Leifsonia shinshuensis</i>	99.01		
A2			<i>Nocardia</i>	<i>Nocardia nova</i>	99.49	

Table 2. The PGPTs of the isolated endophyte.

Strain number	Strain name	Phosphate solubilization	Siderophore	ACC deaminase	IAA production ($\mu\text{g ml}^{-1}$)
B1	<i>Bacillus aryabhatai</i>	–	++	–	–
B2	<i>Bacillus paranthracis</i>	–	–	–	16.72 \pm 3.05
B3	<i>Bacillus cereus</i>	–	–	–	–
B4	<i>Bacillus flexus</i>	–	++	+	35.02 \pm 3.02
B5	<i>Bacillus kochii</i>	–	+++	+	14.56 \pm 1.53
B6	<i>Paenibacillus tylopili</i>	+	–	–	6.98 \pm 0.87
B7	<i>Solibacillus isronensis</i>	+	++	–	11.64 \pm 1.07
B8	<i>Brevibacterium frigoritolerans</i>	+	++	+	8.48 \pm 0.76
B9	<i>Bacillus siamensis</i>	–	+	+	–
B10	<i>Bacillus nealsonii</i>	–	–	–	2.80 \pm 0.75
B11	<i>Lysinibacillus fusiformis</i>	–	++	–	15.11 \pm 1.32
B12	<i>Bacillus subtilis subsp. stercoris</i>	–	+	+	–
PB1	<i>Trinickia diaoshuihuensis</i>	–	–	–	4.70 \pm 0.35
PB2	<i>Limnobacter thiooxidans</i>	–	–	–	–
A1	<i>Leifsonia shinshuensis</i>	–	–	+	–
A2	<i>Nocardia nova</i>	–	++	+	25.39 \pm 0.43

(P–) solubilization activity and production capacity of siderophores were indicated by the medium dissolving halo/plaque. The larger the value, the stronger the ability. Note: +++ denotes >2; ++ denotes 1.5–2; + denotes 1–1.5; <1, – means no ability. The ability to use ACC was represented by '+' and '–', where '+' means possesses ACC enzyme activity and '–' means no ACC enzyme activity.

In particular, the IAA content produced by strains B4 and A2 was significantly higher than that produced by other strains (Figure 3b). Unfortunately, we did not find any superproliferative bacteria with all the aforementioned growth-promoting abilities; only endophytes B4, B8 and A2 exhibited three of the growth-promoting abilities.

Aluminum tolerance ability of identified endophytes

Tea plants are typical aluminum hyperaccumulation woody plants. Under the 2.0-mM aluminum treatment, the aluminum content reached 1000 mg kg⁻¹. Even the free Al³⁺ content

was shown to be >200 mg kg⁻¹ in tea roots (Fu et al. 2020). Since these endophytic bacteria were mainly isolated from roots grown under 2.0 mM aluminum, we set different aluminum concentrations (from 0 to 200 mg l⁻¹) to test the aluminum tolerance of these bacteria.

The results with both liquid and solid media indicated that most endophytes had strong aluminum tolerance and could grow under 100 mg l⁻¹ aluminum (Table 3). Among them, A1 (*Leifsonia shinshuensis*) could still grow even at 200 mg l⁻¹ aluminum. At \leq 100 mg l⁻¹ aluminum, A1 grew normally, with OD₆₀₀ values being 0.8–1.0 units of that of the control

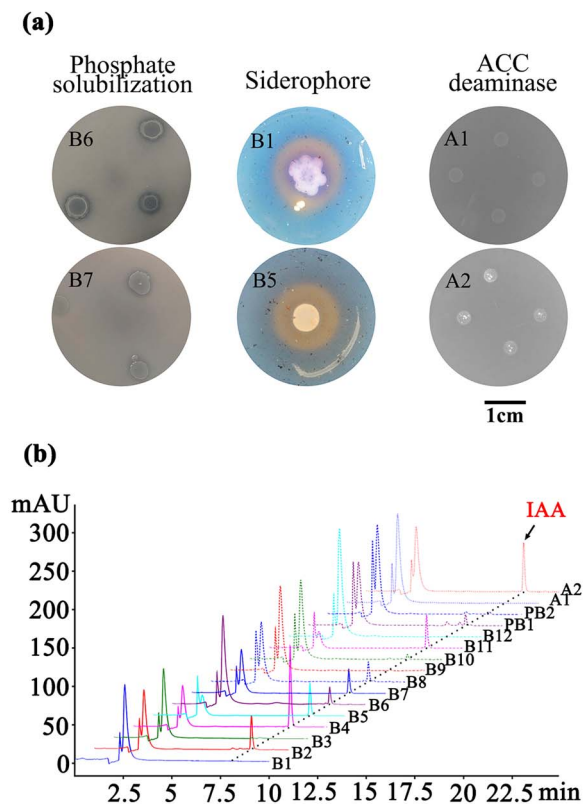


Figure 3. PGP activity screening of endophytic bacteria from the root system of Shuchazao. (a) Schematic diagram of qualitative and accelerating experiments with some probiotics. Scale bars, 50 cm. (b) HPLC determination of indoleacetic acid production by isolated bacteria.

(Table 3 and Figure 4). Compared with that of A1, the aluminum tolerance of A2 (*Nocardia nova*) was slightly weaker. From 20 to 100 mg l⁻¹, the OD₆₀₀ values of A2 were 0.5–0.8 units of that of the control. At 200 mg l⁻¹ aluminum, A2 hardly grew (Table 3 and Figure 4). When the aluminum concentration was <40 mg l⁻¹, the growth of B4 was basically unaffected. Then, with increasing aluminum concentration, the growth ability was weakened, and at 200 mg l⁻¹, there was basically no growth (Table 3 and Figure 4). Regarding B8, its aluminum resistance was weaker than that of B4. From 0 to 100 mg l⁻¹, with increasing aluminum concentration, the growth ability became weaker, and at 200 mg l⁻¹, it barely grew (Table 3 and Figure 4). In comparison, B7 had the weakest aluminum resistance, followed by B5, B9 and B8 (Table 3). Therefore, by combining PGPTs and aluminum tolerance ability, follow-up plant–endophyte interaction research will give priority to the use of bacterial strains A1, A2 and B4.

Effect of endophytic bacteria on the growth of wheat seedlings without/with aluminum

The above results indicated that some isolated endophytes had a certain ability to promote plant growth. For example,

B4 and A2 had strong IAA production ability, and some bacteria, such as A1, had strong aluminum tolerance ability. To clarify whether these bacteria played an important role in plant growth, we first explored the optimal bacterial concentration.

The bacterial concentration gradient treatment results showed that when treated with different concentrations of A1 or B4 bacterial solution, there were certain differences in wheat seedlings, especially in wheat roots (Figure S4a available as Supplementary data at *Tree Physiology Online*). Under treatment with different concentrations of A1, the germination rate of wheat seeds first increased with increasing concentration of the bacterial solution and then decreased. When the A1 bacterial concentration was 10⁵ c.f.u. ml⁻¹, the germination rate was the highest. For B4 bacteria, the change in germination rate was similar. However, from 10⁵ to 10⁷ c.f.u. ml⁻¹, the germination rate was relatively stable (Figure S4b available as Supplementary data at *Tree Physiology Online*). Different bacterial concentrations also affected the root elongation of wheat seedlings, although the change was not particularly obvious. In comparison, when the concentration of the A1 or B4 bacterial solution was 10⁶ c.f.u. ml⁻¹, the growth of wheat roots was better (Figure S4c available as Supplementary data at *Tree Physiology Online*). The growth of lateral roots of wheat seedlings was significantly promoted under the five concentrations of A1 or B4 bacteria. For A1 bacteria, the optimum concentration was 10⁷ c.f.u. ml⁻¹, while for B4, it was 10⁶ c.f.u. ml⁻¹ (Figure S4d available as Supplementary data at *Tree Physiology Online*). Therefore, based on the various indicators, 10⁶ c.f.u. ml⁻¹ bacterial concentration was selected for subsequent growth promotion experiments (Figure S4 available as Supplementary data at *Tree Physiology Online*).

Bacteria A1, A2 and B4 and the mixture of the three strains were further used to study the effect of endophytes on the growth of wheat roots. The results indicated that whether it was a single strain or a SynCom system, certain promotion effects on the growth of wheat roots were observed (Figure 5a and b). Under different endophyte treatments, the germination rate of wheat seeds increased slightly, but the root length of wheat was remarkably increased. When treated with the bacterial mixture of the three strains, the increase was even greater (Figure 5c and d). Furthermore, lateral root elongation was the most significant trait. After these endophyte treatments, both the number of lateral roots and the length of lateral roots increased significantly (Figure 5). For example, the number of lateral roots (>1 cm) treated with A1, A2 or B4 was almost 1.5 times higher than that of the control.

Wheat is an aluminum-sensitive plant. When treated with 0.4 mM aluminum, wheat growth was significantly inhibited, especially in roots, to 30% of that of the control. However, the inhibition of wheat growth by aluminum could be significantly reduced by A1 and B4 bacteria, especially A1

Table 3. Aluminum tolerance ability of the isolated endophyte in LB liquid medium under different Al³⁺ concentrations.

Strain number	Relative vitality at different concentrations of Al ³⁺ (mg l ⁻¹)					
	0	20	40	60	100	200
B1	+++	+++	+++	+++	+++	–
B2	+++	+++	+++	+++	+++	–
B3	+++	++	++	++	++	–
B4	+++	+++	+++	++	+	–
B5	+++	++	++	++	–	–
B6	+++	+++	+++	+++	++	–
B7	+++	++	–	–	–	–
B8	+++	++	+	+	+	–
B9	+++	+	+	+	+	–
B10	+++	++	++	+	+	–
B11	+++	+++	+++	++	++	–
B12	+++	+++	++	++	++	–
PB1	+++	+++	+++	+++	++	–
PB2	+++	+++	+++	+++	++	–
A1	+++	+++	+++	+++	++	+
A2	+++	++	++	++	++	–

The aluminum tolerance ability of the each endophyte was calculated by OD₆₀₀ of each Al³⁺ concentration to 0 mg l⁻¹. Note: +++ denotes 0.8–1.0; ++ denotes 0.5–0.8; + denotes 0.2–0.5; – denotes 0–0.2.

(Figure 6a and b). Analysis of aluminum in wheat roots showed that a very small amount of aluminum accumulated in the control wheat roots and that a large amount of aluminum accumulated in the aluminum-treated wheat roots, whereas the amount of aluminum was significantly reduced in the aluminum-combined bacteria-treated wheat roots (Figure 6c). In addition, compared with wheat supplemented with B4, wheat supplemented with the A1 strain accumulated less aluminum, with A1 exhibiting a stronger aluminum tolerance than B4 (Figure 6c and d). The detoxification abilities of strains A1 and B4 were consistent with their aluminum resistance (Figure 6).

Through the treatment of wheat seeds and wheat seedlings, we found that these isolated endophytic bacteria with growth-promoting characteristics could promote the growth of wheat, especially the growth of lateral roots, and alleviate the toxicity of aluminum to aluminum-sensitive wheat plants.

Effect of endophytic bacteria on the growth of tea cuttings without/with aluminum

The endophytic bacteria with PGPTs were mainly isolated from tea roots under the treatment with 0.4 or 2.0 mM Al³⁺, and the roots of tea plants under the aluminum treatment grew better, leading to the following question: will these endophytes promote the rooting of tea plants? Endophytic bacterium A1 (*L. shishuensis*) was selected to research the effect of endophytic bacteria on the rooting of tea cuttings.

After 6 months of cultivation, the growth of the tea cuttings showed a significant difference, including both the shoot and root systems (Figure 7). For the shoot system, compared

with that of the control, the shoot weight was increased by 2.5 times after A1 endophyte treatment. Meanwhile, the leaf number was significantly increased, from an average of 3.5 to 4.4. The effect of endophyte A1 on shoot length was not as great as that on leaf number (Figure 7a and b). For the root system, the root weight, root number, root volume and root surface area increased significantly, increasing by >1.5 times compared with that of the control (Figure 7a and c). By contrast, the effect of endophyte A1 on root length was less pronounced.

Unlike that of wheat, the growth of tea cuttings was indeed promoted when treated with 0.4 mM aluminum, but it was slightly less effective than the A1 bacteria treatment (Figure 7c and d). When aluminum and bacteria were administered simultaneously, the growth of tea seedlings was still promoted, and the effect was better than that with A1 bacteria alone (Figure 7d).

In short, the A1 endophyte promoted the growth and development of lateral roots, thereby promoting the growth of roots and tea plants (Figure 7).

Discussion

Effect of aluminum on tea plant growth

Tea plants are typical aluminum-tolerant plants that can grow normally in a 2-mM aluminum environment. Research on the effect of aluminum on tea plants has mainly focused on aluminum tolerance, including the complexation of organic acids (Morita et al. 2008), complexation of polyphenols (Nagata et al. 1992, Fu et al. 2020) and modification of cell walls

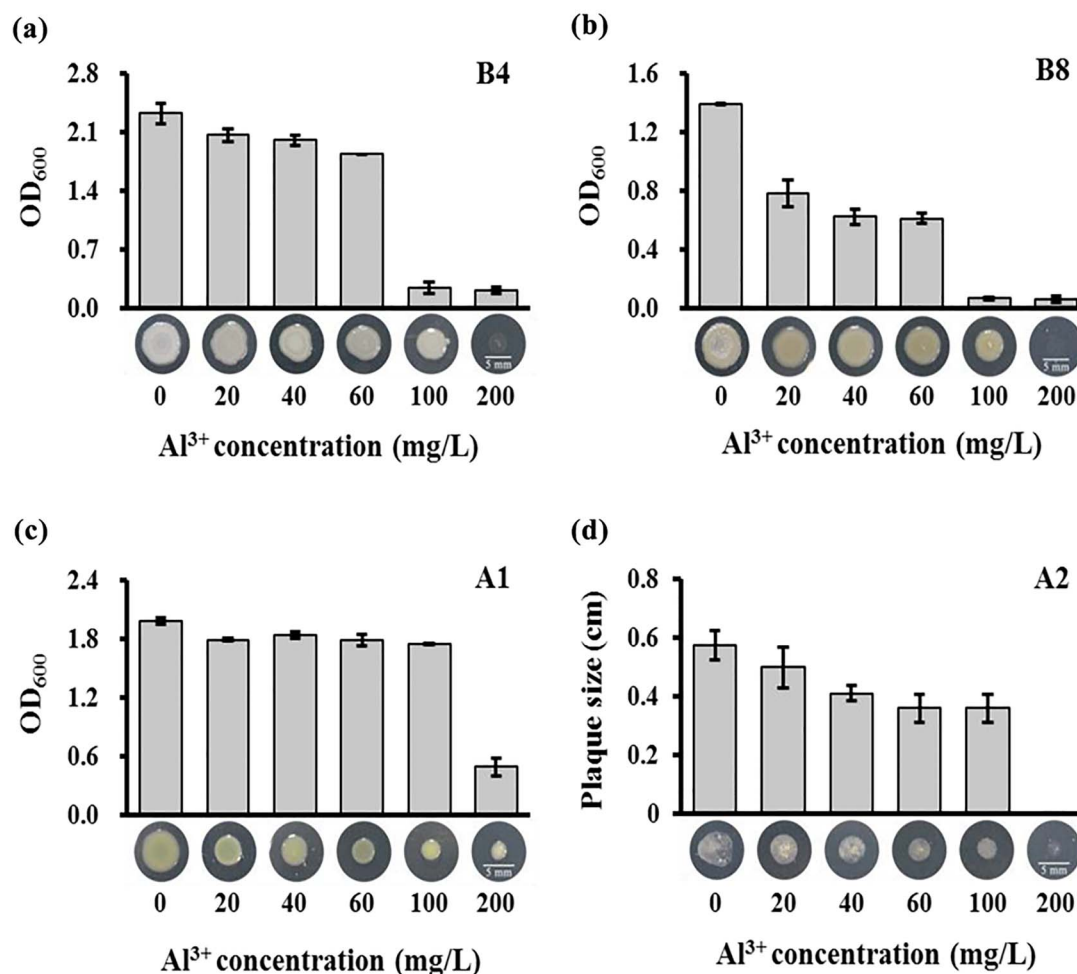


Figure 4. Aluminum tolerance ability of the selected endophyte in both LB liquid and solid media under different Al^{3+} concentrations. For A1, B4 and B8, the histogram and colony photographs represent the growth of bacteria in liquid and solid media, respectively. For A2, both the histogram and colony photographs represent the growth of A2 on solid media.

(Li et al. 2017, Safari et al. 2018). Moreover, unlike the case with other plants, low-concentration aluminum has a promoting effect on the growth of tea trees. It can even be said that aluminum is essential for the root growth and development of tea plants (Safari et al. 2018, Fu et al. 2020, Sun et al. 2020). However, the mechanism by which aluminum promotes the growth of tea plants has not been clarified.

Some endophytes can promote the growth of plant hosts, and some plant growth-promoting bacteria with heavy metal tolerance have been shown to enhance phytoremediation (Santoyo et al. 2016, Han et al. 2018, Rho et al. 2018, Wang et al. 2018). Does aluminum enhance the distribution of aluminum-tolerant and growth-promoting bacteria, thereby promoting the growth of tea roots? Meta-16S rDNA analysis showed that *Burkholderia* and Actinobacteria were enriched after aluminum treatment (Figures 1 and 2). Moreover, aluminum-tolerant endophyte A1 (Actinobacteria) promoted the growth of tea cuttings,

especially lateral roots (Figure 7). In other words, aluminum indeed promoted the growth of tea plants indirectly by promoting the distribution of aluminum-tolerant and growth-promoting endophytic bacteria (Figure 8). Our research thus provides a new aspect for research on the mechanism by which aluminum promotes tea plant growth.

Application of endophytic bacteria in agricultural production

In recent years, some studies have indicated that the root microbiota has the function of promoting the growth of host plants and enhancing plant metal resistance and disease resistance (Hiruma et al. 2016, Duran et al. 2018, Kwak et al. 2018, Zhang et al. 2019). For example, some microorganisms were reported to promote the absorption and utilization of nitrogen and phosphorus in rice and *Arabidopsis* (Hiruma et al. 2016, Zhang et al. 2019), and some promoted the growth and development of host plants by producing IAA

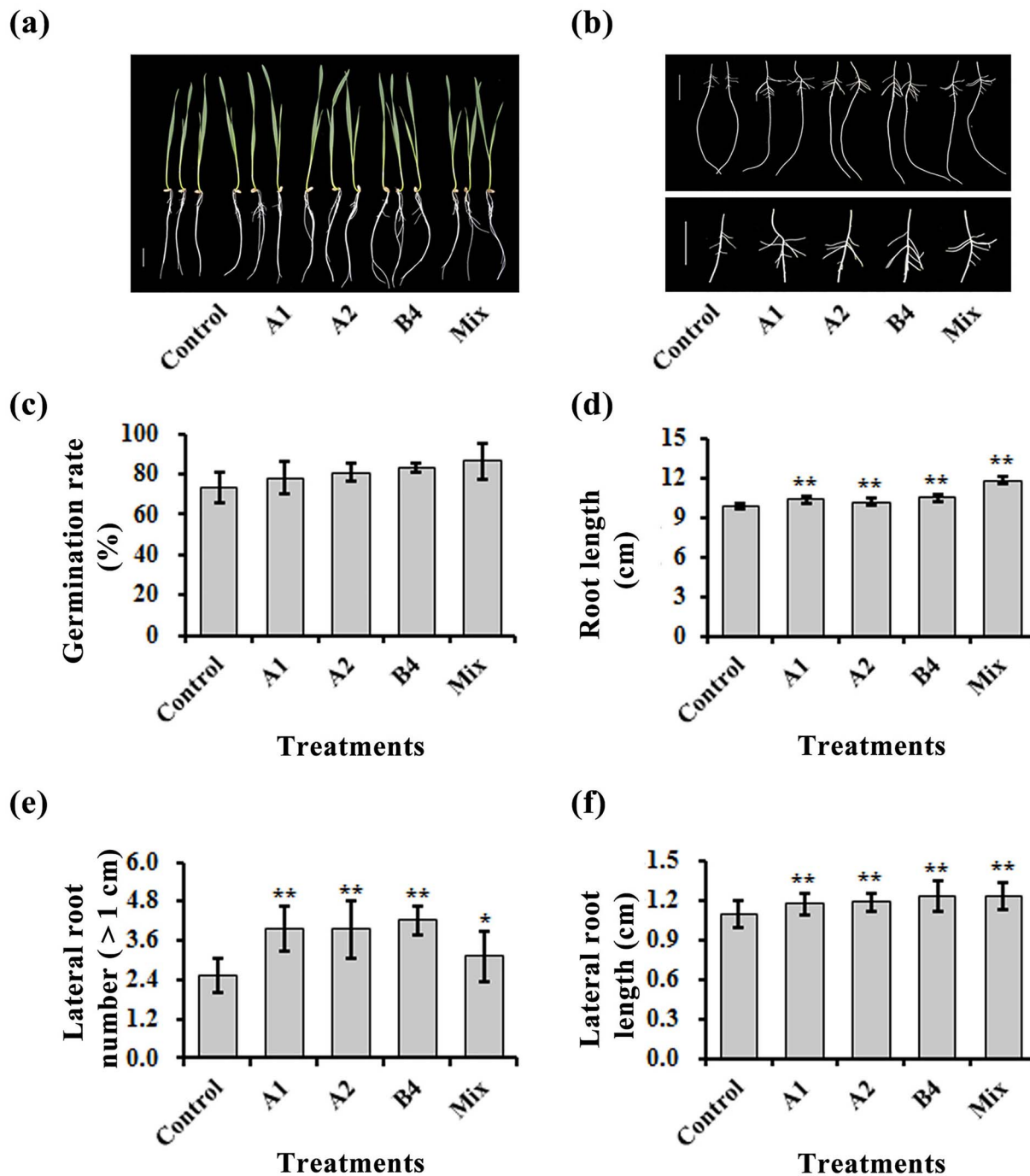


Figure 5. Effects of A1 (*L. shinshuensis*), A2 (*N. nova*) and B4 (*Bacillus flexus*) and a mixture of the three strains on the growth of wheat roots. (a and b) Phenotype of wheat seedlings and magnified root treatment by different endophytes A1, A2 and B4 and a mixture of the three strains after 7 days, respectively. (c) Germination rate of wheat seeds under different endophyte treatments. (d) The length of wheat root. (e) The number of lateral roots that were longer than 1 cm. (f) The lateral root length. The asterisks indicate the significance level (* $P < 0.05$, ** $P < 0.01$) based on the LSD honestly significant difference test.

(Santoyo et al. 2016, Duran et al. 2018, Yan et al. 2018). In the different growth and development stages of tea plants, there are obvious differences in the types, quantity, distribution

and biological functions of endophytic bacteria, which also play a role in promoting growth and the formation of tea flavor (Yan et al. 2018).

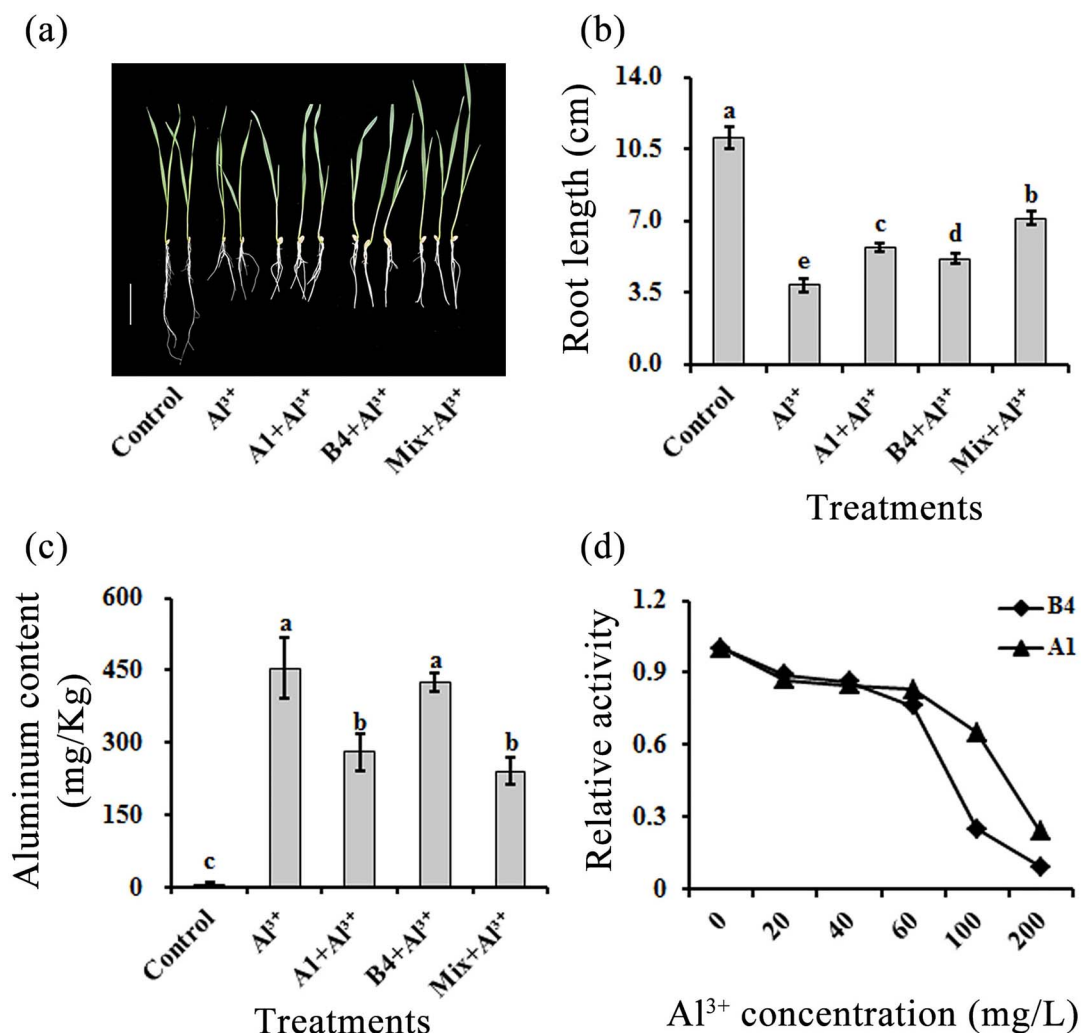


Figure 6. Effects of A1 (*L. shinsuensis*), B4 (*Bacillus flexus*) and a mixture of the two strains on the growth of wheat roots under aluminum treatment. (a) Phenotype of wheat after treatment with 0.4 mM aluminum or combined with the selected strain. (b) The effect on the root length of wheat. (c) The accumulation of aluminum in wheat roots. (d) Relative activity of strains A1 and B4 under different concentrations of aluminum. The letters indicate the significance level based on the LSD honestly significant difference test.

In this study, we isolated 16 bacterial strains from tea roots. The PGPT analysis of these 16 strains indicated that they were all growth-promoting endophytes with different growth-promoting abilities (Table 2 and Figure 3), including phosphate solubilization ability, siderophore production, ACC utilization ability and IAA production ability. Endophytes B4, B8 and A2 had three proliferative abilities. The results of plant–endophyte interaction analysis showed that these PGPT endophytes promoted the growth of both wheat and tea cuttings, especially their lateral roots (Figures 5 and 7). Some plant growth-promoting bacteria with heavy metal tolerance (cadmium, lead or arsenic) were previously shown to enhance phytoremediation (Sheng et al. 2008, Han et al. 2018, Wang et al. 2018). Among the 16 strains, except for B5 and B7, all bacteria tolerated aluminum concentrations > 100 mg l⁻¹, especially A2,

which tolerated 200 mg l⁻¹ aluminum (Table 3 and Figure 4). Further experiments showed that both A1 and B4 reduced the toxicity of aluminum to aluminum-sensitive plant wheat. Moreover, their detoxification ability was consistent with their aluminum resistance (Figure 6). The mechanism by which these bacteria promote the growth of tea seedlings needs further study.

Research on the interactions between root microbiota and host plants involves the fields of microbial ecology and plant molecular biology. Ecological research usually uses a holistic approach to study the root microbiome, which is essential to reveal the condition of the root microbiome, for example, the metagenome (Yan et al. 2018). By contrast, studies using reductionist methods attempt to understand these functions and mechanisms by decomposing the interactions between the root

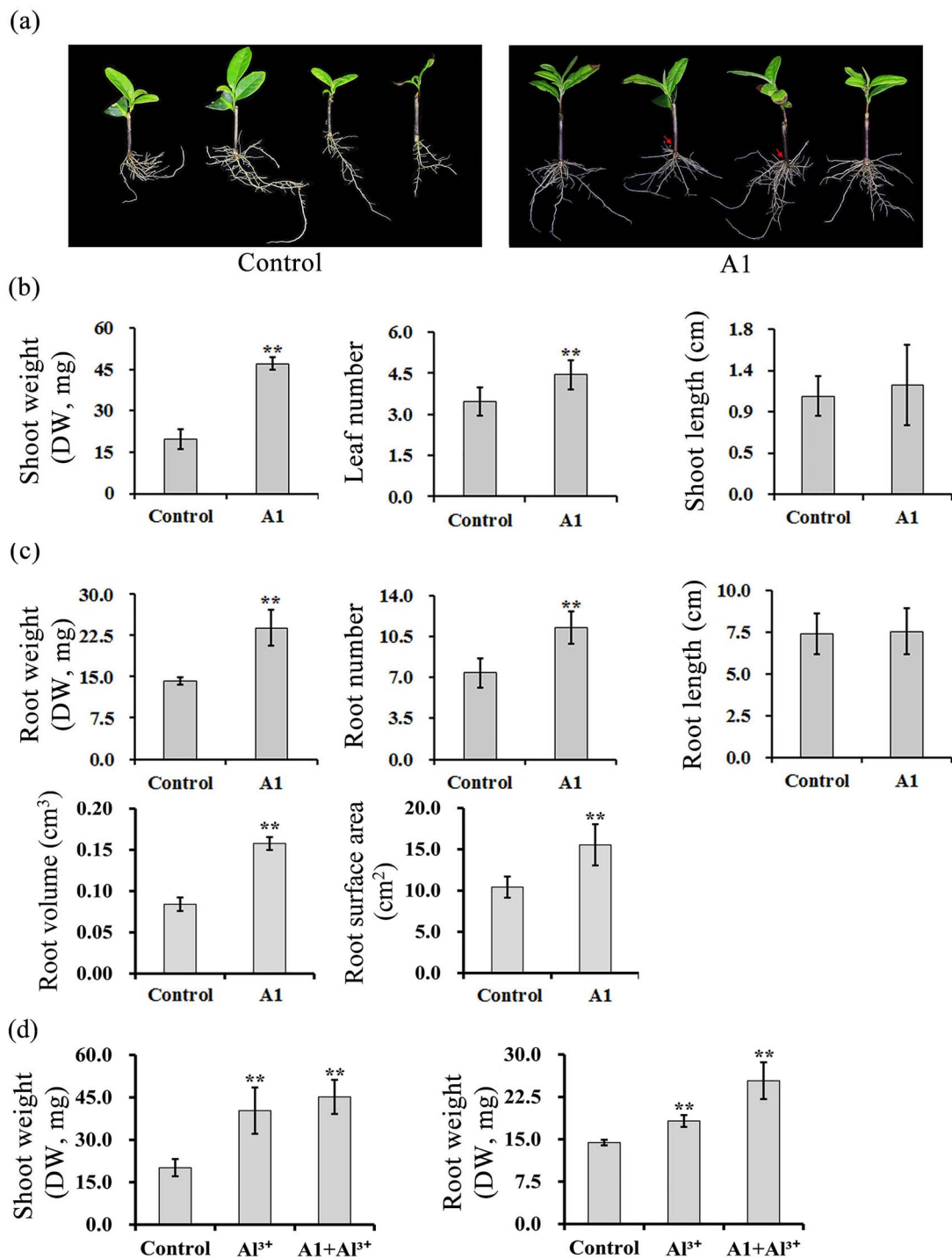


Figure 7. Effects of the A1 (*L. shinshuensis*) strain on the growth of tea cuttings without/with aluminum. (a) Phenotype of tea cuttings after treatment with the A1 strain. (b) The effect of strain A1 on the shoots of tea cuttings. (c) The effect of strain A1 on the roots of tea cuttings. (d) The effect of aluminum and aluminum combined with the A1 strain on the roots of tea cuttings. The asterisks indicate the significance level (* $P < 0.05$, ** $P < 0.01$) based on the LSD honestly significant difference test.

microbiome and the plant host into experimentally controllable factors (YX Liu et al. 2019). Through high-throughput sequencing analysis, we found that *Actinobacteria* and *Burkholderiales* were significantly enriched in the high aluminum group (2 mM)

(Figures 1 and 2). After isolation and plant–bacterial interaction experiments, *Actinobacteria* (A1 and A2) and *Firmicutes* (B4) were proven to have the characteristics of promoting root growth, and SynCom had a better growth promotion

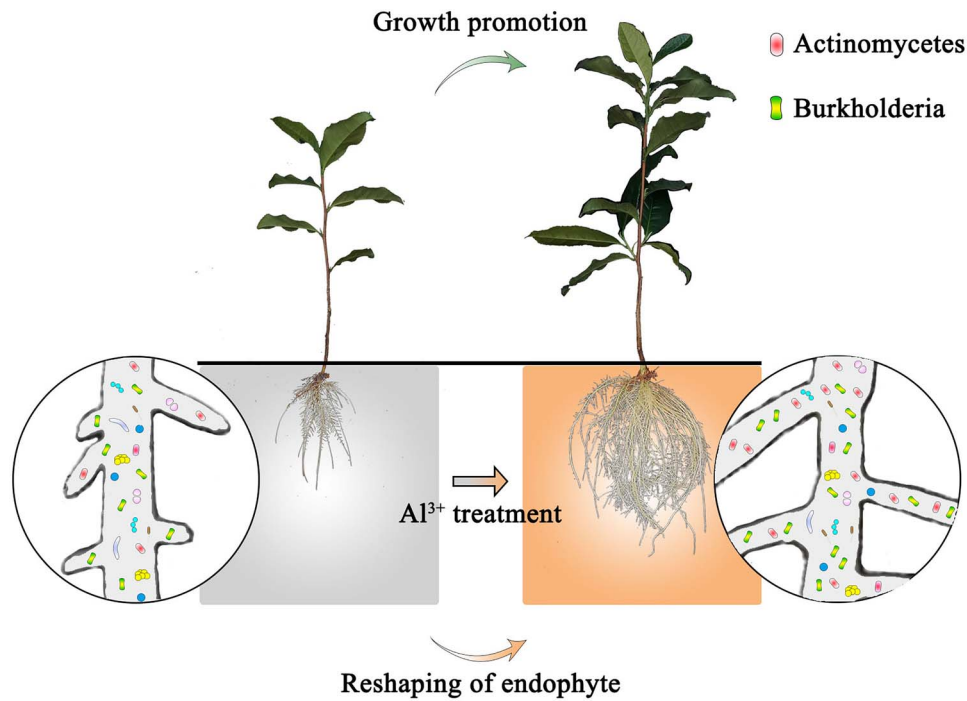


Figure 8. Proposed model of aluminum-tolerant and growth-promoting bacteria promoting the growth of tea roots under aluminum treatment.

effect (Figure 5). Combining ecological research with SynCom approaches will provide functional and mechanistic insights into the interactions between tea plants and the microbiome.

The difficulty of rooting in tea plant cultivation and breeding

Cutting breeding, as one of the most convenient and economical clonal breeding techniques, has been widely used for tea breeding (Nakamura et al. 2001, Erturk et al. 2008, Mello et al. 2016, Ruter 2018, Lowe et al. 2019, XM Liu et al. 2019). The advantages of cutting breeding are maintaining the excellent characteristics of the mother plant, large plant production and fast plant formation (Zhao et al. 2020). In most cases, the rooting of tea cutting is difficult (XM Liu et al. 2019). Research on how to promote the rooting of tea plants has important production and practical significance for the cultivation and breeding of tea plants. At present, the production and breeding of tea plants mainly rely on cutting breeding. However, there are still some bottlenecks in tea cultivation and breeding, such as the low survival rate of tea cuttings, long time required for seedlings growth and poor drought resistance of tea plants (Nakamura et al. 2001, Mello et al. 2016, Ruter 2018, Lowe et al. 2019, XM Liu et al. 2019). The existence of these problems is mainly due to the difficulty in rooting tea cuttings, and the root system of tea cuttings is not as developed as that of tea seedlings. The ABT rooting powder is a commonly used root-promoting agent in production, but the rooting rate in tea breeding still needs

to be improved (XM Liu et al. 2019). Our results also showed that the rooting of tea cuttings was generally difficult to achieve. Fortunately, the bacteria that we screened significantly improved the rooting and root biomass of tea cuttings (Figure 7). This research lays the foundation for the selection of tea plant endophytic strains that can be used for cultivation and breeding and that have significant root-promoting ability.

Conclusions

Burkholderia was enriched in tea roots after aluminum treatment, and *Actinomycetes* represented the dominant strains in hydroponic tea seedlings treated with aluminum. Then, 16 different endophytes were obtained and identified from these tea roots. Among them, B4 (*Bacillus nealsonii*), B8 (*Brevibacterium frigoritolerans*) and A2 (*N. nova*) bacteria each had three growth-promoting traits. Bacteria A1 (*L. shinshuensis*) had the strongest aluminum tolerance ability, up to 200 mg l⁻¹ aluminum. The plant–bacteria interactions showed that endophytes A1, A2, B4 and their SynCom promoted the growth and development of wheat lateral roots and alleviated aluminum stress. Moreover, endophyte A1 promoted the growth of tea cuttings, especially lateral roots, with/without aluminum. Taken together, aluminum could enhance the distribution of aluminum-tolerant and growth-promoting bacteria, thereby promoting the growth of tea roots.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Authors' contributions

The presented study was conducted in collaboration by all authors. X.J., S.Z., L.G. and T.X. conceived and designed the experiments. W.-W.L., X.J., Z.F., M.H., G.C. and J.W. performed the experiments. X.J., W.-W.L. and W.-W.D. analyzed the data. X.J., W.-W.L. and L.G. wrote the manuscript. All authors reviewed the manuscript.

Conflict of interest

None declared.

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