Citric Acid in Rice Root Exudates Enhanced the Colonization and Plant Growth-Promoting Ability of \textit{Bacillus altitudinis} LZP02

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ABSTRACT Exploration of the underlying mechanisms of plant-microbe interactions is very important. In the present study, citric acid in the root exudates of rice significantly enhanced the colonization of \textit{Bacillus altitudinis} LZP02 in the rhizosphere. According to the results of transcriptome and reverse transcription-quantitative PCR or analyses, citric acid increased the expression of several genes involved in bacterial chemotaxis and biofilm formation in \textit{B. altitudinis} LZP02. In addition, citric acid also increased the expression of several genes associated with \textit{S}-adenosylmethionine biosynthesis and metabolism. Interestingly, the secretion of citric acid by rice roots could be increased by inoculation with \textit{B. altitudinis} LZP02. The result indicated that citric acid might be a vital signal in the interaction between rice and \textit{B. altitudinis} LZP02. Further verification showed that citric acid enhanced the plant growth-promoting ability of \textit{B. altitudinis} LZP02.

IMPORTANCE In a previous study, the mechanism by which citric acid in rice root exudates enhanced the colonization of \textit{Bacillus altitudinis} LZP02 was discovered. The present study verified that citric acid increased the recruitment and rice growth-promoting ability of \textit{B. altitudinis} LZP02. These findings serve as an interesting case for explaining the underlying mechanisms of plant-microbe interactions. Henceforth, citric acid and \textit{B. altitudinis} LZP02 could be exploited for the development of sustainable agronomy.

KEYWORDS promotes plant growth, chemotaxis, citric acid, interaction

Chemotaxis is the ability of motile soil bacteria to sense and adjust their movements along gradients of compounds and constitutes a positive role during rhizosphere colonization (1, 2). Plant colonization by plant growth-promoting rhizobacteria (PGPR) is a highly relevant process for the establishment of both green and sustainable agricultural development (3). The plant rhizosphere is a unique position for plant-microbe interactions. The bacteria density orientation in the rhizosphere is aided by root exudates (1). Chemotaxis toward root exudates activates rhizobacterial recruitment and the establishment of bacterium-root interactions. Root exudates promote the growth of soil PGPR and support biofilm formation by microbes (4). Studies have shown that, compared with bacterial strains in which the biofilm is sufficient, the colonization of biofilm-deficient strains was much lower (5). The cell motility relies on the rotation of the flagellum of bacteria; thus, enhancement of flagellar assembly may lead to successful root colonization (6). Organic acids and amino acids are the main components of primary metabolites in root exudates and are secreted and passively lost from roots (7). A previous study has shown that chemotactic responses of \textit{Bacillus velezensis} B26 were induced by organic acids and citric acids enhanced the gene expression connected to the biofilm (8). Another study demonstrated that \textit{Escherichia coli} and \textit{Bacillus subtilis} movement toward amino acids can be utilized quickly (9). Malic acid, citric acid, and succinic acid have been shown to significantly promote \textit{B. amyloliquefaciens} T-S...
recruitment, enhancing the rhizobacteria population (10). Moreover, *Bacillus subtilis* RR4 can move toward malic acid released from rice roots (11).

The bacterial chemotaxis pathway of rhizospheric *Bacillus* includes methyl-accepting chemotaxis proteins (MCPs) that sense signals from root exudates (12). This interaction produces a stimulus that modulates CheA kinase activity (13, 14). The effector protein CheY of the two-component system is rapidly phosphorylated to interact with the flagellar motor and causes changes in the direction of bacterial movement (15). In *pseudo* solanacearum *Ps29*, McpC and McpP mediate the chemotaxis response toward citrate (16). In *Bacillus subtilis*, McpA was the major chemoreceptor response to phenol (17). However, in *Bacillus velezensis*, McpA is a principal chemoreceptor for the chemotactic response to d-galactose in cucumber root exudates (18). The interactions between root exudates and MCPs are crucial for bacterial motility and colonization of the plant root (13). However, the MCPs that mediate chemotaxis toward root exudates differ across bacterial species, and the ligands of the homologous MCPs vary across different bacteria (19).

*Bacillus altitudinis* LZP02 is an efficient plant growth-promoting rhizobacterium that interacts closely with plant roots. In a previous study, we comprehensively identified the chemoattractants of *B. altitudinis* LZP02 in rice root exudates. Compared with the different kinds and concentrations of chemoattractants, 100 μmol L⁻¹ citric acid significantly enhanced the motility and colonization of *B. altitudinis* LZP02 on rice roots (20). In this study, we hypothesized that citric acid promoted the colonization of *B. altitudinis* LZP02 on rice roots by inducing bacterial chemotaxis, thus improving the growth-promoting ability of *B. altitudinis* LZP02 on rice roots.

**RESULTS**

**Effect of citric acid on the colonization of *B. altitudinis* LZP02 on rice roots.** The changes in the number of *B. altitudinis* LZP02 colonies on the rice roots are shown in Fig. 1. Scanning electron microscopy (SEM) images at 50,000× magnification showed that the NP treatment (roots inoculated with *B. altitudinis* LZP02 for 12 h after soaking in a 100 μM sterile citric acid aqueous solution) markedly increased the number of *B. altitudinis* LZP02 colonies compared with those under the SP treatment (roots inoculated with *B. altitudinis* LZP02 for 12 h after soaking in sterile water) (Fig. 1A and B). The number of *B. altitudinis* LZP02 colonies on rice roots was significantly reduced in the CI treatment (roots inoculated with 100 μM sterile citric acid for 24 h after soaking in a *B. altitudinis* LZP02 solution) compared with the CK treatment (roots inoculated with sterile water for 24 h after soaking in a *B. altitudinis* LZP02 solution) (Fig. 1C and D). The number of *B. altitudinis* LZP02 colonies in the NP treatment was 1.97-fold higher than that in the SP treatment (Fig. 1E). In addition, the number of *B. altitudinis* LZP02 colonies in the CI treatment was 1.46-fold lower than that in the CK treatment (Fig. 1F). These results confirmed that 100 μmol L⁻¹ citric acid resulted in significant recruitment of *B. altitudinis* LZP02.

**Transcriptome analysis after citric acid treatment for 30 min.** The total number of differentially expressed genes (DEGs) detected in CN1 (sterile citric acid added to the *B. altitudinis* LZP02 solution to a final concentration of 100 μmol L⁻¹) and CK1 (control treated with the same amount of sterile water) was 3,808. The number of upregulated and downregulated DEGs in CN1 was 428 and 405 (fold change ≥2 or ≤0.5; P ≤ 0.05), respectively, for a total of 833 genes differentially expressed between CK1 and CN1 (Fig. 2A). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was subsequently performed. As shown in a bubble diagram (Fig. 2B), ABC transporters and bacterial chemotaxis pathways were the most significantly enriched.

Gene Ontology (GO) enrichment analysis revealed 5 GO terms associated with bacterial chemotaxis and flagellar assembly (Fig. 2C). Specifically, there were 3, 3, 3, and 1 DEGs enriched taxis (GO:0044330), chemotaxis (GO:0006935), locomotion (GO: 0040011), and bacterial-type flagellum-dependent cell motility (GO:0071973), respectively; all of these genes were upregulated. The KEGG analysis showed that 10 DEGs associated with bacterial chemotaxis were upregulated (Table S1 in supplemental materials). Among their 7 protein
products (Fig. 3), MCP is a methyl-accepting chemotaxis protein. It is worth noting that 3 mcpA and 1 mcpC, which are involved in MCP protein synthesis, were upregulated (Table S1). CheR is a Per-Arnt-Sim (PAS) domain-containing protein; CheB is a chemotaxis response regulator protein-glutamate methylesterase; CheA is a chemotaxis protein; FliG is a flagellar motor switch protein; MotB is a flagellar motor protein; and RbsB is a chemoreceptor for chemotaxis. Two DEGs were associated with biofilm formation, and both of these genes were upregulated (Fig. 2D). The results indicated that several genes associated with bacterial chemotaxis, flagellar assembly, and biofilm formation were affected by citric acid within a short amount of time. These results showed that citric acid increased the colonization ability of B. altitudinis LZP02 in the rice rhizosphere.

**Transcriptome analysis in response to citric acid treatment for 12 h.** GO functional annotation analysis was performed. The common DEGs were assigned to 30 GO terms (level 2) assigned to 3 different categories: 10 biological processes, 9 cellular components, and 11 molecular functions. As shown in Fig. 4A, the common DEGs were
significantly enriched in the following top 10 terms: catalytic activity (GO:0003824), binding (GO:0005882), membrane part (GO:0044425), cellular process (GO:0009987), metabolic process (GO:0008152), cell part (GO:0044464), transporter activity (GO:0005215), membrane (GO:0016020), protein-containing complex (GO:0032991), and localization (GO:0051179). Moreover, a large percentage of DEGs were involved in catalytic activity (34.4%), which belongs to the molecular function category.

As shown in the results of the KEGG pathway enrichment analysis (Fig. 4B), the cysteine and methionine metabolism, oxidative phosphorylation, and pyrimidine metabolism pathways were significantly enriched (P < 0.05). In addition, 11, 7, and 8 DEGs were involved in three pathways; all these genes were significantly upregulated (Table S2).

The DEGs were classified via Clusters of Orthologous Groups (COG) functional annotation. The DEGs were involved in translation, ribosomal structure and biogenesis, amino acid transport, metabolism, inorganic ion transport, and metabolism and transcription (Fig. 4C; Table S3).

The results of the GO enrichment analysis are shown in Fig. 4E. In the biological process category, 12 and 18 DEGs were associated with rRNA binding (GO:0019843) and structural

**FIG 2** Transcriptome analysis of *B. altitudinis* LZP02 at 30 min after inoculation with citric acid. (A) Scatterplot of differentially expressed genes in the CK1 and CN1 treatments; the abscissas and ordinates represent the gene expression levels in the CK1 and CN1 treatments, respectively. Each dot represents a specific gene. (B) KEGG enrichment analysis of DEGs. (C) GO functional enrichment of DEGs. Significantly enriched (P < 0.05) GO categories are shown. The abscissa represents the GO term, the left ordinate represents the number of DEGs (column), and the right ordinate represents the significance level of the enrichment (dot). The biological process (BP), cellular component (CC), and molecular function (MF) categories are represented by green, yellow and blue, respectively. (D) Biofilm formation process in *B. altitudinis* LZP02 in response to citric acid treatment. The yellow background color represents upregulated genes, while the green background color represents no differential expression.
constituents of the ribosome (GO:0003735), respectively. In the cellular components category, 18 DEGs were associated with the ribosome (GO: 00056840). In the molecular function category, 6, 6, 6, 18, 6, and 7 DEGs were associated with carboxylic acid transport (GO:0046942), amino acid transport (GO:0006865), organic acid transport (GO:0015849), translation (GO:0006412), the L-methionine biosynthetic process from methylthioadenosine (GO:0019509), and the S-adenosylmethionine metabolic process (GO:0046500), respectively. All these terms were significantly enriched. Information about these genes is included in Table S4.

The results indicated that citric acid mainly affects the expression of genes involved in organic acid transport, transcription, translation, L-methionine biosynthesis, S-adenosylmethionine metabolism, and oxidative phosphorylation in the bacteria after 12 h.

Effects of citric acid on the growth of *B. altitudinis LZP02*. To assess the influence of citric acid on *B. altitudinis* LZP02, the growth of *B. altitudinis* LZP02 in the presence of 100 μM citric acid was analyzed. As shown in Fig. 4D, the growth of *B. altitudinis* LZP02 in the LZP02-CA treatment was obviously different from that in the control. The bacterial trains entered the logarithmic phase earlier, and the optical density (OD) value was greater.

Transcriptome differences in response to citric acid treatment at 30 min and 12 h. Many of the DEGs in the bacterial strain were upregulated in response to citric acid treatment at different treatment times. The upregulated DEGs were involved in different pathways, but the expression of genes involved in the same pathway was different. As shown in Fig. 5A, 6 genes (*mcpA, mcpC, cheR, cheB, cheA, and motB*) were involved in the bacterial chemotaxis pathway, and 2 genes (*kinE and kinA*) were associated with biofilm formation. According to the transcriptome results of N1 (30-min citric acid treatment), these genes were significantly upregulated (log2[FC] > 1). According to the transcriptome results of N2 (12-h citric acid treatment), 2 genes associated with bacterial chemotaxis (*mcpA and motB*) were insignificantly upregulated. Four genes associated with bacterial chemotaxis (*mcpC, cheR, cheB, and cheA*) were statistically insignificantly downregulated. Two genes associated with biofilm formation (*kinE and kinA*) were statistically insignificantly downregulated.

Reverse transcription-quantitative PCR analysis. To confirm the RNA-seq data, the expression of several genes, including 10 DEGs in N1 and 8 DEGs in N2, was determined. The expression levels of *flgG* and *flmM* in N2 were significantly decreased in citric acid-treated bacteria, and the expression levels of the other 16 DEGs were significantly

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**FIG 3** Bacterial chemotaxis pathway in *B. altitudinis* LZP02 in response to citric acid treatment. The figure was made in I-Sanger (https://cloud.majorbio.com). The nodes associated with *B. altitudinis* LZP02 are shown in yellow. The red boxes represent upregulated genes. MCP, methyl-accepting chemotaxis proteins; CheR, PAS domain-containing protein; CheB, chemotaxis response regulator protein-glutamate methyltransferase; CheA, chemotaxis proteins; FlgG, flagellar motor switch protein; MotB, flagellar motor protein; RbsB, chemoreceptor for chemotaxis.
increased. These results are consistent with the results of the transcriptomic analysis (Fig. 5B, C; Tables S1, S2, and S4).

**Induction of citric acid production in rice roots.** The concentration of citric acid in the plant rhizosphere provides chemoattractant functions and induces biofilm formation. The concentration of citric acid in the rice root exudates was measured after incubation for 1 to 5 days. The results showed that root exudates from the uninoculated rice (CK treatment) produced 1.20 μmol L⁻¹ citric acid in 1 day; however, LZP02 treatment resulted in 3.85 μmol L⁻¹ citric acid in 1 day, which was 3 times higher than that of the control. At 3 to 5 days, the concentration of citric acid was significantly increased in the LZP02 treatment compared with the CK (Fig. 6B). These results indicated that the secretion of citric acid was induced by *B. altitudinis* LZP02. Taken together, these findings suggest that citric acid might be an important signal by root exudates.

**FIG 4** Transcriptome analysis of *B. altitudinis* LZP02 at 12 h after inoculation with citric acid and growth curve determination. (A) GO functional annotations of DEGs. The ordinate represents the GO term, the bottom abcissa represents the percent (column), and the upper abcissa represents the number of DEGs (dot). (B) KEGG enrichment analysis of DEGs. (C) COG functional annotations of DEGs. (D) Growth curves of *B. altitudinis* LZP02 and the bacteria treated with citric acid (LZP02-CA). (E) GO functional enrichment of DEGs. Significantly enriched (*P* < 0.05) GO categories are shown in the figure. The ordinate represents the GO term, the bottom abcissa represents the significance level of enrichment (column), and the top abcissa represents the number of DEGs (dot).
Plant growth-promoting trait analysis. The germination index of rice was determined. As shown in Fig. 7A, the germination index of rice increased gradually across the 4 treatments. In addition, the germination index of rice was significantly increased in the combined treatment of citric acid and Bacillus altitudinis LZP02 (NPJ) compared with the SP treatment. The gibberellin (GA) secretion of Bacillus altitudinis LZP02 treated with citric acid (CA treatment) was significantly increased compared with that under the CK treatment (Fig. 7B). The effect of citric acid and Bacillus altitudinis LZP02 on the growth of rice was subsequently explored. The growth-promoting effects of these treatments were different from those of the SP treatment. Compared with the SP treatment, the NPJ treatment resulted in the highest growth. Scans of the rice roots revealed that the 8 parameters of the roots of rice in the NPJ treatment increased significantly. The effect of NP treatment was also relatively greater because citric acid can increase the germination index of rice and shorten the germination time of seeds (Fig. 7C). The results showed that the combined treatment of citric acid and Bacillus altitudinis LZP02 promoted rice growth.

Eight growth traits were measured under different conditions. The growth traits
included root length (Fig. 8A), shadow area (Fig. 8B), surface area (Fig. 8C), root volume (Fig. 8D), number of connections (Fig. 8E), number of nodes (Fig. 8F), number of root tips (Fig. 8G), and number of forks (Fig. 8H). Compared with those under the SP treatment, the root length, shadow area, surface area, root volume, number of connections, number of nodes, number of root tips, and number of forks under the SPJ treatment (10 mL of *B. altitudinis* LZP02 plus sterile water added to the water-soaked seeds) increased by 5.05-fold, 4.63-fold, 3.54-fold, 2.39-fold, 3.90-fold, 4.08-fold, 4.46-fold, and 5.16-fold, respectively; those under the NP treatment increased by 4.44-fold, 5.38-fold, 3.27-fold, 2.12-fold, 3.92-fold, 4.54-fold, 5.18-fold, and 5.29-fold, respectively; and those under the NPJ treatment increased by 8.50-fold, 6.72-fold, 4.56-fold, 4.17-fold, 9.40-fold, 10.11-fold, 11.51-fold, and 10.61-fold, respectively. The experiment showed that rice root growth was enhanced under the SPJ, NP, and NPJ treatments. Notably, the effect of rice growth promotion was the most significant under the combined treatment of citric acid and *B. altitudinis* LZP02.

**DISCUSSION**

Organic acids in plant root exudates have a significant effect on root colonization of PGPR and increase the occurrence of plant-microbe interactions (21, 22). The present study highlights the importance of citric acid in plant growth-promoting rhizobacterium. *B. altitudinis* LZP02 is a beneficial plant growth-promoting rhizobacterium with a strong ability of rice rhizosphere colonization and promote rice growth (23). Our previous study showed that the citric acid within rice root exudates induced the chemotaxis of *B. altitudinis* LZP02, and 100 μM was determined to be the optimal concentration for bacterial chemotaxis (20). Here, we concentrated on the interaction between citric acid and *B. altitudinis* LZP02 at the molecular level and on rice growth-promoting trait analysis. In this study, SEM clearly indicated that the introduction of citric acid could increase the number of *B. altitudinis* LZP02 colonies in the rice roots. These results were confirmed by reverse verification, such that when citric acid was added to the culture media, the bacteria on the roots collapsed. The quantification results were consistent with the observations from the SEM images (Fig. 1). These results confirmed that 100 μmol L⁻¹ citric acid can lead to significant recruitment of *B. altitudinis* LZP02. In agricultural production, rice roots secrete citric acid, which induces chemotaxis of bacterial strains in the soil. The results demonstrated that citric acid in rice root exudates enhanced the colonization of the bacterial strain LZP02.

Flagellum-mediated motility plays an important role in various physiological activities, such as bacterial chemotaxis, biofilm formation, and host colonization (24–27). We further revealed that citric acid considerably increased the expression of genes
related to chemotaxis and flagellar biosynthesis in *B. altitudinis* LZP02, thus enhancing its motility (20) and colonization in the rice root (Fig. 2). In this study, KEGG enrichment analysis of the transcriptome discovered that 12 DEGs associated with bacterial chemotaxis and biofilm formation were significantly upregulated in *B. altitudinis* LZP02 after 30 min of citric acid treatment (Table S1). These genes encode 8 proteins (e.g., MCP, a methyl-accepting chemotaxis protein; FliG, a flagellar motor switch protein; and MotB, a flagellar motor protein) (Fig. 3 and Table S1). The phenotypic results were consistent with the results of the transcriptome analysis. Bacterial chemotaxis depends on MCP sensing and binding to chemoeffectors, followed by transmitting the perceived signal to downstream proteins that play a role in the chemotaxis signaling system (28). It is well known that this process is rapid (29). We found that the expression of these genes associated with bacterial chemotaxis and biofilm formation differed in *B. altitudinis* LZP02 after 12 h of citric acid treatment (Fig. 5). These findings supported the results of the transcriptome analysis after 30 min of citric acid treatment. Similarly, another study found that genes involved in quorum sensing, chemotaxis, and biofilm formation...
in *Paenibacillus polymyxa* SC2 were significantly upregulated after pepper root exudate treatment for 30 min (30).

A previous study showed that D-galactose from cucumber roots could induce biofilm formation of *B. velezensis* SQ9 in an *mcpA*-dependent manner (31). Moreover, flagellum-related genes were significantly upregulated in *Pseudomonas koreensis* GS after treatment with *Streptomyces pactum* Act12 cell-free filtrate, while rhizosphere colonization and competition were enhanced (32). A previous study found that a *motA*-deficient strain showed lower colonization ability in wheat roots, while the biocontrol effects that worked against it were reduced (33). Chemotaxis is the first step during rhizosphere colonization. These results confirmed that an increase in the expression of flagellum-mediated motility genes after citric acid treatment may promote root colonization by *B. altitudinis* LZP02 in the rice rhizosphere.

Competition is more prevalent in free-living microbes in habitats where resources are relatively scarce, thus improving the physiological activity of bacteria and supporting the impact of plant growth promotion. Here, the bacteria entered the logarithmic phase earlier after citric acid treatment, and the OD value was larger (Fig. 4D). Soil microorganisms confirmed rapid and complete metabolism with citric acid as a source of carbon and energy. Biostimulation of citric acid was confirmed, which showed an increase in dehydrogenase and phosphatase activities (34). S-adenosylmethionine (SAM) is an important physiologically active substance. As a methyl donor, aminopropyl donor and precursor of sulphydryl compounds, SAM is involved in a range of biochemical reactions in all living organisms (35). Examples include the synthesis of nucleic acids, proteins, phospholipids, and vitamins and the mutual transformation of cysteine, glutathione, polyamines, and taurine (36–38). Our GO enrichment analysis of the transcriptome data showed that 7 DEGs associated with *S*-adenosylmethionine biosynthesis and metabolism were significantly upregulated in *B. altitudinis* LZP02 after 12 h of citric acid treatment (Fig. 4E and Table S4). According to a previous study, SAM is known to participate in a number of essential metabolic pathways in plants (39). Whether such interaction mechanisms occur in the rhizosphere remains unknown. In vivo, SAM is formed by the transfer of an adenosine group of ATP to L-methionine, catalyzed by *S*-adenosylmethionine synthase (*metK*), which depletes ATP in the cells. Therefore, the level of intracellular ATP is one of the factors limiting the biosynthesis of SAM (36, 40). It is worth noting that 7 DEGs involved in the oxidative phosphorylation pathway were significantly upregulated in *B. altitudinis* LZP02. The results showed that the intracellular ATP level of *B. altitudinis* LZP02 significantly increased after treatment with citric acid for 12 h (Fig. 4B and
Therefore, citric acid might improve the physiological activity of bacteria by increasing the expression of genes involved in SAM biosynthesis and metabolism.

Moreover, *B. altitudinis* LZP02 caused an increase in the secretion of citric acid from the rice roots (Fig. 6B). These results indicate that citric acid might be a momentous signal in the interaction between plants and *B. altitudinis* LZP02, which is worthy of further study (31). Similarly, the α-galactose content was shown to be 3-fold higher in the root exudates of *B. velezensis* SQR9-colonized cucumber than in those of uninoculated cucumber (31), and *B. subtilis* RR4 isolated from the rice rhizosphere induces malic acid biosynthesis in rice roots (11). Our experiments showed that citric acid secretion in rice decreased after 2 days of *B. altitudinis* LZP02 treatment. We speculate that higher concentrations of citric acid were utilized by *B. altitudinis* LZP02 (Fig. 6).

Finally, the combined use of citric acid and *B. altitudinis* LZP02 treatment had a synergistic effect on plant growth promotion, including on both seed germination and growth traits, as observed during our experiments. The results showed that citric acid alone increased germination and plant growth. Additionally, the application of CA has been reported to enhance tomato seed germination and root length (41). However, the mechanism underlying the effect of low-molecular-weight organic acids on plant seeds is worthy of further study. Seed germination is the first step to ensuring grain yield and quality (42–44). A sufficient germination index and postgermination growth promote subsequent cultivation (45, 46). Moreover, bioactive GA promotes seed germination in a number of plant species (47, 48). Our results showed that citric acid promotes GA secretion by *B. altitudinis* LZP02. When rice seeds were soaked in citric acid, the addition of *B. altitudinis* LZP02 increased the germination index and enhanced rice growth traits (Fig. 7 and 8).

**Conclusion.** A schematic diagram of the interaction between citric acid and *B. altitudinis* LZP02 is shown in Fig. 9. The citric acid in rice root exudates enhanced the colonization of *B. altitudinis* LZP02 by increasing the expression of genes involved in the bacterial chemotaxis and biofilm formation pathways. The citric acid promotes GA secretion by *B. altitudinis* LZP02. The combination of both citric acid and *B. altitudinis* LZP02 promoted seed germination and rice growth. Moreover, *B. altitudinis* LZP02 increased the secretion of citric acid from the rice roots. The results confirmed that the
interaction between citric acid and *B. altitudinis* LZP02 could be exploited for the development of sustainable agriculture.

**MATERIALS AND METHODS**

**Bacterial strain, media, plant material, and growth conditions.** *B. altitudinis* LZP02 is a rice rhizobacterium (GenBank: CP075052). *B. altitudinis* LZP02 was grown at 30°C in Luria-Bertani (LB) media (peptone, 10 g L⁻¹; yeast extract, 5 g L⁻¹; and NaCl, 8 g L⁻¹) for 10 h, collected via centrifugation at 9,569 × g, and stored in 15% glycerol at −80°C. Rice seeds (*Oryza sativa* ‘Longpeng 46’) were used in this study. Murashige-Skoog (MS) liquid media was purchased from Sigma ALDRICH (Shanghai) TRADING Co., LTD.

**SEM.** Rice seedlings roots of a similar size were rinsed with sterile water. The roots were soaked in 100 μmol L⁻¹ sterile citric acid aqueous solution for 9 h (NP). The roots of the control group were soaked with an equal volume of sterile water (SP). All the seedlings were placed in MS liquid media (50 mL) that included *B. altitudinis* LZP02 (4 × 10⁷ CFU/mL) for 12 h. In another contrasting experiment, the roots of seedlings were soaked in *B. altitudinis* LZP02 solution (OD₆₀₀ = 0.5) for 1 h. The treated seedlings were subsequently transferred to MS liquid media. Sterile citric acid solution was added to the MS liquid media to a final concentration of 100 μmol L⁻¹ (CL), and the control group was treated with the same amount of sterile water (CK). All the seedlings were transplanted into the media for 24 h. All of the operations were performed under temperature (22 ± 2°C). SEM was performed as previously described (49). The length of the root segments was 0.4 to 1 cm, and the root segments were fixed in 2.5% glutaraldehyde for 12 h. Then, a 40%, 50%, 60%, 70%, 80%, 90%, and 100% ethanol series was used to dehydrate the root segments. After dehydration, the root segments were allowed to dry under natural conditions. Two- to three-millimeter-thick samples were prepared. The different parts of roots were observed, and images were obtained (S-3400; Hitachi, Japan). Fibrous roots (0.01 g) of the same size were used in the above-described experiment. The samples were then placed in 1 mL of sterile water, vortexed for 30 min, and counted with a hemocytometer.

**Growth curve assays.** Growth curves of *B. altitudinis* LZP02 (CK) were constructed, and the control strains and strains treated with 100 μmol L⁻¹ citric acid (LZP02-CA) in LB media were measured under the same conditions (120 rpm, 30°C) with shaking. Absorbance measurements at 600 nm were performed every 2 h. Three replicates were included for each treatment.

**Transcriptome analysis of *B. altitudinis* LZP02. (i) Sample preparation.** For this experiment, *B. altitudinis* LZP02 was treated with sterile citric acid for 30 min. The steps for sample preparation included *B. altitudinis* LZP02 being grown at 30°C in media (peptone, 10 g L⁻¹; NaCl, 8 g L⁻¹) to an OD = 0.7. In the next step, sterile citric acid was added to the *B. altitudinis* LZP02 solution to a final concentration of 100 μmol L⁻¹ (CN1), while the control was treated with the same amount of sterile water (CK1). The treated bacteria were centrifuged after the culture oscillated for 30 min.

In addition, *B. altitudinis* LZP02 was treated with sterile citric acid for 12 h. The steps for sample preparation included the addition of sterile citric acid to the media (peptone, 10 g L⁻¹; NaCl, 8 g L⁻¹) to a final concentration of 100 μmol L⁻¹ (CN2), while the control received an equal volume of sterile water (CK2). Each treatment was inoculated with 1 mL of broth culture of *B. altitudinis* LZP02 for 8 h. The culture was incubated at 30°C and shaken at 120 rpm for 12 h. The bacterial cells in each group were harvested by centrifugation (8,000 rpm, 4°C), and samples were sent directly to Majorbio Biopharm Technology Co., Ltd. (Shanghai, China) for transcriptome sequencing.

**(ii) RNA extraction, library construction, and sequencing.** Total RNA was extracted using TRIzol reagent (Invitrogen) and quantified using an ND2000 (NanoDrop Technologies). Only high-quality RNA (OD₂₆₀/₂₃₀ = 1.8–2.0; OD₂₆₀/₂₃₀ ≥ 2.0; relative intensity noise ≥ 6.5; 28S:18S ≥ 1.0; ≥ 100 ng/μL; and ≥ 2 μg) was used to construct the sequencing library.

The RNA-seq library was prepared according to instructions of the TruSeq RNA sample preparation kit from Illumina (San Diego, CA), with 2 μg of total RNA used. Double-stranded cDNA was synthesized and subjected to end repair, phosphorylation, and polyadenylation. The libraries were screened for cDNA target fragments of 200 bp followed by PCR amplification of 15 PCR cycles. After quantification via TBS380, the paired-end RNA-seq library was sequenced by an Illumina HiSeq x10 instrument (2 × 150-bp read length).

**(iii) Bioinformatics analysis.** High-quality reads in each sample were aligned to the reference genome of *B. altitudinis* LZP02 (this study; NCBI accession number CP075052) using Bowtie2 (50, 51). The expected number of fragments per kilobase of transcript per million base pairs sequenced of each gene was calculated (52).

The edgeR (https://www.bioconductor.org/), DESeq2 (http://bioconductor.org/), and DESeq packages (http://www.r-project.org/) were used to identify DEGs across samples or groups (53, 54). The genes with a fold change ≥ 2 and a P < 0.05 in each comparison were regarded as significant DEGs (55).

GO enrichment analysis of DEGs was performed using GOATools (https://github.com/tanghaibao/GOatools) (56). The statistical enrichment of DEGs in KEGG pathways was determined using KOBASE 2.0 software (https://doi.org/10.18170/DVN/ZKCO43) (57).

**Validation of transcriptome data by reverse transcription-quantitative PCR.** To verify the RNA-seq results, 18 DEGs from the RNA-seq analysis were selected, and reverse transcription-quantitative PCR (qRT-PCR) was performed to confirm their expression changes. Moreover, qRT-PCR was performed on the samples of RNA-seq. Single-stranded cDNA was generated from the total RNA using HiScript (Vazyme) followed by quantitative real-time PCR (ABI7300; Applied Biosystems, USA). The primers and internal control used for qRT-PCR are listed in Table S5 and Table S6. Each qRT-PCR mixture (20 μL) included 10 μL of 2× ChamQ SYBR Color qPCR Master Mix, 0.8 μL of forward primer (5 μM), 0.8 μL of reverse primer (5 μM), 0.4 μL of 50× ROX Reference Dye 1, 2 μL of template (cDNA), and 6 μL of ddH₂O.
PCR was performed as follows: 5 min at 95°C followed by 40 cycles of 5 s at 95°C and 30 s at 55°C. This study was performed by using the 2-ΔΔct method (S8). Each measurement was repeated three times.

**Quantification of citric acid concentration.** Sterile rice seeds were cultivated until the seedling stage was reached. Rice seedlings of similar size were transplanted into MS liquid medium (50 mL). In the treatment group, the media for *B. altitudinis* LZP02 were washed away and added to MS liquid media to a final concentration of 4 x 10^7 CFU/mL, while the control group received an equal volume of sterile water. Three replicates were included for each treatment. The citric acid concentration in rice roots was measured using test kits (Nanjing Jiancheng Biological Engineering Institute, Nanjing, China). In this experiment, rice seedlings were grown in a growth chamber; the roots were inoculated with *B. altitudinis* LZP02, and the root exudates were collected for quantitative analysis (Fig. 6A).

**Analysis of plant growth-promoting traits.** The rice seeds were disinfected with H2O2 and rinsed six times with sterile water.

The sterilized seeds were soaked in a 100 μmol L⁻¹ citric acid solution and sterile water for 2 h each. Then, the seeds were transferred to the growth chamber. Ten milliliters of *B. altitudinis* LZP02 (4 x 10^7 CFU/mL) (NP) plus sterile water (NP) was added to the seeds soaked in citric acid. In addition, 10 mL of *B. altitudinis* LZP02 (4 x 10^7 CFU/mL) (NP) plus sterile water (NP) was added to the water-soaked seeds. The seeds were in controlled light (16/8-h light/dark cycle), luminance (30,000 lx), and temperature (22 ± 2°C) conditions. All of the experimental operations were aseptic. The germination index and eight root parameters were observed and determined.

**Determination of GA secretion.** The determination of GA secretion by *B. altitudinis* LZP02 and the strains treated with citric acid was measured under the same conditions, with shaking (59).

**Statistical analysis.** Analysis was conducted via SPSS 24.0 or GraphPad Prism 6.01. One-way ANOVA and t-test were used to statistical analysis. The details are described in the figure legends.

**Data availability.** The raw sequence reads of the transcriptome have been deposited in NCBI under the accession number PRJNA849272.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**

PDF file, 0.1 MB.

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The authors declare that there are no conflicts of interest.

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