***Bacillus pumilus* LZP02 Promotes Rice Root Growth by Improving Carbohydrate Metabolism and Phenylpropanoid Biosynthesis**

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Elucidation of the underlying mechanisms of plant growth promotion of rhizobacteria is very important. This study explored the mechanism by which *Bacillus pumilus* LZP02 promotes growth in rice roots through proteomic, transcriptomic, and metabolomic techniques. The results showed that *B. pumilus* LZP02 promoted the absorption of phosphorous, calcium, and magnesium ions by colonization of rice roots and enhanced peroxidase, catalase, superoxide dismutase, and $\text{Ca}^{2+}\text{Mg}^{2+}$ adenosine triphosphatase activities and chlorophyll contents in rice. The proteomic results showed that most of the differentially expressed proteins were involved in carbohydrate metabolism and that the biosynthesis of other secondary metabolites was also increased. According to RNA-seq and reverse transcription-quantitative PCR analyses, expression of some genes involved in carbohydrate metabolism and phenylpropanoid biosynthesis was upregulated in rice roots. Regarding metabolomics, phenylpropanoid biosynthesis, starch and sucrose metabolism, the pentose phosphate pathway, and glyoxylate and dicarboxylate metabolism were increased. The results indicated that *B. pumilus* LZP02 promoted the growth of rice roots by enhancing carbohydrate metabolism and phenylpropanoid biosynthesis.

Keywords: *Bacillus pumilus*, biofertilizer, carbohydrate metabolism, phenylpropanoid biosynthesis, rice

Applying large amounts of chemical fertilizers to increase the yield of plants can lead to severe environmental pollution (Suresh Rao et al. 2016). Biofertilizers are sustainable alternative technologies that can enhance productivity without ecological

impacts (Nayak et al. 2019). Plant growth-promoting rhizobacteria (PGPRs) colonize plant roots and have beneficial effects (Hermosa et al. 2012). Indeed, PGPRs can be used as biofertilizers, and these microorganisms play an important role in creating a favorable ecological environment in the rhizosphere, reducing the use of chemical fertilizers and pesticides, inhibiting the occurrence of pests and diseases, and ensuring the sustainable development of modern agriculture while achieving the goal of increased production (Li et al. 2020; Naik et al. 2019; Z. Wang et al. 2019b).

Rice (*Oryza sativa*) is one of the most important stable food crops in the world. In Asia, more than two billion people alone derive 80% of their energy needs by consuming rice (Chaudhari et al. 2018). PGPRs have been applied to rice in various forms to enhance growth and yield. For example, *Burkholderia pyrrocinia* BRM 32113 resulted in significant increases in growth promotion and reduced area under the disease progress curve in rice (Arriel-Elias et al. 2019). *Azospirillum brasilense* can colonize rice roots and promote growth (Thomas et al. 2019), and *Paenibacillus polymyxa* Sx3 significantly promoted growth and suppressed bacterial leaf blight in rice (Abdallah et al. 2019). In addition, *Bacillus pumilus* enhanced the tolerance of rice to a combination of NaCl and high-boron stress (Khan et al. 2016). The *B. pumilus* LZP02 strain used in the present study is a phosphate-dissolving bacterium that produces auxin and gibberellin and has the ability to synthesize iron carriers (Liu et al. 2018b; H. Wang et al. 2019). However, in-depth research on the promotion mechanism of PGPRs in the rice rhizosphere has not been carried out to date, even though elucidation of such mechanisms is extremely important and urgently needed. In particular, systematic studies of gene expression regulation combined with metabolic regulation are lacking, and the paucity of research in this area is a bottleneck that restricts the accurate design of strains, the optimization of interaction conditions, and the regulation of PGPR physiological functions.

In this study, *B. pumilus* LZP02 was added to the culture medium of rice plants, after which root growth was measured and colonization of rice roots was observed by scanning electron microscopy (SEM). Atomic absorption spectrometry was used to detect rice root nutrient absorption, and determination of the root catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and adenosine triphosphatase (ATPase) activities and chlorophyll contents was performed. These indicators

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were then combined with proteomic, transcriptomic, and metabolomic analyses to evaluate the mechanism underlying the effect of *B. pumilus* LZP02 on rice root growth.

RESULTS

Effect of *B. pumilus* LZP02 on the growth of rice roots.

The effect of *B. pumilus* LZP02 on the growth of rice roots was explored to determine the optimal inoculum concentration for a plant growth-promotion effect, and S-3 (inoculated *B. pumilus* LZP02 at 4×10^7 CFU/ml) showed a higher effect (Fig. 1A). *B. pumilus* LZP02 markedly increased the number of rice fibrous roots in the S-3 treatment (Fig. 1B and C).

Compared with the control (CK) (0 CFU/ml), the length of S-3 roots increased by 62.9% (Fig. 2A), the shadow area increased by 63.2% (Fig. 2B), the surface area increased by 63.1% (Fig. 2C), the volume increased by 52.7% (Fig. 2D), the number of connections increased by 67.3% (Fig. 2E), the number of nodes increased by 68.2% (Fig. 2F), the number of root tips increased by 63.0% (Fig. 2G), and the number of forks increased by 73.5% (Fig. 2H). These results indicate that rice root growth was significantly enhanced under the S-3 condition.

The colonization of rice root by *B. pumilus* LZP02 was confirmed using 2.0- and 5.0-k magnification SEM, whereas the strain was not found on the roots of CK (Fig. 3).

Effect of *B. pumilus* LZP02 on the physiological characteristics of rice roots.

The effects of *B. pumilus* LZP02 on different physiological characteristics of rice roots are shown in Figure 4. The nitrogen, phosphorous, calcium, and magnesium contents of S-3 increased by 27.2, 24.6, 18.1, and 28.3%, respectively, compared with those of CK (Fig. 4A); meanwhile, the root activity increased by 39.2% (Fig. 4B), POD activity increased by 29.6% (Fig. 4C), CAT activity increased by 56.3% (Fig. 4D), and SOD activity increased by 59.1% (Fig. 4E). The contents of sulfur, sodium, iron, and manganese were not significantly changed. The contents of potassium and zinc were significantly reduced. The ATPase activity also exhibited significant changes; the Na^+K^+ -ATPase of S-3 was reduced by 76.7% compared with that of CK, but the $\text{Ca}^{2+}\text{Mg}^{2+}$ -ATPase of S-3 increased by 56.3% (Fig. 4F); this result is consistent with the results for ion absorption. There were also significant changes in the chlorophyll content of rice leaves, whereby the chlorophyll a and chlorophyll b levels in S-3 increased by 42.2 and 50.8%, respectively, compared with those of CK (Fig. 4G). These results indicate that S-3 significantly enhanced different activities in rice that can be related to plant growth promotion.

Proteomic differences between CK and S-3.

The total number of quantified proteins in CK and S-3 was 1,360. The number of upregulated and downregulated proteins in S-3 was 103 and 640, respectively, adding a total of 743 differentially expressed proteins (DEPs) between CK and S-3 (Fig. 5A). To obtain a global picture of the proteomic changes, these 743 DEPs were annotated by gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. The results of the GO analysis showed that the most-represented categories were metabolic processes, cells, cell parts, cellular processes, binding, and catalytic activity, accounting for 12.96, 12.70, 12.68, 12.38, 11.60, and 11.47% of the DEPs, respectively (Fig. 5B). A KEGG pathway analysis was then performed to investigate the main biological pathways in which these DEPs were enriched. The results of the KEGG_B analysis showed that most proteins are related to carbohydrate metabolism, which accounted for 28.41% of the

DEPs. This was followed by amino acid metabolism and translation, which accounted for 14.35 and 12.12% of the DEPs, respectively (Fig. 5C). Among the KEGG_C biological process terms, 15 had the largest number of DEPs. These terms included 13 metabolic processes, including one within biosynthesis of other secondary metabolites (phenylpropanoid biosynthesis), one within nucleotide metabolism (purine metabolism), three within amino acid metabolism (glutathione metabolism, cysteine and methionine metabolism, and glycine, serine, and threonine metabolism), and eight within carbohydrate metabolism (fructose and mannose metabolism, starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, the pentose phosphate pathway, glycolysis/gluconeogenesis, glyoxylate, and dicarboxylate metabolism, pyruvate metabolism, and the citrate cycle [trichloroacetic acid cycle]) (Fig. 5D). These processes are related to the growth of plants.

Transcriptomic differences between CK and S-3.

Through the KEGG_C biological process based on the transcriptional analysis, the differentially expressed genes (DEGs) were mainly concentrated in fructose and mannose metabolism, starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, glyoxylate and dicarboxylate metabolism, phenylpropanoid biosynthesis, and the pentose phosphate pathway (Fig. 6A). This is consistent with the proteome data. As shown in Figure 6B, DEGs were observed among these processes ($P \leq 0.05$). Compared with CK, the S-3 treatment resulted in the differential expression of 14 genes. Under the S-3 treatment, most genes (*Os02g0207900*, *Os05g0135500*, *Os09g0298200*, *Os07g0577300*, *XLOC_000876*, *Os08g0495800*, *Os01g0174300*, *Os03g0115100*, *Os08g0526100*, *Os03g0102100*, *Os12g0291100*, *Os02g0714200*, and *Os08g0154300*) showed increased expression, whereas the expression of *XLOC_029271* was reduced. Details regarding the genes are presented in Supplementary Table S1. Two DEGs (upregulated) participate in phenylpropanoid biosynthesis, three (two upregulated and one

Fig. 1. Effect of strain *Bacillus pumilus* LZP02 on the growth of rice roots at 48 h after inoculation with *B. pumilus* LZP02. **A**, Effects of different strain concentrations on the growth of rice roots (colony-forming units per milliliter), control (CK) = 0, S-1 = 1×10^7 , S-2 = 2×10^7 , S-3 = 4×10^7 , S-4 = 6×10^7 , S-5 = 8×10^7 , S-6 = 1×10^8 , or S-7 = 1.2×10^8 . **B**, Effect of CK treatment on rice fibrous roots at 48 h after inoculation with *B. pumilus* LZP02. **C**, Effect of S-3 treatment on rice fibrous roots at 48 h after inoculation with *B. pumilus* LZP02.

downregulated) participate in starch and sucrose metabolism, seven (upregulated) participate in fructose and mannose metabolism, amino sugar and nucleotide sugar metabolism, and the pentose phosphate pathway, and two (upregulated) participate in glyoxylate and dicarboxylate metabolism.

To confirm the RNA-seq data, the expressions of some genes, including *Os02g0207900* and *Os05g0135500* in phenylpropanoid biosynthesis, *Os09g0298200*, *Os01g0174300*, and *Os08g0526100* in amino sugar and nucleotide sugar metabolism, *Os07g0577300*, *XLOC_000876*, and *XLOC_029271* in sucrose and starch metabolism, *Os08g0495800* and *Os03g0115100* in fructose and mannose metabolism, *Os03g0102100* and *Os12g0291100* in glyoxylic and dicarboxylic metabolism, and *Os02g0714200* and *Os08g0154300* in the pentose phosphate pathway were measured by reverse transcription-quantitative PCR (RT-qPCR). Consistent with the results of the transcriptomic analysis, the expression levels of genes, including *Os09g0298200*, *Os08g0495800*, *Os01g0174300*, *Os03g0115100*, *Os08g0526100*, and *Os03g0102100*, were changed by *B. pumilus* LZP02 (Fig. 6C).

Metabolomic differences among CK, S-3, and J (only *B. pumilus* LZP02 at 4×10^7 CFU/ml without rice).

In order to find out the different metabolites in rice root exudates inoculated with *B. pumilus* LZP02, the metabolomics of CK, S-3, and J were analyzed in Murashige and Skoog liquid medium. The significantly altered metabolites ($P < 0.05$) in the CK, S-3, and J treatments were selected for subsequent analysis and were summarized. The principal component analysis (PCA) results for the CK, S-3, and J treatments

suggested significant changes in metabolites (Fig. 7), and the differential metabolites between the CK- and S-3-treated plants are illustrated in Figure 8A. The results identified 13 differential metabolites related to phenylpropanoid biosynthesis, starch and sucrose metabolism, the pentose phosphate pathway, and glyoxylate and dicarboxylate metabolism ($P < 0.05$); sucrose, ferulic acid, oleic acid, acetyl-coA, and ribose

Fig. 3. Scanning electron microscopy images of rice roots at 48 h after inoculation with *Bacillus pumilus* LZP02. **A**, Control (CK) treatment under 2.0-k magnification. **B**, S-3 treatment under 2.0-k magnification. **C**, CK treatment under 5.0-k magnification. **D**, S-3 treatment under 5.0-k magnification.

Fig. 2. Effect of *Bacillus pumilus* LZP02 on the growth of rice roots under S-3 treatment at 48 h after inoculation with *B. pumilus* LZP02. **A**, Root length and **B**, root shadow area (sum of shadow area of the tiled roots per plant). **C**, Root surface area (total root surface area per plant). **D**, Root volume (total root volume per plant). **E**, Number of root connections (total number of overlapping points of different roots of a plant by plane projection). **F**, Number of root nodes (total number of root nodes per plant). **G**, Number of root tips (total number of root tips per plant). **H**, Number of root forks (sum of the number of branching roots per plant). Asterisks (**) indicate $P < 0.01$ ($n = 3$).

5-phosphate were the most significantly different metabolites between CK and S-3 (Fig. 8B). The differential metabolites between S-3- and J treatments are shown in Figure 8C. According to the data, nine of the differential metabolites are associated with phenylpropanoid biosynthesis, starch and sucrose metabolism, the pentose phosphate pathway, and glyoxylate and dicarboxylate metabolism ($P < 0.05$), and 3-feruloylquinic acid and feruloyl tyramine were the most significantly different between S-3 and J (Fig. 8D). The malic acid and ferulic acid contents increased in S-3, whereas the malic acid, ferulic acid, and 3-feruloylquinic acid contents increased in J. These findings confirmed the reliability of the metabolomic, transcriptomic, and proteomic analyses at the metabolic, transcriptomic, and protein levels, respectively.

DISCUSSION

Currently, the demand for rice is gradually increasing, and the growth of rice roots directly affects yield. PGPRs are an important functional group of beneficial bacteria that can be used for plant growth promotion (Lemessa and Zeller 2007). *B. pumilus* LZIP02 promoted the growth of rice roots (Figs. 1 and 2). The SEM results clearly indicated the growth status of bacteria and roots and demonstrated that *B. pumilus* LZIP02 colonizes rice roots (Fig. 3). *B. pumilus* LZIP02 also improved physiological indicators in rice (Fig. 4), similar to arbuscular mycorrhizal fungi in fava bean (Pereira et al. 2019) and *Pedobacter* sp. strain CC1 and *Bacillus safensis* B106 in strawberry (Morais et al. 2019).

Based on the proteomic results (Fig. 5), the DEPs were primarily involved in phenylpropanoid biosynthesis, starch and sucrose metabolism, the pentose phosphate pathway, and glyoxylate and dicarboxylate metabolism. Details regarding the DEPs are provided in Supplementary Table S2. Fifteen DEPs involved in starch and sucrose metabolism were highlighted, including hexokinase, cytosolic starch phosphorylase, cell-wall invertase, β -glucosidase, vacuolar acid invertase, and fructose-6-phosphate 1-phosphotransferase. The expression level of β -glucosidase, which plays a role in hydrolyzing sucrose to glucose (Pang et al. 2017), was significantly increased, and the level of *Os07g0577300*, encoding a glycoside hydrolase, was also increased in the transcriptome (Fig. 6B and C). The expression of *XLOC_029271* was increased in the absence of phosphorus (Zhu et al. 2015). However, the results showed that *B. pumilus* LZIP02 downregulated the expression of *XLOC_029271* (Fig. 6B and C) and improved phosphorus absorption (Fig. 4A). It could be speculated that the improved phosphorus absorption resulted in the downregulation of *XLOC_029271*. Sucrose and glucose-6-phosphate, which are important intermediate metabolites in starch and sucrose metabolism, provide a large amount of energy and are closely linked to many other reactions (Ohashi et al. 2018); both exhibited significant changes in the metabolome in the present study (Fig. 8). Therefore, *B. pumilus* LZIP02 could enhance the starch and sucrose metabolism as an intermediate reaction process to provide the energy needed for biological reactions in rice roots (Fig. 9), which was similar to the effect of *Pseudomonas putida* in maize (SKZ et al. 2018). Through the

Fig. 4. Effects of *Bacillus pumilus* LZIP02 on the physiological characteristics of rice roots at 48 h after inoculation with *B. pumilus* LZIP02. **A**, Effect of S-3 treatment on the absorption of different ions; **B**, Effect of S-3 treatment on rice root activity; **C**, peroxidase (POD) activity; **D**, catalase (CAT) activity; **E**, superoxide dismutase (SOD) activity; **F**, adenosine triphosphatase (ATPase) activity; **G**, chlorophyll concentration. Two asterisks (**) indicate $P < 0.01$ and one (*) indicates $P < 0.05$ ($n = 3$).

proteomic analysis, 20 DEPs were identified in glyoxylate and dicarboxylate metabolism, and the isocitrate lyase level increased significantly. Isocitrate lyase is one of the key enzymes in the unique metabolic process of the glyoxylate cycle, and it catalyzes the decomposition of isocitrate to form glyoxylic acid and succinic acid (Bhusal et al. 2017; Lu et al. 2005). The changes in expression of the *Os12g0291100* gene in glyoxylic and dicarboxylic metabolism (of the transcriptome) and the glyoxylic acid content (of the metabolome) were consistent with that of isocitrate lyase (of the proteome). Oleic acid is decomposed by β -oxidation to form acetyl-CoA (Salam et al. 1989). Glyoxylic acid produces malic acid through acetyl-CoA and dehydrogenation of malic acid produces oxaloacetate, which constitutes the glyoxylate cycle (Lu et al. 2005). Important metabolites in pathways, namely, oleic acid, acetyl-CoA, glyoxylic acid, malic acid, and oxaloacetate, were all significantly upregulated (Fig. 8B). In addition, the malic acid content increased significantly in both S-3 versus CK and S-3 versus J (Fig. 8). The increase in metabolites may provide the material required for starch and sucrose metabolism. The proteomic analysis identified 15 DEPs in the pentose phosphate pathway, and ribose 5-phosphate isomerase precursor, an important enzyme in the pentose phosphate pathway, was significantly upregulated (Fig. 5). With regard to the metabolomic results, ribose 5-phosphate was significantly upregulated, as was also confirmed by the proteomic results (Fig. 9). In transcriptomics, the gene *Os02g0714200* encoding pyrophosphate-fructose-6-phosphate phosphotransferase was significantly upregulated (Fig. 6B and C). Overexpression of cytosolic invertase results in upregulation of carbohydrates while increasing flux through the pentose phosphate pathway (Centeno et al. 2008). Based on our results, an increase in the flux of the pentose phosphate pathway could provide glucose as an energy source for rice. Overall, upregulation of some metabolic pathways, including starch and sucrose metabolism, the

pentose phosphate pathway, and glyoxylate and dicarboxylate metabolism, led to upregulation of carbohydrate metabolism in rice roots (Fig. 9). Therefore, the results indicated that *B. pumilus* LZP02 could promote the growth of rice roots by enhancing carbohydrate metabolism.

A total of 28 DEPs in phenylpropanoid biosynthesis, including cytosolic aldehyde dehydrogenase, 4-coumarate:coenzyme A ligase, cinnamyl alcohol dehydrogenase, phenylalanine ammonia-lyase, β -glucosidase, cytochrome, and POD, were identified (Fig. 5; Supplementary Table S2). 4-Coumarate:coenzyme A ligase is a key enzyme in the phenylpropanoid metabolism pathway in monoalcohols and flavonoid biosynthesis and is related to the synthesis of lignin and ferulic acid in rice (Gui et al. 2011; Sun et al. 2013). Cinnamyl alcohol dehydrogenase is involved in the synthesis of lignin and catalyzes the formation of precursors of lignin monomers from a variety of different cinnamaldehydes (Fan et al. 2009). POD, which is present in peroxisomes, participates in the synthesis of lignin from carbohydrates, thereby protecting cells (Hrubcová et al. 2000). Significant increases were observed in the expression levels of 4-coumarate:coenzyme A ligase, cinnamyl alcohol dehydrogenase, and POD in this study (Supplementary Table S2), suggesting that *B. pumilus* LZP02 could promote lignin synthesis and resilience in rice roots (Fig. 9). At the same time, expression of gene *Os05g0135500*, encoding POD, was increased in the transcriptome (Fig. 6B and C). Phenylalanine ammonia-lyase (Liu et al. 2018a) and β -glucosidase (Pengthaisong and Ketudat Cairns 2014) are also important enzymes in the phenylpropanoid biosynthesis pathway. The expression level of phenylpropanoid biosynthesis pathway components correlates positively with the lignin content in *Toona sinensis* (Sui et al. 2019). Additionally, an increase in the expression of genes that participate in phenylpropanoid biosynthesis increases resistance to fungal pathogens in avocado (Xoca-Orozco et al. 2019). Ferulic acid is an intermediate metabolite that participates in the synthesis of coniferyl alcohol

Fig. 5. Effect of *Bacillus pumilus* LZP02 on the proteome of rice roots at 48 h after inoculation with *B. pumilus* LZP02. **A**, Differential analysis scatter plot of proteins in control (CK) and S-3 treatments; the abscissa and ordinate represent the expression levels of S-3 and CK treatments, respectively. **B**, Gene ontology analysis of the differentially expressed proteins (DEPs) between CK and S-3. **C**, KEGG_B analysis of the DEPs between CK and S-3. **D**, KEGG_C analysis of the DEPs between CK and S-3 (only the first 15 are shown).

from coniferaldehyde and, ultimately, in the synthesis of lignin and it also exhibits antioxidant activity to protect DNA (Jia et al. 2019). The metabolomic data showed that the addition of *B. pumilus* LZP02 to rice resulted in a significant increase in ferulic acid; the ferulic acid content was significantly increased in both S-3 versus CK and S-3 versus J (Fig. 8). This may be because rice root metabolites and LZP02 metabolites can promote the growth of other organisms (Rasmussen et al. 2020; Toju et al. 2018). Therefore, *B. pumilus* LZP02 may enzymatically stimulate phenylpropanoid biosynthesis to promote growth in rice roots (Fig. 9).

The study has demonstrated that *B. pumilus* LZP02 colonizes rice roots and promotes growth in rice roots by improving carbohydrate metabolism and phenylpropanoid biosynthesis (Fig. 9).

MATERIALS AND METHODS

Materials.

B. pumilus LZP02 was isolated previously from the rice rhizosphere in northeast China. *B. pumilus* LZP02 was cultured in beef extract-peptone medium for 24 h, which was collected by centrifuging at $5,600 \times g$ and was stored with 15% glycerol at -80°C . *B. pumilus* LZP02 was cultured in Luria-Bertani medium for 8 h, and the final cell concentration was 10^9 CFU/ml (Supplementary Fig. S1). The culture was centrifuged, and the cells were suspended in stroke-physiological saline solution (NaCl 0.9%) at 10^9 CFU/ml until use.

Rice seeds (*Oryza sativa* Longgeng 46) were provided by the Rice Research Institute of Heilongjiang Academy of Agricultural Sciences. The seeds were arranged randomly in a growth chamber, with controlled light (cycles of 16 h of light and 8 h of dark), temperature ($22 \pm 2^{\circ}\text{C}$) and relative humidity (around 70%). Sterile water was added twice per week until the seedling stage.

Growth and root analysis.

Rice seedlings of similar size were selected and were planted in 50 ml of Murashige and Skoog liquid medium. Rice seedlings were treated in the two-leaf one-heart period with *B. pumilus* LZP02. The final concentrations of *B. pumilus* LZP02 in each treatment were as follows: 0 CFU/ml (CK), 1×10^7 CFU/ml (S-1), 2×10^7 CFU/ml (S-2), 4×10^7 CFU/ml (S-3), 6×10^7 CFU/ml (S-4), 8×10^7 CFU/ml (S-5), 1×10^8 CFU/ml (S-6), or 1.2×10^8 CFU/ml (S-7). All the operations were performed under aseptic conditions.

To determine the *B. pumilus* LZP02 concentration most suitable for rice seedling root growth, data on the roots, including root length, root shadow area, root surface area, root

Fig. 7. A principal component analysis score plot for the metabolomics data in control (CK), S-3, and J treatments at 48 h after inoculation with *Bacillus pumilus* LZP02 through a metabolomic analysis.

Fig. 6. Effect of *Bacillus pumilus* LZP02 on the transcriptome of rice roots at 48 h after inoculation with *B. pumilus* LZP02. **A**, KEGG_C analysis of differentially expressed genes in the control (CK) and S-3. **B**, Log₂ fold change values of differentially expressed genes in the transcriptome. **C**, Gene expression of differentially expressed genes by reverse transcription-quantitative (RT-q)PCR. Two asterisks (**) indicate $P < 0.01$ and one (*) indicates $P < 0.05$ ($n = 3$); red dots indicate that the transcriptome is identical to the RT-qPCR result. A and N m = amino sugar and nucleotide sugar metabolism; F and M m = fructose and mannose metabolism; PPP = pentose phosphate pathway. In B and C, yellow represents upregulated genes, and blue represents downregulated genes.

volume, number of root connections, number of root nodes, number of root tips, and number of root forks, were observed and were measured, at 48 h after inoculation, using a Tuopu root analyzer (RootAnalysis, PM-Tech).

SEM.

SEM was performed as previously described (Rashid et al. 2018). Rice roots in the two-leaf one-heart period were randomly collected from the CK and S-3 treatments at 48 h after inoculation with *B. pumilus* LZP02; 2- to 3-mm-thick samples were adhered to a copper sample table by using a conductive adhesive. A HUS-5GB-type vacuum coating apparatus was used to plate the sections, and the samples were subjected to SEM (S-3400, Hitachi) to observe different parts of the rice root system and obtain images.

Physiological index determination.

The POD, SOD, CAT, and ATPase activities in rice roots were measured using corresponding test kits (Nanjing Jiancheng Biological Engineering Institute). The ion absorption content was determined using an ion absorption meter (Yoshihara et al. 2019). The chlorophyll content was determined according to the method described by Sakaiguchi et al. (2019). Root activity was determined according to the method described by Whalley et al. (2017).

Proteomic assay.

Protein extraction of rice roots inoculated with CK and S-3 treatments cultured for 48 h was performed using the cold

acetone method. Roots (2 g) were cut into approximately 5 cm pieces, were ground to a powder in liquid nitrogen, and were then dissolved in 2 ml of lysis buffer (8 M urea, 2% sodium dodecyl sulfate [SDS], 1× protease inhibitor cocktail [Roche Ltd.]), followed by sonication on ice for 30 min and centrifugation at $9,520 \times g$ for 30 min at 4°C. Each treatment was repeated three times. The supernatant was transferred to a fresh tube. For each sample, proteins were precipitated overnight with ice-cold acetone at -20°C. The precipitates were washed with acetone three times and were redissolved in 8 M urea, followed by sonication on ice. Protein quality was examined by SDS-polyacrylamide gel electrophoresis.

A BCA protein assay kit (Pierce) was used to determine the protein concentration of the supernatant. A 100-μg sample of protein per condition was transferred into a new tube, and the volume was adjusted to 100 μl with 8 M urea; 11 μl of 1 M DL-dithiothreitol was added, and the samples were incubated at 37°C for 1 h and were transferred into a 10-K ultrafiltration tube (Merck Millipore). To remove the urea, the samples were washed three times, using 100 mM triethylammonium bicarbonate with centrifugation. A total of 120 μl of 55 mM iodoacetamide was added to the samples, which were then incubated at room temperature for 20 min, protected from light. The proteins were digested overnight with sequence-grade modified trypsin (Promega).

Each sample was divided into three equal parts and was resuspended in 30 μl of solvent C (C: water with 0.1% formic acid, D: ACN with 0.1% formic acid), was separated by nanoLC (liquid chromatography), and was analyzed by on-line electrospray tandem mass spectrometry (MS/MS). The

Fig. 8. Analysis of differential and specific metabolites of different treatments at 48 h after inoculation with *Bacillus pumilus* LZP02. **A**, Heat map analysis of S-3 versus control (CK) differential metabolites. **B**, Significantly different metabolites in phenylpropanoid biosynthesis, starch and sucrose metabolism, the pentose phosphate pathway, and glyoxylate and dicarboxylate metabolism between CK and S-3. **C**, Heat map analysis of S-3 versus J differential metabolites. **D**, Significantly different metabolites in phenylpropanoid biosynthesis, starch and sucrose metabolism, the pentose phosphate pathway, and glyoxylate and dicarboxylate metabolism between S-3 and J.

experiments were performed using an Easy-nLC 1000 system (Thermo Fisher Scientific) connected to a Q Exactive mass spectrometer (Thermo Fisher Scientific) equipped with an online nanoelectrospray ion source. A 10- μ l peptide sample was loaded onto the trap column (Thermo Scientific Acclaim PepMap C18, 100 μ m $\times$ 2 cm) with a flow rate of 10 μ l/min for 3 min and was subsequently separated on an analytical column (Acclaim PepMap C18, 75 μ m $\times$ 15 cm) with a linear gradient from 3% D to 32% D in 120 min. The column was re-equilibrated under the initial conditions for 10 min. The column flow rate was maintained at 300 nl/min. An electrospray voltage of 2 kV against the inlet of the mass spectrometer was used. The mass spectrometer was operated in the data-dependent acquisition mode to switch automatically between MS and MS/MS acquisition. Full-scan MS spectra (m/z 350 to 1,550) were acquired with a mass resolution of 35 K, followed by sequential high-energy collisional dissociation MS/MS scans with a resolution of 17.5. The dynamic exclusion time was set as 20 s.

MS/MS spectra were extracted, charge-state deconvoluted, and deisotoped. The MS data were then transformed into MGF files with Proteome Discovery 1.2 (Thermo) and processed by MaxQuant v1.5. PEAKS DB was set up for a search using trypsin as the digestion enzyme with a parent ion tolerance of 7.0 ppm. Carbamidomethylation was specified as a fixed modification. Oxidation (M) and acetylation (protein N-term) were specified as variable modifications. Peptides were filtered with a 1% false discovery rate (FDR).

Protein identifications were accepted if they contained at least two identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. iBAQ was employed for peptide and protein abundance calculations. Proteins with a fold change in a comparison >1.2 or <0.83 and unadjusted significance level $P < 0.05$ were considered to be differentially expressed. The proteins were annotated against the GO and KEGG databases to identify functions. Significant GO and KEGG functions and pathways were examined for DEPs with $P \leq 0.05$.

RNA-seq.

For this experiment, roots (2 g each treatment) from CK and S-3 cultured for 48 h after incubation with *B. pumilus* LZP02

were sent to Gene Denovo Biotechnology Co. for RNA extraction, library construction, RNA-seq, and primary data analysis. Each treatment was repeated three times. In brief, RNA was extracted from the rice root system, using a TRIzol kit according to manufacturer instructions (Promega), and was mixed in equal quantities for sequencing. After total RNA was extracted, eukaryotic messenger RNA (mRNA) was enriched with oligo (dT) beads, and prokaryotic mRNA was enriched by removing ribosomal RNA (rRNA), using the Ribo-Zero magnetic kit (Epicentre). The enriched mRNA was fragmented into short fragments using fragmentation buffer and was reverse transcribed into cDNA with random primers. Second-strand cDNA was synthesized by using DNA polymerase I, RNase H, dNTP, and buffer. The cDNA fragments were purified with the QiaQuick PCR extraction kit, were end-repaired and poly(A)-treated, and were ligated to Illumina sequencing adapters. The ligation products were size-selected by agarose gel electrophoresis, were PCR-amplified, and were sequenced using Illumina HiSeqTM 2500 (Gene De Novo Biotechnology Co.).

Reads obtained from sequencing include raw reads containing adapters or low-quality bases, which affect the subsequent assembly and analysis. Thus, to obtain high-quality clean reads, the reads were further filtered as follows: i) reads containing adapters were removed, ii) reads containing more than 10% unknown nucleotides (N) were removed, and iii) low-quality reads containing more than 50% low-quality (Q value ≤ 20) bases were removed. The short-read alignment tool Bowtie2 (Langmead and Salzberg 2012) was used for mapping reads to the rRNA database, and the rRNA-mapped reads were removed. The remaining reads were further used for assembly and analysis of the transcriptome. The rRNA-removed reads of each sample were mapped to the reference genome by TopHat2 (version 2.0.3.12) (Kim et al. 2013). After alignment with the reference genome, unmapped reads (or those mapped very poorly) were realigned with Bowtie2, and the enriched unmapped reads were split into smaller segments, which were then used to find potential splice sites. The section and the section position of these short segments were predicted as well. A set of splice sites was built with the initial unmapped reads by TopHat2, without relying on known gene annotations (Trapnell et al. 2010). Transcript reconstruction was carried out with Cufflinks software (Trapnell et al. 2012).

Fig. 9. Schematic diagram of *Bacillus pumilus* LZP02-mediated promotion of the growth of rice roots, which was summarized through the genes and proteins that were significantly expressed upon treatment. The solid black arrows indicate that metabolites at both ends were detected, and the dashed arrows indicate that metabolites at both ends were not detected or only metabolites at one end of the arrow were detected.

The edgeR package (available online) was used to identify DEGs across samples or groups. We considered genes with fold change ≥ 2 and FDR < 0.05 in a comparison as significant DEGs. The DEGs were then subjected to enrichment analysis of functions and KEGG pathways.

RT-qPCR analysis.

Rice roots from CK and S-3 cultured for 48 h after incubation with *B. pumilus* LZP02 were collected, and total cellular RNA was extracted using a UNIQ-10 Column TRIzol total RNA isolation kit (Sangon Biotech) according to the manufacturer protocol. Each treatment was repeated three times. The purified RNA was reverse transcribed into complementary DNA (cDNA) by RevertAid Premium RT (Thermo Scientific), and the cDNA samples were stored at -70°C until use. Each RT-qPCR mixture (20 μl) contained 10 μl of 2 $\times$ SG Fast qPCR master mix (High Rox) (*Taq* DNA polymerase, SYBR green I dye, MgCl_2 , dNTP mix), 0.4 μl of forward primer (10 μM), 0.4 μl of reverse primer (10 μM), 2 μl of cDNA template (diluted 10 times), and 7.2 μl of double distilled H_2O . RT-qPCR was carried out using a StepOne Plus real-time PCR instrument (ABI). Gene expression was determined from three replicates of a single real-time PCR experiment. The primers used for RT-qPCR are shown in Supplementary Table S3. The expression ratio was recorded as the fold difference in the quantity of real-time PCR products from CK and S-3. The relative abundance for each assayed mRNA versus a constitutively expressed gene (16S rRNA) was determined according to the $2^{-\Delta\Delta\text{Ct}}$ method (Z. Wang et al. 2019a and b).

Metabolomic assay in medium.

The metabolomics of CK, S-3, and J were analyzed in Murashige and Skoog liquid medium. For this assay, 50-ml samples of CK, S-3, and J treatments were collected in 50-ml EP tubes. After the addition of 1,000 μl of extract solvent (acetonitrile-methanol-water, 2:2:1, containing internal standard), the samples were vortexed for 30 s, were homogenized at 45 Hz for 4 min, and were sonicated for 5 min in an ice-water bath. The homogenization and sonication cycle was repeated three times, followed by incubation at -20°C for 1 h and centrifugation at $8,044 \times g$ and 4°C for 15 min. The resulting supernatants were transferred to LC-MS vials and stored at -80°C until ultra-high performance liquid chromatography (UHPLC)-QE Orbitrap/MS analysis. The quality control (QC) sample was prepared by mixing equal aliquots of the supernatants from all the samples. LC-MS/MS analyses were performed using a 1290 UHPLC system (Agilent Technologies) with a UPLC HSS T3 column (2.1×100 mm, 1.8 μm) coupled to a Q Exactive instrument (Orbitrap MS, Thermo). Mobile phase A was 0.1% formic acid in water for positive ion mode and 5 mmol of ammonium acetate per liter in water for negative ion mode; mobile phase B was acetonitrile. The elution gradient was set as follows: 0 min, 1% B; 1 min, 1% B; 8 min, 99% B; 10 min, 99% B; 10.1 min, 1% B; and 12 min, 1% B. The flow rate was 0.5 ml/min. The injection volume was 2 μl . The QE mass spectrometer was used due to its ability to acquire MS/MS spectra on an information-dependent basis during an LC/MS experiment. In this mode, the acquisition software (Xcalibur 4.0.27, Thermo) continuously evaluates the full-scan survey MS data as it collects and triggers the acquisition of MS/MS spectra depending on preselected criteria. Electrospray ionization source conditions were set as follows: sheath gas flow rate, 45 Arb; aux gas flow rate, 15 Arb; capillary temperature, 320°C ; full MS resolution, 70,000; MS/MS resolution, 17,500; collision energy, 20/40/60 eV in the NCE model; spray voltage, 3.8 kV (positive) or -3.1 kV (negative).

MS raw data (.raw) files were converted to the mzML format using Proteo Wizard and were processed by R package XCMS

(version 3.2), including retention time alignment, peak detection, and peak matching. The data were filtered based on the following criterion: the number of samples containing a metabolite was less than 50% of the total number of samples in a group (QC samples were also taken as a group). Normalization to an internal standard (Roberts et al. 2012) was subsequently performed for each sample. Next, missing values were replaced by half of the minimum value found in the dataset by default (Xia et al. 2009). The preprocessing results were used to generate a data matrix that consisted of the retention time, mass-to-charge ratio (m/z), and peak intensity. OSI-SMMS (version 1.0) (Dalian Chem Data Solution Information Technology Co., Ltd.) was applied for peak annotation after data processing with an in-house MS/MS database.

For preliminary visualization of differences between different groups of samples, the unsupervised dimensionality reduction method PCA was applied to all samples using R package models. PCA is a statistical procedure that converts hundreds of thousands of correlated metabolite variables into a set of linearly uncorrelated variables called principal components.

A variable importance in the projection (VIP) score of the (O) PLS model was applied to rank the metabolites that best distinguished the two groups. The VIP threshold was set to 1. In addition, the t test was used in univariate analysis for screening differential metabolites. Metabolites with a P value of t test < 0.05 and $\text{VIP} \geq 1$ were considered differential metabolites between the two groups.

Metabolites were mapped to KEGG metabolic pathways for pathway enrichment analysis, identifying significantly enriched metabolic pathways or signal transduction pathways associated with the differential metabolites compared with the entire background. The calculation formula is as follows:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{m}{i} \binom{N-M}{n-i}}{\binom{N}{n}}$$

where N is the total number of metabolites with KEGG annotations, n is the number of differential metabolites in N , M is the total number of metabolites annotated to specific pathways, and m is the number of differential metabolites in M . The calculated P value was subjected to FDR correction, with $\text{FDR} \leq 0.05$ as a threshold. Pathways meeting this condition were defined as significantly enriched pathways associated with the differential metabolites.

Statistical analyses.

All statistical analyses were performed using SPSS 22.0 (IBM). The differences in the means were determined using Turkey's test, with $P < 0.05$ or $P < 0.01$ ($n = 3$) denoting statistical significance.

AUTHOR-RECOMMENDED INTERNET RESOURCES

edgeR package: <https://www.rdocumentation.org/packages/edgeR>
R package models: <http://www.r-project.org>

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