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🌐 The effects of *Rhodopseudomonas palustris* on the improvement of agronomic traits and key enzyme-coding genes related to polysaccharide biosynthesis in *Codonopsis pilosula*

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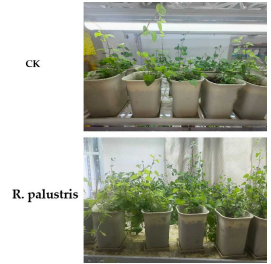
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Abstract

1. Activation and Purification of *Rhodopseudomonas palustris* 2. Soil treatment 3. Germination promotion of *Codonopsis pilosula* 4. Measurement of agronomic traits and polysaccharide content of *Codonopsis pilosula* 5. Transcriptome sequencing of *Codonopsis pilosula* 6. Transcriptome data analysis methods 7. Verification of expression level by qRT-PCR

Materials

	A	B
	UV-1900i UV-Visible Spectrophotometer	Shimadzu Corporation, Japan
	Multifunctional Pulverizer	Yongkang Hongtaiyang Electromechanical Co., Ltd., China
	765 UV-Visible Spectrophotometer	Shanghai Instrument Analysis Instrument Co., Ltd., China
	H1750R Benchtop High-Speed Refrigerated Centrifuge	Hunan Xiangyi Laboratory Instrument Development Co., Ltd., China
	SB-5200DTDN Ultrasonic Cleaner	Ningbo Xinzhi Biotechnology Co., Ltd., China
	HH-S2 Constant Temperature Water Bath	Zhengzhou Great Wall Scientific Industrial and Trade Co., Ltd., China
	SB-1200 EYELA Water Bath Rotary Evaporator	Shanghai Eyela Instrument Co., Ltd., China
	NanoDrop 2000 Spectrophotometer	Thermo Scientific, USA)
	Agilent 2100 Bioanalyzer	Agilent Technologies, Santa Clara, CA, USA

1 Activation and Purification of *Rhodopseudomonas palustris*

- 1.1 *Rhodopseudomonas palustris* was isolated from activated sludge at a wastewater treatment plant and was isolated, identified, and preserved by the Department of Traditional Chinese Medicine, Shanxi Medical University. The research group used a photosynthetic bacteria culture medium (sodium acetate 1640 mg, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 75 mg, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 200 mg, EDTA 20 mg, yeast extract 1000 mg, K_2HPO_4 900 mg, $(\text{NH}_4)_2\text{SO}_4$ 1320 mg, KH_2PO_4 600 mg, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 11.8 mg, trace elements 1 mL, deionized water 1000 mL, pH 6.8–7.2). The bacteria were activated and purified by culturing under the conditions of $28 \pm 2^\circ\text{C}$, light intensity 1200 Lux, and continuous light for 24 hours for 3 days. The viable bacterial count was $\geq 2.0 \times 10^8$ CFU/mL.

2 Soil treatment

- 2.1 The soil used for planting *Codonopsis pilosula* was purchased from Shandong Jiuwu Agricultural Technology Co., Ltd., China. The organic matter content is $\geq 50\%$, and the pH is neutral. Before the experiment, the soil was sterilized in an oven at 120°C for 30 minutes, then cooled to room temperature. Distilled water was used to mix the soil, ensuring it could be "held into a clump, then easily broken apart with a light pinch." The homogenized soil was precisely weighed at 500g and placed into pots with a height of 14 cm, a bottom width of 7.5 cm, and a top width of 10.2 cm, and set aside for use.

3 Germination promotion of *Codonopsis pilosula*

3.1

All experimental instruments used were sterilized. The *Codonopsis pilosula* seeds were wrapped in gauze and placed in distilled water at $50\text{--}55^\circ\text{C}$, then cooled to room temperature. The soaked seeds were placed in Petri dishes and incubated at room temperature (25°C) in the dark. Distilled water was sprayed daily to maintain moisture. Once the seeds had germinated, they were transferred to seedling trays. When the seedlings reached 10 cm in height, uniform seedlings were selected and transplanted into prepared flower pots. Every two weeks, the control group received 10 mL of distilled water for root irrigation and foliar spraying, while the experimental group received 10 mL of *Rhodopseudomonas palustris* for root irrigation and foliar spraying. The *Codonopsis pilosula* was harvested after 90 days as experimental material.

4

Measurement of agronomic traits and polysaccharide content of *Codonopsis pilosula*

4.1 After sampling, a portion of the samples was thoroughly washed with distilled water and the surface moisture was absorbed with blotting paper. A tape measure was used to measure the root length and plant height of *Codonopsis pilosula*. A vernier caliper was used to measure the diameter of the main root, the number of branches on the main stem, leaf length, leaf width, and the diameter of the main stem. The number of leaves, lateral roots, and main stem branches were counted manually. The samples were then dried at 45–50°C, ground, and sieved through a 60-mesh sieve. The polysaccharide content of *Codonopsis pilosula* was determined using the phenol-sulfuric acid method.

5 Transcriptome sequencing of *Codonopsis pilosula*

5.1 The other portion of the samples was washed with nuclease-free water, surface moisture was absorbed with blotting paper, and the samples were wrapped in aluminum foil. These samples were rapidly frozen in liquid nitrogen and stored in a -80°C freezer. Using roots, stems, and leaves from two treatments, with three biological replicates as materials, each tissue was placed in a mortar and ground in liquid nitrogen. Total RNA was extracted using TRIzol reagent. Agarose gel electrophoresis (gel concentration: 1.2%; 0.5×TBE electrophoresis buffer; 150v for 15 minutes) was performed to assess the integrity of the RNA. The purity of the RNA was detected using a spectrophotometer (ThermoScientific, USA) under OD260/OD280 conditions. The VAHTS Universal V6 RNA-seq Library Prep Kit was used to construct the transcriptome libraries. Sequencing was performed on the Illumina Novaseq6000 sequencing platform, generating 150bp paired-end reads. The raw data (raw reads) in fastq format were processed using Trimmomatic[1] to remove reads containing poly-N and low-quality reads, resulting in clean reads. The clean reads were then assembled into expressed sequence tags (contigs), and the transcripts were de novo assembled using Trinity[2] software. Based on sequence similarity and length, the longest Unigene was selected, and the obtained Unigenes were annotated separately in functional databases such as the Non-Redundant Protein Database (NR), Clusters of Orthologous Groups (KOG), Gene Ontology (GO), Swiss-Prot Protein Database (Swiss-Prot), evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Protein Family Database (Pfam).

6 Transcriptome data analysis methods

6.1 The number of reads aligned to Unigenes in each sample was obtained using the software bowtie2[3], and using eXpress[4] software to calculate the FPKM[5] values of Unigenes. DESeq2[6] software was used to calculate the differential multiples and the negative binomial distribution (NB) test was used to determine the significance of the differences. By default, DEGs were screened based on $q < 0.05$ and fold change ≥ 2 , which was considered statistically significant. The spliced Unigenes were used as the

database, and the expression abundance of each Unigene in each sample was identified by sequence similarity alignment. Principal component analysis (PCA) was performed using Unigene expression to investigate the distribution of samples. Based on the hypergeometric distribution, R software was used to analyze the GO enrichment and KEGG pathway enrichment of DEGs, respectively. The DEGs involved in the biosynthesis of Codonopsis polysaccharides were up-regulated after *R. palustris* treatment, and the FPKM values of the key DEGs were analyzed.

7 Verification of expression level by qRT-PCR

- 7.1 Each tissue sample was placed into a mortar and ground in liquid nitrogen. Total RNA was extracted using the RNAQUEOUS KIT, Ambion-1912, and stored in a -80°C freezer for later use. A 0.5 microgram portion of RNA was taken for reverse transcription to synthesize the first strand of cDNA, which was then placed in a -80°C freezer after synthesis was completed. The qRT-PCR primers were designed according to each nucleotide sequence fragment using Premier 5.0 software. The PCR mix was prepared using the 2×PerfectStart™ Green qPCR SuperMix kit and then subjected to 45 cycles of PCR amplification under the following cycling conditions: 94°C for 30 seconds, followed by 94°C for 5 seconds and 60°C for 30 seconds. The reaction was processed on the Roche LightCycler 480 II for detection. The expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method, and the expression amount was calculated accordingly. In this experiment, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference gene to validate the expression levels of key enzyme genes (TRINITY_DN19905_c0_g1_i5_6, TRINITY_DN14015_c0_g1_i3_1, TRINITY_DN22021_c1_g1_i5_1, TRINITY_DN17387_c0_g1_i13_7) from the transcriptome database of *C. pilosula* through quantitative real-time PCR (qRT-PCR) analysis.



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