

Study on Transformation of The Gene of Resveratrol Synthase into *Cordyceps militaris*

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Abstract: Resveratrol synthase (RS) is a key enzyme in Resveratrol (Res) synthesis pathway. RS genes have been transformed and expressed in many plants and microorganisms, and play biological roles in plant metabolism and regulation. In this study, the full length sequence of resveratrol synthase RS gene was transformed into *Cordyceps militaris* by *Agrobacterium tumefaciens* infestation with 1179 bp. The RS gene was integrated into *Cordyceps militaris* genome by PCR, Southern blot hybridization, Northern hybridization and other molecular biological tests. Objective To improve the content of RS gene in into *Cordyceps militaris* by transgenic technology, improve the resistance of *Cordyceps militaris* and promote the content of resveratrol downstream products, provide a theoretical basis for studying the biological function and regulation mechanism of RS gene metabolites, and provide an effective way to improve species, resistance and increase the yield of metabolites.

1 Introduction

Resveratrol (Res) is a natural polyphenol plant antitoxin with significant anti-cancer activity, mainly found in *Polygonum cuspidatum*, grape and *Cordyceps militaris*. Resveratrol has antioxidant, anti proliferation, apoptosis promoting, anti-aging and immune regulatory effects, and is widely used in the treatment of cancer and immune system diseases [1-6]. Resveratrol was first obtained from the root extract of *Veratrum lancifolium*, and has been obtained from more than 70 plants. The results showed that the contents of *Polygonum cuspidatum*, grape was higher. Resveratrol synthase (RS) is a key enzyme that catalyzes the reaction of malonyl coenzyme A and coumarinyl coenzyme A, and the final product of the catalysis is resveratrol [7]. The catalytic reaction of RS enzyme is a series of condensation reactions, and also an important regulatory enzyme affecting resveratrol biosynthesis [8,9]. RS gene is transformed into corresponding plants or microorganisms through gene transformation technology, which has improved the stress resistance of plants or microorganisms and the yield of resveratrol [10]. In 1990, Hain et al first transformed resveratrol synthase RS gene of peanut into tobacco and detected the expression of resveratrol in transgenic tobacco suspension cells [11]. Zheng Shigang and others reported that the disease resistance and yield of RS transgenic crops were relatively improved [12]. Lo et al. measured the contents of three Res related metabolites in the crude extract of *Arabidopsis thaliana* with RS gene of sorghum, namely Res trans hexyl glucoside, diglucoside and cis hexyl glucoside, by liquid chromatography tandem mass

spectrometry (LC/MS/MS). The results showed that the accumulation of cis hexyl glucoside and cis hexyl glucoside in Res was large, and trans hexyl glucoside could not be detected [13]. RS transgenic fruit and vegetable crops, such as apple (*Pyrus malus* L.) and strawberry (*Fragaria vesca* L.), have significantly improved their stress resistance, such as disease resistance and insect resistance. Some researchers have transferred RS gene into Chinese herbal medicine, such as *Salvia miltiorrhiza*. The RS gene transformed *Salvia miltiorrhiza* not only has increased stress resistance, but also has enhanced pharmacological activity. It can be seen that RS gene has a good application prospect in the transformation of plant and microbial functions.

2 Experimental materials and methods

2.1 Experimental materials

2.1.1. Medium for *Cordyceps militaris* culture and RS gene transformation

CYM medium: glucose 20g/L, tryptone 2g/L, yeast extract 2g/L, potassium dihydrogen phosphate 0.46g/L, magnesium sulfate heptahydrate 0.5g/L, potassium hydrogen sulfate 1.0g/L. Screening medium: CYM+0.1 mg/L IBA+2 mg/L PPT+350 mg/L Cb; Fruit body culture medium: 20g rice, 15g sorghum rice, 30mL sterile water.

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2.1.2. Strain and expression vector

Agrobacterium tumefaciens, LBA4404(Rif R, SmR), expression vector pCAMBIA3300, RS gene recombinant vector pCAMBIA3300-RJ39-RS-Tnos were constructed and preserved by the State Key Laboratory of Plant Genetic Engineering and Protein Engineering, Academy of Life Sciences, Peking University.

2.1.3. Primers, enzymes and reagents

Primers for RS gene amplification: RSF: 5'-CGGGATCCGCCATGGCTTCAGTTGAGAAATTTA G-3'; RSR: 5'-GTGAGCTCGAAGGGTAAACCATTCTCTTTTAT-3', synthesized by Shanghai Sangong Biotechnology Co., Ltd; T4 DNA ligase, Taq DNA polymerase, DNA restriction endonuclease, pMD18T carrier, dNTPs, gel recovery kit, etc. were purchased from Takara Company (Japan); Kanamycin, streptomycin and rifampicin were equally purchased from Beijing Dingguo Biotechnology Co., Ltd; Sequencing was completed by Shanghai Bioengineering Co., Ltd.

2.1.4. *Cordyceps militaris*

Isolation and identification of *Cordyceps militaris* species from Changbai Mountain wild fungi.

2.2. Test method

2.2.1. Isolation and identification of *Cordyceps militaris* fungi

2.2.1.1. Separation and purification of *Cordyceps militaris*

Wipe and disinfect the wild *cordyceps militaris* collected from Changbai Mountain with 75% alcohol, pick up the spores on the fruiting bodies, inoculate them on the CYM medium, pick out the robust mycelia, inoculate them on the CYM solid medium, and conduct subculture for many times until the purified mycelia of cordyceps fungi are obtained.

2.2.1.2. Genomic DNA extraction of *Cordyceps militaris* fungi

Take *Cordyceps* mycelium, inoculate it on CYM liquid medium, place it in a constant temperature shaker at 28°C, shake it at 150r/min for 5 to 7 days, and obtain a single mycelium of *Cordyceps* fungi. The fungal genomic DNA extraction kit was used to extract the DNA of *Cordyceps* mycelia.

2.2.1.3. Molecular biological identification of mycelium DNA

PCR reaction system (50μL) : Mycelial DNA 2μL; ITS 3'Primer 2μL; ITS 5' primer 2μL; TagE 25μL;

ddH₂O 19μL. PCR reaction conditions: 94°C pre denaturation for 4 min; Denaturation at 94°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 1.5 min, 30 cycles; Extend at 72°C for 10 min and keep at 4°C for 1 min. PCR product electrophoresis, gel recovery of electrophoresis products, connection of gel recovery products, transformation of *Escherichia coli*, and PCR detection of bacterial fluid; Send the PCR amplified positive clone of the bacterial solution to the Sequencing Department of Bioengineering (Beijing) Co., Ltd. for sequencing.

2.2.2. *Agrobacterium* mediated transformation

The plasmid pCAMBIA3300-RJ39-RS-Tnos was transformed into the competent state of *Agrobacterium tumefaciens* EHA104 by liquid nitrogen freeze-thaw method (liquid nitrogen freeze-thaw for 3min, and maintained at 37°C for 5min). The genetic transformation of *Cordyceps militaris* was carried out by *agrobacterium* co culture method. The positive single colony was selected and inoculated in CYM liquid medium, and the culture was oscillatory at 28°C for 3 days to the logarithmic growth period.

2.2.3. Identification and detection of RS transgenic *Cordyceps sinensis*

2.2.3.1. PCR detection of RS transgenic *Cordyceps militaris*

The mycelia of RS transgenic *Cordyceps militaris* and non RS transgenic *Cordyceps militaris* were selected, and the genomic DNA of transformants and non transformants was extracted by CTAB method. The PCR reaction system (20μL) Is: template DNA 1μL; Primer RSF1μL; Primer RSR1μL; ten×buffer 2μL; dNTPs (2.5 mmol/L) 2μL; Taq DNA polymerase (10000 U/mL) 0.2μL. The reaction conditions were: pre denaturation at 94°C for 4 min; 94°C for 30 s, 50°C for 1 min for 30 s, 72°C for 1 min for 30 s, 30 cycles; Then 72°C for 10min. PCR products were electrophoretic on 0.7% agarose gel, and the size of the target gene was observed by gel imager.

2.2.3.2. Detection of RS transgenic *Cordyceps militaris* by Southern blot hybridization

CTAB (cetyltrimethylammonium bromide) method was used to extract the total DNA of *Cordyceps militaris*, PCR, membrane transfer, preparation of probes, hybridization and detection [14].

2.2.3.3. Northern hybridization test of RS transgenic *Cordyceps militaris*

Trizol method was used to extract total RNA from young leaves of transgenic and non transgenic *Cordyceps militaris*, electrophoresis, membrane transfer, probe preparation, hybridization and detection [14].

3 Results & Discussion

3.1 PCR identification of transgenic *Cordyceps militaris*

Resveratrol synthetase RS gene was cloned from *Parthenocissus tricuspidata* from the State Key Laboratory of Plant Genetic Engineering and Protein Engineering, Academy of Life Sciences, Peking University. The gene sequence is 1179bp long and encodes 392 amino acids. The plant expression vector pCAMBIA3300-RJ39-Tnos was constructed with strawberry fruit specific promoter RJ39 (Fig. 1).

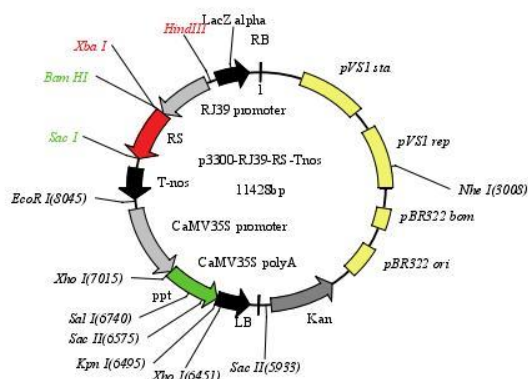


Fig. 1 ExpReSSION vector pCAMBIA3300-RJ39-RS-Tnos

The plant expression vector pCAMBIA3300-RJ39-RS-Tnos was transformed into *Agrobacterium tumefaciens* LBA4404. The marker gene was selected as herbicide resistance gene (bar). Through *Agrobacterium tumefaciens* mediated *Cordyceps militaris*, 50 resistant strains were obtained. Genomic DNA was extracted, and PCR amplification was performed with primers RSFP and RSRP. Electrophoresis results showed that 15 strains were positive, with a conversion rate of 30%. The PCR amplification band size of RS gene positive *Cordyceps militaris* was about 1.1kb, while the negative control did not. The PCR results of some RS transgenic *Cordyceps militaris* plants are shown in Figure 2.

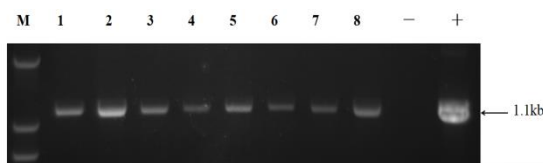


Fig. 2 PCR identification of transgenic pCAMBIA3300-RJ39-RS-TNOS *Cordyceps militaris*. 1-8: Positive Result of PCR identification of transgenic *Cordyceps militaris*. -: DNA of wild-type *Cordyceps militaris* was the negative control template.+: Plasmid pCAMBIA3300-RJ39-RS-Tnos was the positive control of the template. M: DL2000marker.

3.2 Southern blot hybridization detection of transgenic *Cordyceps militaris*

Seven of the 15 RS positive strains were randomly selected for Southern blot hybridization. The total DNA of *Cordyceps militaris* was extracted by CTAB method, PCR, membrane transfer, probe preparation, hybridization and detection. Results Six strains were all transgenic *Cordyceps militaris* strains, and one was RS negative strain (Fig. 3).

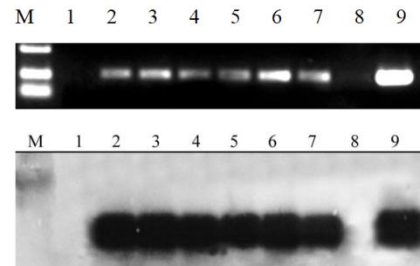


Fig. 3 PCR amplification and PCR-Southern hybridization Results of RS transgenic *Cordyceps militaris*. 1: RS-negative; 2-7: RS positive; 8: negative control (non-gm materials); 9: positive control; M: DL2000marker.

3.3. Northern hybridization detection of transgenic *Cordyceps militaris*

The total RNA of RS transgenic *Cordyceps militaris* with positive PCR identification was extracted and transferred to the Hybond N+membrane by formaldehyde denaturation electrophoresis. The RS gene was hybridized with digoxin labeled full-length RS gene fragment as probe, and the transcriptional level of RS gene in transgenic *Cordyceps militaris* was detected by Northern hybridization. Northern blotting analysis results showed that the exogenous RS gene could be normally transcribed in the transgenic *Cordyceps militaris* mycelia, while non transgenic materials did not have hybridization signals (Fig. 4).

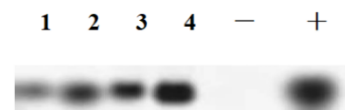


Fig. 4 Northern Blotting analysis Results. 1-4: RS positive; -: negative control (non-gm material) ; +: positive control.

4 Conclusions

Resveratrol (Res for short), a secondary metabolite of plants, is a kind of plant protectant, which has a defensive effect on pathogenic bacteria. Studies have found that Res has anti-cancer, anti-inflammatory, cardiovascular disease prevention and other health functions, but the content of Res in plants is very low. The biosynthetic mechanism of Res has been clarified, so it is possible to use microorganisms to express Res heterologously. Res biosynthesis is catalyzed by phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-coumaric acid: coenzyme A ligase (4CL) and resveratrol synthase (RS). Resveratrol

synthase is the most critical enzyme in Res biosynthesis, which is responsible for catalyzing the production of Res from 1 molecule coumarinyl coenzyme A and 3 molecules malonyl coenzyme A [15]. In this study, the nucleotide sequence of resveratrol synthase RS gene cloned from *Parthenocissus tricuspidata* is 1179 bp long and encodes 392 amino acids [16]. The homology between this gene and the resveratrol synthase gene of *Cordyceps militaris* FM955393 registered in GenBank is 84.41%. Since promoters play an important role in regulating gene expression and physiological and biochemical characteristics of receptor plants, this experiment used strawberry fruit specific promoter RJ39 to construct pCAMBIA3300-RJ39-RS-Tnos plant expression vector and transform *Cordyceps militaris*. After screening on selected culture medium, 18 transformants were verified and 6 positive transformants were obtained through PCR, Southern and Southern hybridization. The RS gene expression of *Parthenocissus tricuspidata* was obtained from the fruiting body of *Cordyceps militaris*, which improved the economic value of *Cordyceps militaris*. In recent years, great progress has been made in the research on the function, development and utilization of resveratrol. With the development of sustainable agriculture and the continuous demand of human for drugs and health products, it is urgent to use plant genetic engineering technology to increase the yield of resveratrol. With the continuous cloning and improvement of resveratrol synthase RS gene, and the inheritance improvement of crops, drugs and microorganisms and the cultivation of new varieties by using RS gene are also in order, but how RS gene participates in stress response and its mechanism of action need to be further studied. In a word, the demand of Res in food, health care, medicine and other industries can only be met by transferring resveratrol synthase RS gene to improve the nutritional added value of receptor plants and the production of Res in recombinant microorganisms.

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