Overexpression of Ribonucleotide Reductase Small Subunit, RNRM, Increases Cordycepin Biosynthesis in Transformed Cordyceps militaris

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[ABSTRACT] Cordycepin was the first adenosine analogue used as an anticancer and antiviral agent, which is extracted from Cordyceps militaris and hasn’t been biosynthesized until now. This study was first conducted to verify the role of ribonucleotide reductases (RNRs, the two RNR subunits, RNRL and RNRM) in the biosynthesis of cordycepin by over expressing RNRs genes in transformed C. militaris. Quantitative real-time PCR (qRT-PCR) and western blotting results showed that the mRNA and protein levels of RNR subunit genes were significantly upregulated in transformant C. militaris strains compared to the control strain. The results of the HPLC assay indicated that the cordycepin was significantly higher in the C. militaris transformants carrying RNRM than in the wild-type strain, whereas the RNRML was preferentially downregulated. For the C. militaris transformant carrying RNRL, the content of cordycepin wasn’t remarkably changed. Furthermore, we revealed that inhibiting RNRs with Triapine (3-AP) almost abrogated the up-regulation of cordycepin. Therefore, our results suggested that RNRM can probably directly participate in cordycepin biosynthesis by hydrolyzing adenosine, which is useful for improving cordycepin synthesis and helps to satisfy the commercial demand of cordycepin in the field of medicine.

(KEY WORDS) cordycepin biosynthesis; ribonucleotide reductases gene; Agrobacterium tumefaciens-mediated transfection

Introduction

Cordycepin, 3′-deoxyadenosine (3′-dDNA), is an analogue of 2′-dDNA, which was first isolated from the culture filtrate of Cordyceps militaris by Cunningham et al. in 1950. It is also the first nucleoside analogue used as a chemotherapeutic agent for cancer treatment [1-2]. Cordycepin is also an active component of traditional Chinese medicine that is reputed to alleviate a large variety of ailments [3]. Many studies have found that cordycepin has various bioactivities, including antitumor, antiviral, antioxidant, and anti-inflammatory activities, with broad therapeutic potential in immunological, hepatic, renal and cardiovascular systems and as a clinical anti-fungal agent [4-9]. In addition, much effort was made to obtain cordycepin mainly from the fruiting body or mycelia of C. militaris and other Cordyceps spp [8]. However, due to its medicinal usage and the miniscule amount in these natural resources (< approximately 2.54 mg/g), the market price of cordycepin has increased up to approximately $12 000 kg⁻¹ [7].

Adenosine has been demonstrated to be the direct precursor of cordycepin biosynthesis, indicating that cordycepin might be processed by the reduction reaction starting from adenosine [8-9]. However, the biosynthetic mechanism of cordycepin is not well understood. With transcriptome sequencing technology, the biosynthesis pathway and putative genes involved in cordycepin synthesis were predicted in Ophiocordyceps sinensis and C. militaris [10], including ribonucleotide reductases (RNRs), adenosine kinase, adenylyl kinase, 5′-nucleotidase, etc [11-12]. Among them, RNRs are key and necessary enzymes for the hydrolysis of adenosine di-
phosphate (ADP) to deoxyadenosine diphosphate (dADP) in the 2'-deoxyadenosine metabolic pathway. It was also the only reductase for adenosine in *C. militaris* [13]. However, whether RNRs are involved in the biosynthesis of cordycepin has not been confirmed yet. Taken together, we proposed the hypothesis that the adenosine precursor was used in the synthesis of cordycepin through a reduction reaction and that RNRs might act as the potential reduction agent.

Moreover, *C. militaris* is a highly medicinal fungus due to its generation of various metabolites, particularly cordycepin. Owing to its medical properties being similar to those of *O. sinensis*, which is not readily available and costly, *C. militaris* is widely used as a substitute for *O. sinensis*. Today, it is widely used as a substitute for *O. sinensis*. To determine the sensitivity of *C. militaris*, the 35S1 promoter fragment was amplified from *C. militaris* strain JM4 mediated by A. tumefaciens AGL-1 was successfully constructed with high efficiency.

To examine the role of RNRs in the cordycepin biosynthesis mechanism, two genes encoding the cDNA of RNR subunits (RNR small subunit, RNRM; RNR large subunit, RNRL) were transformed separately or together into *C. militaris* and overexpressed in the transformants. Meanwhile, a certain concentration of 3AP, an inhibitor of ribonucleotide reductase, was used to treat *C. militaris* transformants and the wild-type strain. Furthermore, the cordycepin concentration was examined by HPLC. Our study provides a new resource for cordycepin biosynthesis, which is established for breeding the high-producing cordycepin strain.

**Methods and materials**

**Materials and Strains**

Fresh *C. militaris* (CICC14015) was provided by Sichuan Jiuyuan Biotechnology Co., Ltd. (Sichuan, China). *Escherichia coli* strain DH5α (Beijing, China) was used for the propagation of plasmid DNA, The *Agrobacterium tumefaciens* strain GV3101 (Beijing, China), pCAMBIA1300 containing a hygromycin B resistance gene (*Hyg B*) and T-DNA containing the kanamycin resistance gene were provided by Prof. Songhu Wang (Chengdu Institute of Biology of Chinese Academy of Sciences, Chengdu, China).

**RNA isolation, construction of recombinants**

Total RNA was extracted from *C. militaris* by using Trizol reagent (Ambion) according to the manufacturer's protocol. Using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara), total RNA was used as a template to reverse transcribe into cDNA. Based on the CDS sequences of RNRL (GenBank Accession Number: 573978269) and RNRM (GenBank Accession Number: 573986094), primer sequences (F/R) were introduced with *Xba* I and *Hind* III sites and *Kpn* I and *Sma* I-*Xba* I sites, respectively (Table 1). In addition, for the expression analysis, the FLAG tag (sequence: 5′-GAT-TACAAGGATGACCAAGCATAAG-3′) was added to the 3′ terminus of the RNRL and RNRM cDNA.

To effect the expression of RNRL and RNRM in *C. militaris*, the 35S1 promoter fragment was amplified from pCAMBIA2300 as a template with specific primers F/R introduced sites (*Eco* R I and *Kpn* I) and then fused into the pCAMBIA1300 vector to construct the pCAMBIA1300:35S1 expressing vector. The cDNA fragments of RNRL and RNRM were digested and fused into the pCAMBIA1300:35S1 vector to generate the pCAMBIA1300:RNRL and pCAMBIA1300:RNRL constructs, respectively. 35S2 was also amplified from pCAMBIA2300 as a template with specific primers F/R introduced *Sma* I and *Xba* I sites and fused into the 3′ terminus of RNRL in the RNRL construct, and then RNRL was digested and inserted into it generate pCAMBIA1300:RNRL constructs. These constructs were confirmed by sequencing and used for the transformation of *C. militaris*.

**Transformation of Cordyceps militaris by ATMT**

The conidiospores were gently washed from the fresh fruiting body of *C. militaris* with sterile ddH2O, and the conidial number was calculated hematocyte counter. To determine the sensitivity of *C. militaris* to hyg B, the conidiospores of *C. militaris* grown on PDA with different concentrations of hyg B (0, 200, 400, 600, 800 and 1200 µg/ml) were assayed.

The recombinants harboring RNRL, RNRM and

<table>
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<th>Gene name</th>
<th>Sequence</th>
<th>GenBank accession number</th>
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<tr>
<td>35S1-F</td>
<td>5′-CGGAATTCACGATGATGCCGTACCCCTACTCCA-3′</td>
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<td>5′-CGGGTGACCCGATCTAGCTTGCGTCTC-3′</td>
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<td>5′-CGGGTGACCCGATCTAGCTTGCTCCTCGTAATCCGCTGACATGACG-3′</td>
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<td>RNRM-R</td>
<td>5′-CTCTAGATCCCCCGGGGAGTACCTCTGTGATCGTACCCCTACTCCA-3′</td>
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Table 1: Names and sequences of primers for 35S and RNR

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RNRML plasmids were transformed into *C. militaris* conidiospores by ATMT according to a previous study \[17\]. To change the medium more conveniently, we coated with filter paper on induction medium (IM medium) and CO-IM medium \[18\]. Then, the filter paper was transferred to a new selective PDA solid resistant plate containing 800 μg/ml hyg B 800, 100 μg/ml cephalosporin C380, 60 μg/ml streptomycin sulfate and 60 μg/ml penicillin and incubated for 2- to 5-d at 23 °C. Following such culture conditions, individually positive *C. militaris* transformants were screened and transferred to a new PDA-resistant plate for incubation at 23°C for 7 d, shaken at 200 rpm. All experiments were independently repeated in triplicate.

Approximately 3 individual transformants from each PDA-resistant plate were transferred to a flask containing 6 ml LB liquid medium (containing 50 μg/ml kan, 25 μg/ml rif, and 50 μg/ml gent). The flask was incubated at 28°C on a 200 rpm shaker until the OD 600 was up to 1.5. Then, the transformants were collected by centrifugation, and the OD 600 was adjusted to 0.15 - 0.3. Finally, the transformants were incubated at 28°C in IM medium, shaking at 200 rpm until the OD 600 reached 8.0.

### The analysis of the transformants

Approximately 3 individual transformants were selected from the three Hyg B-resistant plates and incubated in 100 ml liquid PDA medium at 23°C for 7 d. Genomic DNA for PCR detection was extracted from approximately 100 mg *C. militaris* hyphae and 30 s and 72°C for 1 min, and 72°C for 5 min. The PCR reactions were repeated in triplicate.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Amplification Length</th>
<th>T_M Range(°C)</th>
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<tr>
<td>qRNRL-F</td>
<td>5'-CGAAGTCCTGCTAACAACATGGCAGCG-3'</td>
<td>234 bp</td>
<td>63°C</td>
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<tr>
<td>qRNRL-R</td>
<td>5'-TTGTCTCTGACTGCCCCTCAACC-3'</td>
<td>228 bp</td>
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<td>qRNRM-F</td>
<td>5'-GAAGACCCGGTGCTCCAGAAAAGG-3'</td>
<td>246 bp</td>
<td>63°C</td>
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<td>5'-AGAAGTCCTGCTTGCGCCCGC-3'</td>
<td>234 bp</td>
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<td>β-actin-F</td>
<td>5'-ATATCTTTATCTCCTCCCTTAC-3'</td>
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<td>β-actin-R</td>
<td>5'-GGCGTGCGGGAAGGCAGAAG-3'</td>
<td>246 bp</td>
<td>61°C</td>
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Table 2  Quantitative real-time PCR primers for RNRL, RNRM and β-actin

Illumina. T_M = Melting temperature

**qRT-PCR analysis for gene expression in transformants**

All RNA samples were extracted separately from the positive transformants and control cordyceps strains with Tri-zol reagent (Ambion) and then treated with RT reagent Kit with gDNA Eraser (Takara) to generate cDNA. qRT-PCR was performed using a CFX Connect™ Optics Module instrument (BIORAD) and carried out with 2 × Ultra SYBR Mixture (CWBO) according to the manufacturer’s instructions. Each sample was repeated three times. The gene-specific and β-actin primers (housekeeping gene) are listed in Table 2. The mRNA expression level was calculated with 2^−ΔΔCt and normalized to that of β-actin \[20\]. No significant differences in β-actin levels were examined between different groups, and all experiments were independently repeated in triplicate.

**Western blot analysis of gene expression in transformants**

Three individual transformants (RNRM, RNRL and RNRML) were ground by liquid nitrogen. Approximately 100 mg samples were added to a 100 μL 2×SDS PAGE loading buffer and boiled at 100°C for 10 min and then centrifuged at 12000 rpm for 5 min. After dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 25 μl. supernatant, blocking, incubation and washing were performed \[21-22\]. Then, membranes were incubated with horseradish peroxidase (FLAG)-conjugated goat anti-rabbit IgG (Trans-Gen, Beijing, China; diluted 1:2000 with 5% nonfat milk in TBST) for 1 h at room temperature. Finally, the protein bands were detected after adding Pierce™ ECL western blotting substrate. All experiments were independently repeated in triplicate.

**Analysis of cordycepin content by HPLC**

Approximately 3 individual *C. militaris* transformants were cultured in PDA liquid medium containing 800 μg/ml Hyg B for different time courses (5, 10 and 15 d). For inhibitor experiments, three concentration gradients (0, 25, and 50 μM/ml) were added to *C. militaris* medium. The transformant and wild-type strains were incubated with a selected reliable RNR inhibitor, triapine (3-AP, 25 μM/ml) for 10 d, respectively. Then, the *C. militaris* hyphae were collected and
dried at approximately 65°C for 8 h.

The dried samples were ground into a powder, followed by ultrasonic extraction at 37°C and 100 W for 30 min. Samples were filtered through a 0.22 μM syringe filter (Mollex, Merck Millipore Ltd. Ireland). HPLC analysis was performed with a ZORBAX Eclipse Plus C-18 Column (250×4.6 mm, 5 μm, Shiseido Nanospace G4288C, LC-20A, Japan). The standard for cordycepin was purchased from the China Food and Drug Administration Institute (Batch number: 110858-201503) and injected at five sample volumes of 8-80 μg/ml to draw the calibration curve. The standards or samples were separated using a gradient mobile phase consisting of ultrapure water (A) and methyl alcohol (B). The gradient elution program was as follows: 0 ~ 20 min, 5 ~ 10% B; 20 ~ 25 min, 10 ~ 40% B; and 25 ~ 30 min, 40% B, and the flow rate was 1 ml/min at 25°C [23-24]. A standard curve was prepared, and the linear regression equation is shown below: 

\[ Y = 127287x - 157188, \quad R^2 = 0.9992 \quad (n=3) \]

For each sample, the injection volume was 20 μL, and the absorption of the extracts was detected at a wavelength of 260 nm. At least 3 independent measurements were performed.

**Results**

**cDNA Clone, Recombinant construction, and C. militaris Transformation**

The constructs (Fig. 1) were confirmed by sequencing and were transformed into C. militaris conidia. A previous study successfully established the C. militaris genetic transformation system mediated by ATMT [18-20]. According to the optimized C. militaris transformation procedure in a previous study, the transformation efficiency was in the range of 30-600 transformants per 1×10^5 conidia after being cocultivated for 5 d. The sensitivity assay of hygromycin B showed that the optimal inhibitory concentration of hygromycin B on the growth of C. militaris was 800 μg/ml (Fig. 2), which was used for selecting C. militaris transformants in this study. The inhibitory concentration of Hyg B is inconsistent with a previous study, probably due to the difference in the C. militaris

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**Fig. 1**  A: Schematic map of the inserted T-DNA in pCAMBIA1300. LB: Left border; RB, right border; Poly, CaMV 35S poly-A terminator; 35S, CaMV 35S promoter from cauliflower mosaic virus; Hyg R: Hygromycin resistance gene; RNRM: ribonucleoside diphosphate reductase small subunit; RNRL: ribonucleoside diphosphate reductase large subunit; 35S1, 35S promoter cloned from pCAMBIA2300, with the two restriction sites EcoR I and Kpn I; 35S2, 35S promoter cloned from pCAMBIA2300, with the two restriction sites Smal I and Xba I. B: Gel electrophoresis map of 35S1 + RNRM bacteria liquid PCR. C: Gel electrophoresis map of 35S1 + RNRM bacteria liquid PCR.
strain type. After selecting for 5 d, 10 individual positive transformants were screened and cultured in 10 mL PDA liquid medium (200 rpm, 23°C). After culturing for 7 d, the C. militaris strains were stored at −80°C.

Expression analysis of exogenous RNRs in C. militaris transformants

To examine the expression of exogenous RNR genes in C. militaris, qRT-PCR and western blotting were used to detect the mRNA and protein levels of the RNR genes in the transformant strains. The results showed that the mRNA levels of RNRM in the RNRM and RNRML transformant spores increased by 2.93-fold and 2.40-fold (p < 0.01) compared to that of the control, respectively (Fig. 3A). In addition, RNRL mRNA levels also increased by 1.95-fold and 3.07-fold (p < 0.01) in these transformants. Moreover, the protein levels of RNRL and RNRM ligated with FLAG tags were also detected in the transformants by western blotting (Fig. 3B). Therefore, two subunits of the exogenous RNRs were successfully transfected into C. militaris spores.

Cordycepin content assay in C. militaris by HPLC

Using HPLC, cordycepin contents in transgenic C. militaris cultivation for 5, 10 and 15 d were assayed. The results (Fig. 4A) indicated that the content of cordycepin was significantly upregulated approximately 1.6-fold (p < 0.01) in the RNRM transgenic strain compared to the control. For the RNRL transgenic strains, cordycepin content was nearly consistent with that in the control. However, the production of cordycepin decreased by 56% (p < 0.001) compared to the control when cotransfected the two subunits into C. militaris conidiospores. In addition, cordycepin content in the transgenic and control strains treated with 25 μM/ml 3-AP for 5-d was assayed. The results showed that the cordycepin content was reduced approximately 1.8-fold to 2.7-fold in the treated group (p < 0.001) compared to the untreated group (Fig. 4B). In addition, cordycepin synthesis was also weakened in the presence of 3AP in the medium. Our results found that the cordycepin content in the transformants was significantly reduced by approximately 38% to 53% (p < 0.001) compared to wild type (Fig. 4B). The content of 3-AP was determined by
Discussion

A previous study found that the RNRs of *C. militaris* belong to the type 1a protein with α2β2 subunit structures, and sequences are highly conserved [29]. Moreover, sequence analysis revealed that the main catalytically active region of the enzyme was located in the RNRL subunit, not in RNRM. However, the RNRM subunit contained a conserved iron-regulatory site. The large RNRL subunit has a site binding to the substrate and the allosteric effector. The small RNRM subunit is a homodimer with two identical ferrous iron centers and stabilizes a tyrosyl radical, which plays an important role in triggering electron transport during the catalytic process. In addition, the RNR enzyme has substrate specificity that binds to the four NDP substrates by binding with different effectors to alter enzyme activity and substrate specificity [28]. While the human RNR consists of a large subunit RNRML and a small subunit RNRMZ or RNRMZB, the two RNRs and their three subunits have different bioactivities in different stages of tumor onset [27-28]. The above studies indicated that different RNR types could exert different biological effects and that different subunits also have different bioactivities. Therefore, our studies suggested that RNRM might directly regulate cordycepin biosynthesis.

According to most previous studies, adenosine would probably be the coprecursor of cordycepin and 2′-doxyadenosine, suggesting that there is mutual antagonism between the synthesis pathway of cordycepin and that of 2′-doxyadenosine (for the synthesis pathway between cordycepin and 2′-doxyadenosine) [10]. When RNRM and RNRL coexisted, they tended to form RNRs, which demonstrated the reduction of adenosine diphosphate (ADP) to deoxyadenosine diphosphate (dADP) in the 2′-doxyadenosine metabolic pathway. As a result, the coprecursor adenosine was consumed so that the synthesis of cordycepin was negatively influenced in the process. Hence, the cordycepin content was instead reduced when *C. militaris* was transfected with the two subunits together. Triapine (3-AP), a selective reliable RNR inhibitor, can inhibit the deoxygenation process of nucleotides to interfere with DNA synthesis. At present, it is mostly used for anti-tumor cells when the concentration reaches 25 μM, leading to significant regulation [29]. As almost no reports about 3-AP apply to fungi and considering that the highest concentrations of the inhibitor may affect the growth of *C. militaris*, 25 μM/ml concentrations have been determined in this experiment. Taken together, RNRM might directly regulate cordycepin biosynthesis via the reduction of adenosine. The putative roles of RNRs in the predicted synthesis mechanism of cordycepin are illustrated in Fig. 5.

In conclusion, this study was conducted to verify the roles of RNR genes in the cordycepin biosynthesis mechanism. Based on our results and previous studies, we proposed that adenosine would be the coprecursor of cordycepin and 2′-doxyadenosine and that there is mutual antagonism for the synthesis pathway between cordycepin and 2′-doxyadenosine. The RNRL consists of the large subunit (RNRLML) and the small subunit (RNRL), and RNRL and RNRL might tightly positively regulate cordycepin biosynthesis. Further studies would be performed to excavate more regulators in the biosynthesis pathway of cordycepin and reveal its mechanism. Here, we speculate that the overexpression of RNRL is a promising genetic engineering strategy to improve the biosynthesis of cordycepin in *C. militaris*.

Founding

This study was supported by the Natural Sciences Foundation of China Science (81872959, 81373920, 30801522), Sichuan Province Youth Innovation Team Fund (19CXTD0055), China Scholarship Fund (201708570027).
Fig. 5  Model illustrating the biosynthesis pathway of cordycepin. ADEK: adenylate kinase. ADK: adenosine kinase. 5′-NT: 5′-nucleotidase.

References


