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. 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

[CLC Number] Q812

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 anticancer, antiviral, antioxidant, and anti-inflammatory activities, with broad therapeutic potential in immunological, hepatic, renal and cardiovascular systems and as a clinical anti-fungal agent [4-5]. In addition, much effort was made to obtain cordycepin mainly from the fruiting body or mycelia of C. militaris and other Cordyceps spp. [6]. However, due to its medicinal usage and the miniscule amount in these natural resources (< approximately 2.54 mg·g⁻¹), 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 [10-12]. Among them, RNRs are key and necessary enzymes for the hydrolysis of adenosine diphosphate (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*. *C. militaris* has been established in different artificial media [14]. *Agrobacterium tumefaciens*-mediated transformation (ATMT) is a highly efficient transformation method and is widely used in entomopathogenic fungi [13]. Recently, the transformation of *C. militaris* JM4 mediated by *A. tumefaciens* AGL-1 was successfully constructed with high efficiency [14].

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 *C. militaris* 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*-mediated transformation (ATMT) is a highly efficient transformation method and is widely used in entomopathogenic fungi [13]. Recently, the transformation of *C. militaris* JM4 mediated by *A. tumefaciens* AGL-1 was successfully constructed with high efficiency [14].

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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 [19]. Then, the filter paper was transferred to a new selective PDA solid resistant plate containing 800 μg mL⁻¹ hyg B 800, 100 μg mL⁻¹ cephaleosporin C380, 60 μg mL⁻¹ streptomycin sulfate and 60 μg mL⁻¹ 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 r min⁻¹. 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⁻¹ kan, 25 μg mL⁻¹ rif, and 50 μg mL⁻¹ gent). The flask was incubated at 28 °C on a 200 r min⁻¹ shaker until the OD₆₀₀ was up to 1.5. Then, the transformants were collected by centrifugation, and the OD₆₀₀ was adjusted to 0.15–0.3. Finally, the transformants were incubated at 28 °C in IM medium, shaking at 200 rpm until the OD₆₀₀ reached 0.8.

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 from the three Hyg B-resistant plates and incubated in 100 ml LB liquid medium (containing 50 μg mL⁻¹ rif, and 50 μg mL⁻¹ kan, 25 μg mL⁻¹ gent). The flask was incubated at 12000 r·min⁻¹ 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 (TransGen, 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⁻¹ Hyg B for different time courses (5, 10 and 15 d). For inhibitor experiments, three concentration gradients (0, 25, and 50 μmol mL⁻¹) were added to C. militaris medium. The transformant and wild-type strains were incubated with a selected reliable RNR inhibitor, triapine (3-AP, 25 μmol mL⁻¹) for 10 d, respectively. Then, the C. militaris hyphae were collected and dried at approximately 65 °C for 8 h.

**Table 2** Quantitative real-time PCR primers for RNRL, RNRM and β-actin

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Amplification Length</th>
<th>Tₘ Range (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>qRNRL-F</td>
<td>5'-CGACCTCGTCAACACTAGCGACG-3'</td>
<td>234 bp</td>
<td>63</td>
</tr>
<tr>
<td>qRNRL-R</td>
<td>5'-TTGTCTTTCTGACTGGCCTCAACC-3'</td>
<td></td>
<td>63</td>
</tr>
<tr>
<td>qRNRM-F</td>
<td>5'-TGAAGAACCGTGCCACCTATAGC-3'</td>
<td>246 bp</td>
<td>63</td>
</tr>
<tr>
<td>qRNRM-R</td>
<td>5'-AGAAGTTCTGCTTGCCGCCCAG-3'</td>
<td></td>
<td>63</td>
</tr>
<tr>
<td>β-actin-F</td>
<td>5'-TATTTACCTCTCCGACCTC-3'</td>
<td>208 bp</td>
<td>62</td>
</tr>
<tr>
<td>β-actin-R</td>
<td>5'-GGCGTGGGGAAGGCGAACC-3'</td>
<td></td>
<td>61</td>
</tr>
</tbody>
</table>

Illumina. Tₘ = Melting temperature
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 μmol·L$^{-1}$ syringe filter (Mollex, Merck Millipore Ltd. Ireland). HPLC analysis was performed with a ZORBAX Eclipse Plus C-18 Column (250 mm × 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$^{-1}$ 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$^{-1}$ at 25 °C [23-24].

A standard curve was prepared, and the linear regression equation is shown below: $Y = 127287x - 157188, R^2 = 0.9992 \ (n = 3)$. 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 co-cultivated 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$^{-1}$ (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* strain type. After selecting for 5 d, 10 individual

![Fig. 1](image-url)
positive transformants were screened and cultured in 10 mL PDA liquid medium (200 r·min\(^{-1}\), 23 °C). After culturing for 7 d, the \textit{C. militaris} strains were stored at −80 °C.

**Expression analysis of exogenous RNRs in \textit{C. militaris} transformants**

To examine the expression of exogenous RNR genes in \textit{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 \textit{C. militaris} spores.

**Cordycepin content assay in \textit{C. militaris} by HPLC**

Using HPLC, cordycepin contents in transgenic \textit{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 \textit{C. militaris} conidiospores. In addition, cordycepin content in the transgenic and control strains treated with 25 μm·mL\(^{-1}\) 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...
**Fig. 4** Cordycepin content analysis in different transformants (RNRM, RNRL and RNRML) A: incubated for 5, 10 and 15 d. B: incubated with 3-AP treated for 10 d. Control: *C. militaris* strain untransformed with foreign genes; RNRL, *C. militaris* strain transformed with pCAMBIA-35S1-RNRL; RNRL, *C. militaris* strain transformed with pCAMBIA-35S1; RNRML, *C. militaris* strain transformed with pCAMBIA-35S1-RNRML-3-AP treated: *C. militaris* samples were treated with 3-AP (25 μmol·mL⁻¹); 3 AP- untreated: *C. militaris* samples were untreated with 3-AP. All data are means of 5 replicates with error bars indicating SD. *' is significantly different compared to control at ‘P < 0.01’, **'is significantly different compared to control at ‘P < 0.001’ by one-way ANOVA test.

**Discussion**

A previous study found that the RNRs of *C. militaris* belong to the type Ia protein with α2β2 subunit structures, and sequences are highly conserved. 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. While the human RNR consists of a large subunit RNRL and a small subunit RNRMZ or RNRMZB, the two RNRs and their three subunits have different bioactivities in different stages of tumor onset. 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. 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 μmol·L⁻¹, leading to significant regulation. 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 (RNRL) and the small subunit (RNRML), and RNRM 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 RNRM is a promising genetic engineering strategy to improve the biosynthesis of cordycepin in *C. militaris*.

**Acknowledgement**

We would like to thank Dr. WANG Song-Hu (Chengdu Institute of Biology, Chinese Academy of Sciences) for his help in preparing the manuscript.

**References**
Fig. 5  Model illustrating the biosynthesis pathway of cordycepin. ADEK: adenylate kinase. ADK: adenosine kinase. 5′-NT: 5′-nucleotidase


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