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Research Article

Identification of the Genes Involved in the Fruiting Body Production and Cordycepin Formation of *Cordyceps militaris* Fungus

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Abstract A mutant library of *Cordyceps militaris* was constructed by improved *Agrobacterium tumefaciens*-mediated transformation and screened for degradation features. Six mutants with altered characters in *in vitro* and *in vivo* fruiting body production, and cordycepin formation were found to contain a single copy T-DNA. T-DNA flanking sequences of these mutants were identified by thermal asymmetric interlaced-PCR approach. ATP-dependent helicase, cytochrome oxidase subunit I and ubiquitin-like activating enzyme were involved in *in vitro* fruiting body production, serine/threonine phosphatase involved in *in vivo* fruiting body production, while glucose-methanol-choline oxidoreductase and telomerase reverse transcriptase involved in cordycepin formation. These genes were analyzed by bioinformatics methods, and their molecular function and biology process were speculated by Gene Ontology (GO) analysis. The results provided useful information for the control of culture degeneration in commercial production of *C. militaris*.

Keywords *Agrobacterium tumefaciens*, Cordycepin, *Cordyceps militaris*, Degeneration, Fruiting body

Cordyceps militaris is an insect-born fungus with abundant active constituents and a multitude of pharmacological activities [1, 2]. Methods for commercial production of fruiting bodies of this fungus have been established in artificial media [3, 4] or with insects, such as silkworm *Bombyx mori* pupae [5], millworm *Tenebrio molitor* pupae [6] and greater wax moth *Galleria mellonella* larvae [7]. Usually, four pivotal growth periods are identified during cultivating *C. militaris* fruiting bodies, including mycelial culture, color induction, stromal stimulation, and fruiting body production [4, 8]. For the better commercialization of this medical fungus, several improvements are of great importance, including prevention of the culture loss during *in vitro* or *in vivo* fruiting body production, increase of the active constituents (particularly cordycepin) and enhancement of the fungal pathogenicity to the target insects.

Fungal degeneration is detrimental to the culture of this fungus. Detection of the culture degeneration in the early stage may avoid great economic loss. Apart from the reports of phenotypic variation in the degenerative fungal cultures with *Metarhizium anisopliae* [9], little information is available to explain the culture instability of *C. militaris* at the molecular level.

As an entomopathogenic fungus, *C. militaris* not only infects different insect hosts, but also produces fruiting bodies from its hosts. Infection ability is very important in this process. The critical genes involved in the fungal pathogenicity were identified, including adhesin MAD1 [10], Subtilisin-like (Pr1), trypsin-like (Pr2), and chitinases (CHI1) from *M. anisoplia* [11, 12], and two serine protease genes (*csp1* and *csp2*) from *Ophiocordyceps sinensis* [13]. But no reports are available on the molecular control of *C. militaris* growth in the infected insect hosts.

Cordycepin (3′-deoxyadenosine), a nucleoside derivative, is a major bioactive compound found in *Cordyceps* species [14]. High production of cordycepin in *Cordyceps* cultures significantly increases the commercial value of this fungus. Methods to increase the cordycepcin production by optimizing the culture media and conditions were reported recently [15, 16]. However, the genes involved in the cordycepin production in *C. militaris* remain unknown.

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Agrobacterium tumefaciens-mediated transformation (ATMT) was used for insertional mutagenesis in *C. militaris* [4]. In this study, attempts were made to identify the genes involved in *in vitro* and *in vivo* fruiting body production, and cordycepin formation from *C. militaris* mutant library.

MATERIALS AND METHODS

Fungal strains and media. A laboratory and commercial JM4 strain of *C. militaris* from Guangdong Entomological Institute was used. Potato dextrose agar supplemented with 10% peptone (PPDA) was prepared for culturing *C. militaris*. The stock culture of this strain was maintained on PPDA plates at 4°C. *Escherichia coli* strain DH5α was used as a host for the propagation of plasmid DNA. *A. tumefaciens* strain AGL-1 (provided by Prof. Zide Jiang from South China Agricultural University, China) was maintained at 28°C on Luria-Bertani medium.

Fungal transformation. A random T-DNA insertion library of *C. militaris* JM4 was constructed by ATMT method as described previously, with *A. tumefaciens* strain AGL-1 carrying the binary vector pATMT1 with *hyg* gene under *Aspergillus nidulans trpC* promoter [4]. Mutants with altered characters in *in vitro* and *in vivo* fruiting body production, and cordycepin formation were screened. The copy number of T-DNA in transformants was determined by Southern analysis using a PCR-amplified digoxigenin-labeled *hyg* gene probe, and DIG High Prime DNA labeling and Detection Starter Kit (Roche, Basel, Switzerland).

Cultures for fruiting body production. *In vitro* fruiting body production of *C. militaris* was performed with an artificial medium containing 20 g rice, 0.5 g powder of silkworm pupae and 25-mL nutrient solution (glucose 20 g, KH_2PO_4 2 g, $MgSO_4$ 1 g, ammonium citrate 1 g, peptone 5 g, vitamin B1 20 mg, and 1,000-mL distilled water), according to the method by Zheng *et al.* [4]. The phenotypic characters of transformants, including mycelial growth and color in PPDA, together with the stromal and fruiting body formation on the artificial medium, were observed. The fresh weight of fruiting bodies from each bottle was recorded.

In vivo fruiting body production of *C. militaris* in the infected *G. mellonella* larvae was also performed according to the method by Han *et al.* [7]. The mycelia and conidia (at least 10^6 conidia/mL) for infecting insect larvae were prepared by taking a patch of *C. militaris* with hypha body from PPDA plates (1 cm × 1 cm) into 6-mL shaking sterile water. One milliliter of the suspension was introduced into a 100-mL culture bottle with two layers of filter paper and one larva of greater wax moth *G. mellonella*. The bottle was loosely capped and sealed by parafilm, placed at 12~14°C for 2 wk and then at 20°C for fruiting body formation. The infected larvae were recorded every two days. Three replicates were established for each treatment. All the experiments were repeated twice.

Cordycepin assay. Mutants were checked for cordycepin formation by thin layer chromatography (TLC). Cordycepin was extracted according to Haddad *et al.* [17]. Briefly, dried fruiting body (30 mg) of each mutant was mixed with 1-mL of 50% ethanol for 6 hr in the dark at 25°C. The extracts were centrifuged (3,000 rpm, 10 min, 4°C) and the supernatant was used directly for TLC analysis or stored in a refrigerator (4°C) until use.

TLC analyses were performed according to Li *et al.* [18] by using aluminum sheets (5 × 20 cm) precoated with silica gel 60 F254 (layer thickness 0.2 mm). The TLC plates were developed in a horizontal developing chamber. The solvent system includes chloroform/ethyl acetate/isopropanol/water/strong ammonia-water (80/20/60/3/2, v/v/v/v). Chromatographic development of plates was performed at room temperature. After development, the plates were air-dried for 20 min. Separated compounds on the plates were visualized using long-wavelength 254 nm UV illumination, then the photographs of the plates were taken by a Coolpix 990 digital camera (Nikon, Tokyo, Japan). The compounds spots on photographs of the plates were analyzed by Quantity One 4.6.2 program (Bio-Rad, Hercules, CA, USA). Standard cordycepin for TLC analyses was purchased from Sigma Chemical (St. Louis, MO, USA). There were three replicates for each mutant.

Identification of T-DNA flanking sequences and full-length genes. Thermal asymmetric interlaced-PCR (TAIL-PCR) was employed to obtain genomic DNA sequences of *C. militaris* flanking inserted T-DNA from the selected transformants, using a Genome Walking Kit (Takara, China). The PCR products were ligated into pMD19-T vector (Takara). The plasmid DNA was transformed into *Escherichia coli* DH5α. Colony PCR was used to validate positive clones which were subsequently sequenced by Invitrogen Trading (Shanghai) Co. Ltd. (Shanghai, China).

Full-length genes were obtained by 5′- and 3′-rapid amplification of cDNA ends (RACE) and TAIL-PCR. Briefly, total RNA was extracted using the TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). RNA yield was estimated spectrophotometrically, and the integrity of RNA was confirmed via the detection of discrete 18S and 28S ribosomal RNA bands after agarose gel electrophoresis. With gene-specific primers designed to flanking sequences, two partially overlapping cDNA fragments were generated from total RNA of *C. militaris* using RACE (SMARTer RACE cDNA Amplification Kit; Clontech, Palo Alto, CA, USA). The PCR products were sequenced and confirmed as above. If the amplification of cDNA ends of some genes were not obtained by RACE method, TAIL-PCR was also applied to gain the upstream and downstream sequences of the T-DNA flanking fragment.

Bioinformatics analysis of sequences. DNA sequences from RACE PCR or TAIL-PCR were sequenced and assembled respectively. The sequences were compared with those available at the GenBank databases using BLASTn

and BLASTx. Searches for potential open reading frames (ORF) were carried out using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Classification of sequences was performed under Gene Ontology (GO) criteria. For a general approach, the BLAST2GO program (http://www.blast2go.de/) was used for a similarity search.

RESULTS

Fungal transformation. Four hundered to 600 T-DNA-tagged *C. militaris* mutants per 10^5 conidia were generated by *Agrobacterium tumefaciens*-mediated fungal transformation. 34 mutants with degradation features were obtained. Southern blot analysis showed that the mutants contained 1 or 2 copy of T-DNA and more than 64% mutants contained a single copy. Six mutants with a single T-DNA copy involved in altered phenotypes, cordycepin metabolic disorder or decreased entomopathogenic ability were characterized.

Mutants and genes involved in abnormal *in vitro* fruiting body production. Fresh weight decline of the fruiting bodies is one of the signs of *C. militaris* degradation. Average fresh weight of the fruiting bodies from 600 transformants was determined to isolate the degenerated mutants [4]. Two mutants (SA189 and SB60) produced poor fruiting bodies, compared to wild-type *C. militaris* JM4. Moreover, mutant SA11 was found without color change after light induction (Fig. 1).

The T-DNA flanking and full-length sequences of SA11, SA189, and SB60 were obtained by TAIL-PCR or RACE methods (Table 1).

The assembled T-DNA flanking sequence from SA189 was homologous to ATP-dependent helicase gene from *M. anisopliae* (EFZ02692.1) and *Penicillium marneffei* (XP_002144718.1) (Table 1). The putative ATP-dependent helicase of *C. militaris* contained a DEXDc (DEAD-like helicases superfamily) domain and a HELICc (helicase superfamily c-terminal) domain, predicted in SMART database (Simple Modular Architecture Research Tool, http://smart.embl-heidelberg.de/).

The T-DNA flanking sequence of SA60 was homologous to ubiquitin-like activating enzyme E1 gene from *Verticillium alboatrum* (XP_003009099.1). Reverse transcription-PCR was also carried out to confirm the cDNA sequence of

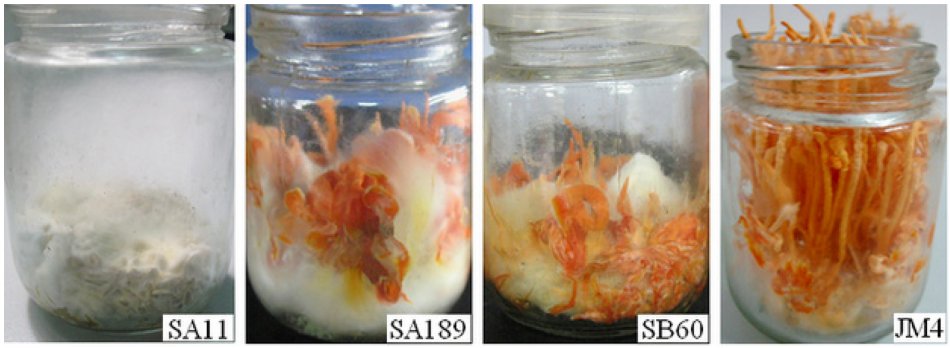

Fig. 1. *In vitro* fruiting body production of the insertional mutants of *Cordyceps militaris* in the artificial medium containing 20 g rice, 0.5 g powder of silkworm pupae and 25-mL nutrient solution (glucose 20 g, KH_2PO_4 2 g, $MgSO_4$ 1 g, ammonium citrate 1 g, peptone 5 g, vitamin B1 20 mg, and 1,000-mL distilled water). Mutant SA11, no color production; mutant SA189 and SB60, poor fruiting body production; JM4, wild-type strain.

Table 1. Homology analysis (BLASTx) of T-DNA-insertion sites in the mutants of *Cordyceps militaris*

Mutant	Altered characters	Putative disrupted gene	Accession No. of obtained gene	Highest identity	E-value	Accession No.
SA11	No *in vitro* fruiting body production	Cytochrome c oxidase subunit I	JQ680973	*Beauveria bassiana* (96%)	0	YP_001876504.1
SA189	No *in vitro* fruiting body production	ATP-dependent helicase	JQ680976	*Metarhizium anisopliae* (69%)	0	EFZ02692.1
SB60	No *in vitro* fruiting body production	Ubiquitin-like activating enzyme E1	JQ680978	*Verticillium alboatrum* (55%)	1E-149	XP_003009099.1
SA125	No *in vivo* fruiting body production	Serine/threonine phosphatase	JQ680975	*Metarhizium anisopliae* (80%)	1E-154	EFY99037.1
SA31	Cordycepin deficiency	Telomerase reverse transcriptase	JQ680977	*Metarhizium anisopliae* (47%)	5E-66	EFZ00131.1
SA145	Cordycepin deficiency	Glucose-methanol-choline oxidoreductase	JQ680974	*Metarhizium anisopliae* (90%)	3E-71	EFY96931.1

ubiquitin-like activating enzyme gene in *C. militaris* (data not shown). The full-length sequences of ubiquitin-like activating enzyme E1 gene (accession No. JQ680978) between genomic DNA (1,804 bp) and cDNA (1,689 bp) were aligned and compared (Table 1).

From the T-DNA flanking sequence of mutant SA11, the full-length cytochrome oxidase subunit I gene (*cox1*, accession No. JQ680973) of *C. militaris* was obtained by TAIL-PCR, containing a 1,593 bp coding region of *cox1* (*CRcox1*) interrupted by a 1,050 bp group I intron coding a LAGLIDADG endonuclease. The transcriptional analysis and molecular characterization of this gene was reported in our previous paper [4].

Mutants and genes involved in *in vivo* fruiting body production. From 600 transformants, no mutant was detected to completely lose infection capacity to *G. mellonella* larvae, while 8 mutants were found with different infection outputs. Mutant SA125 was one of the mutants showing abnormal fruiting body production (no stromal production or no sclerotia formation) in infected *G. mellonella* larvae (Fig. 2). The mutant appeared not to fully use the cadavers as nutrients for development (Fig. 2). The full-length T-DNA flanking sequence of SA125 (accession No. JQ680975) was predicted to be homologous to serine/threonine phosphatase gene from *M. anisopliae* (EFY99037.1) (Table 1).

Mutants and genes involved in cordycepin deficiency. The cordycepin production of 600 mutants was determined by TLC for semi-quantitative analysis. The cordycepin contents of most mutants and wild type JM4 were higher than the standard concentration (0.5 mg/mL). However, the cordycepin contents of five mutants were much lower than that of wild type JM4. Mutant SA31 and SA145 were two of them showing cordycepin deficiency (Fig. 3).

The assembled T-DNA flanking sequences from SA31 and SA145 were predicted to be homologous to telomerase reverse transcriptase gene from *M. anisopliae* (EFZ00131.1)

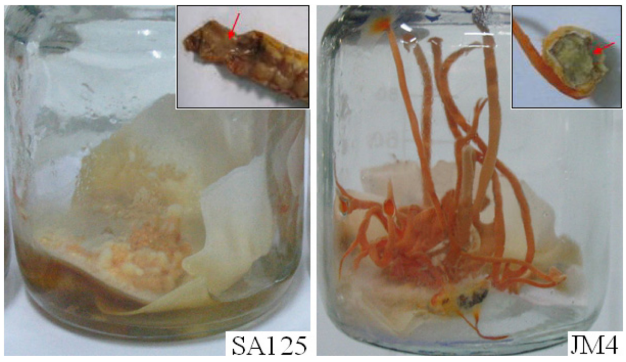

Fig. 2. *In vivo* fruiting body production of the insertional mutants of *Cordyceps militaris* in the infected *Galleria mellonella* larvae. Mutant SA125, no stromal production or sclerotia formation; JM4, wild-type strain.

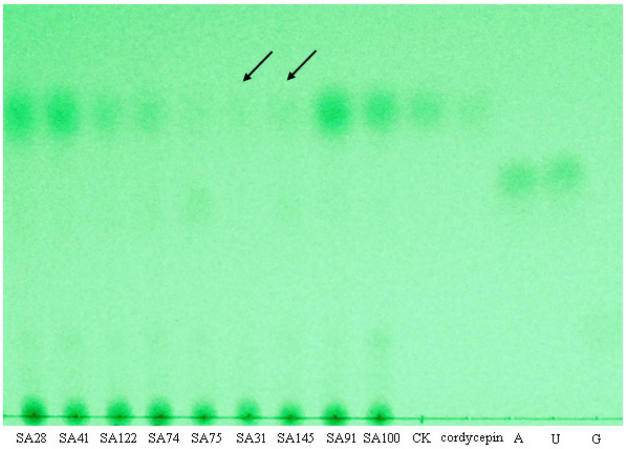

Fig. 3. Cordycepin production determined by thin layer chromatography in the insertional mutants of *Cordyceps militaris*. Among mutants SA28, SA41, SA74, SA75, SA31, SA91, SA100, SA122, and SA145, the cordycepin contents of the mutants SA31 and SA145 were significantly lower than that of wild type JM4. The standard cordycepin, adenoside, uridine and guanine, were marked as cordycepin, A, U, and G. CK, wild type JM4.

and glucose-methanol-choline oxidoreductase gene from *M. anisopliae* (EFY96931.1), respectively.

GO analysis. GO database was used to analyse the putative biological processes and molecular functions of the identified genes. Three genes (ATP-dependent helicase, ubiquitin-like activating enzyme E1 and cytochrome c oxidase subunit I) included the following five categories: metabolic process (GO:0008152), aerobic respiration (GO:0009060), electron transport chain (GO:0022900) and oxidation-reduction process (GO:0055114). Regarding molecular functions, they included the following categories: DNA binding (GO:0003677), ATP binding (GO:0005524), helicase activity (GO:0004386), catalytic activity (GO:0003824), protein binding (GO:0005515), zinc ion binding (GO:0008270), ligase activity (GO:0016874), metal ion binding (GO:0046872), cytochrome-c oxidase activity (GO:0004129), iron ion binding (GO:0005506), electron carrier activity (GO:0009055), and oxidoreductase activity (GO:0016491). The biological processes of the gene (glucose-methanol-choline oxidoreductase) related to the cordycepin production included RNA-dependent DNA replication (GO:0006278) and alcohol metabolic process (GO:0006066); while its molecular functions included DNA binding (GO:0003677), telomeric template RNA reverse transcriptase activity (GO:0003721), RNA binding (GO:0003723), RNA-directed DNA polymerase activity (GO:0003964), flavin adenine dinucleotide binding (GO:0050660), and oxidoreductase activity (acting on CH-OH group of donors) (GO:0016614). The serine/threonine phosphatase gene, influencing *C. militaris* fruiting body production in infected *G. mellifera* larvae, possessed the molecular functions of phosphoprotein

phosphatase activity (GO:0004721) and hydrolase activity (GO:0016787).

DISCUSSION

From *C. militaris* mutant library, several genes involved in mutated characters, such as abnormal fruiting body production in artificial medium and infected insects, or lower cordycepin formation, were successfully identified. The results would provide useful information for the effective control of culture degeneration in commercial production of *C. militaris*.

Fungal culture degeneration is usually irreversible and inheritable, and can result in great commercial losses [19, 20]. In *C. militaris*, culture degeneration was reflected with poor fruiting body production. The degenerate strains usually showed significantly lower protease activity, chitinase activity, dehydrogenase activity, oxidative stress, decolorization activity or/and infection activity to *Galleria* larvae [21]. In the present study, three genes (cytochrome c oxidase subunit I, ATP-dependent helicase and ubiquitin-like activating enzyme E1) were identified to be involved in the poor fruiting body production in the artificial medium.

Cytochrome c oxidase subunit I, encoded by mitochondrial DNA (mtDNA), was the terminal component of the mitochondrial respiratory chain, transfers electrons from reduced cytochrome c to molecular oxygen. Fungi degeneration was closely related to mitochondria, including mitochondrial DNA alterations [9], mtDNA glycation [21] and decreasing activation of dehydrogenase in mitochondria [22]. It was conceivable that the disruption of cytochrome c oxidase subunit I with a group I intron [4] might affect the respiration and growth of *C. militaris* mutant. ATP-dependent helicase was implicated in many cellular processes, including translation initiation [23], and pre-mRNA splicing [24]. Several putative RNA helicases were found in ribosome biogenesis in *Saccharomyce cerevisiae* [25, 26]. The mutation of ATP-dependent RNA helicase caused a severe slow-growth phenotype in *S. cerevisiae* [25]. Here the ATP-dependent helicase mutant caused no fruiting body production of *C. militaris*. *C. militaris* also contained a 1,689 bp gene encoding putative ubiquitin-like activating enzyme E1, an essential gene with extensive sequence similarity to the E1 genes in other fungi. A ubiquitin-protein ligase (E3) specifically attached ubiquitin to the ε-amino group of a lysine residue in the target protein [27]. Ubiquitin-like proteins were signaling messengers that control many cellular functions, such as cell proliferation, apoptosis, the cell cycle and DNA repair [28]. It was interesting that ubiquitin-like activating enzyme E1 in *C. militaris* also controlled the fruiting body production.

No stromal production or sclerotia formation was found in *G. mellonella* larval cadavers infected by mutant SA125. A 1,347-bp serine/threonine phosphatase gene was involved in this mutated phenotype. Serine/threonine phosphatases usually control key biological pathways including early embryonic development, cell proliferation, cell death, circadian rhythm and cancer [28]. It was reported that the kinase homologue involved in fungal pathogenesis was required for full virulence in disparate hosts [29]. Although serine/threonine phosphatase gene did not influence the pathogenic ability of this fungus against *G. mellonella* larvae, it was involved in the fruiting body production in the infected insect larvae.

Cordycepin contents in mutant SA31 and SA145 were scarcely detected by TLC and obviously lower than that in wild type JM4. Two genes, encoding telomerase reverse transcriptase and glucose-methanol-choline oxidoreductase were involved in the cordycepin production in *C. militaris*. The study of telomerase reverse transcriptase mainly focused on human disease. Most telomerase reverse transcriptase gene variants reduced telomerase enzymatic activity *in vitro*. Loss-of-function telomerase gene variants associated with short telomeres were risk factors for sporadic cirrhosis [30]. The expression of telomerase reverse transcriptase in different parts of human body was found associated with cancer [31, 32]. Glucose-methanol-choline oxidoreductase played an important role of cyanohydrin formation and fungal degradation of lignin [33, 34]. It could also oxidize phenolic and nonphenolic benzyl alcohols in *Bjerkandera* [35]. Fungal pyranose oxidase, belonging to glucose-methanol-choline oxidoreductase family, was a flavoenzyme whose preferred substrate among several monosaccharides was D-glucose [36]. Cordycepin might involve in flavin adenine dinucleotide binding and oxidoreductase activity (acting on CH-OH group of donors) according to the gene GO analysis.

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