Screening of Camptothecin Production and SRAP Analysis of Endophytic Fungi from *Camptotheca acuminata* Decne*

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Abstract Endophytic fungi, lived in stems and leaves of host plants, were supposed to be effective or novel sources for therapeutic compounds. *Camptotheca acuminata* Decne is a traditional medical plant in China. About 50 endophytic fungi were isolated from barks of *C. acuminata*. A new fungus producing camptothecin (CPT) was identified as a *Penicillium* based on its morphological features. Ten endophytic fungi of *C. acuminata* were chosen to be amplified using molecular maker sequence related amplified polymorphism (SRAP). The combinations of SRAP primers turned out to be polymorphic and a total of 1 295 polymorphic bands were obtained. These strains were grouped into 3 main clusters and revealed that SRAP could isolate the strains of *C. acuminata* efficiently. It also provided evidence for discussing the genetic diversities of species in *C. acuminata* with SRAP molecular method.

Key words Endophytic fungus *Penicillium* Molecular maker Genetic diversity

1 Introduction

Endophytic fungi, which live within plants' internal tissues or organs without causing any apparent symptoms or diseases in the host plants, may produce same or similar compounds with plants, presumably attributed to the symbiotic relationship with their hosts[1]. A number of endophytic fungi with good bioactivity had been isolated from plants, such as from *Cassia spectabilis*, *Artemisia annua*, *Cynodon dactylon* and so on, since Strobel et al[2] obtained a species of endophytic fungi (*Taxomyces andreanae*) produced paclitaxel from *Taxus brevifolia*[3-5].

Camptotheca acuminata Decne, a traditional medicinal plant, was first found to have pharmacological agent named camptothecin by Wall et al[6]. CPT specifically inhibits DNA topoisomerase I[7] and exhibits preferential toxicity to proliferating cells. At present, some endophytic fungi with anti-tumor activity were isolated from *C. acuminata*. *Xylaria sp.* and *Fusarium solani* were discovered to produce CPT and HCPT[8-9]. New polyketides with significant inhibition on the growth of human-tumor HeLa cells were obtained by Yuan et al[10]. Nevertheless, the use of endophytic fungi for the production of bioactive products remains limited.

Sequence related amplified polymorphism, a PCR-based marker system, was a newer molecular marker first introduced by Li et al[11], and had been successfully applied in genetic diversities of *Tricholoma matsutake*, *Auricularia polytricha* and *Ganoderma strains*[12-14]. However, the application of SRAP has never been discussed in endophytic fungi of *C. acuminata*.

Received April 02. 2011 Accepted May 11. 2011

* Supported by the National Natural Science Foundation of China (30900913) and the Key Research Items from the Ministry of Education of China (03126)

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Thus, the isolation and CPT production of endophytic fungi in *C. acuminata* as well as SRAP analysis were reported.

2 Materials and methods

2.1 Source and isolation of endophytic fungi

Barks of *C. acuminata* were gathered at Zhangjiajie nature conservation area, Hunan Province, China. The barks were surface-sterilized according to the procedures described by Huang et al[15]. The barks were cut into pieces of 0.5 cm^2 and then placed on potato dextrose agar (PDA) supplemented with ampicillin and gentamicin, and purified when incubated on PDA at 28℃.

2.2 Fermentation and analysis of CPT

The strains were fermented in liquid potato dextrose medium at 25℃ for 7 days after been prepared on PDA slants. The cultures were extracted 3 times with chloroform after concentrated under pressure and condensed. The residues were redissolved in methanol.

The residues were analyzed by TLC with developing solvent (chloroform/methanol, 9∶1) and HPLC using a reverse-phase Noca pac C18 column (4×150mm, 4μm) and detection at 254 nm with a gradient program as follows: 30% ~35% acetonitrile for 5 min, 35% ~40% acetonitrile for 7 min (1ml/min). The CPT purchased from Ronghe Pharmaceutical Technology Development Corporation (Shanghai, China) was used as standard.

2.3 DNA extraction and SRAP analysis

Total DNA was extracted from 1.0 g of wet mycelia using the cetyl trimethylammonium bromide (CTAB) method described by Zeng et al[16]. Six forward primers and six reverse primers (Sangon Biotech, Shanghai) were selected for SRAP analysis (Table 1). SRAP amplifications were performed in 25 μl reaction volumes containing the following reagents: 1.0 μg of genomic DNA, 0.5μl of each primer, 1μl dNTPs (2.5 mmol/L), 1.5 U Taq polymerase (BioTeke Corporation, Beijing), 2.5 μl 10×PCR buffer, 2 μl of $MgCl_2$ (25 mmol/L), and sterile double-distilled water. Amplification conditions were: 5 cycles of 94℃ for 3 min, 94℃ for 45 s, 38℃ for 45 s, 72℃ for 1 min; 30 cycles of 94℃ for 45 s, 50℃ for 45 s, 72℃ for 1 min; and a final extension 6 min at 72℃. The amplification products were analyzed by electrophoresis in polyacrylamide gels and stained by silver as described by Bassam et al[17] and photographed. DM 2000 plus DNA (BioTeke Corporation, Beijing) was used as size markers.

Table 1 Primer sequence used for SRAP analysis

me-6	TGAGTCCAAACCGGACA	em-12	GACTGCGTACGAATTCTC
me-8	TGAGTCCAAACCGGACT	em-19	GACTGCGTACGAATTAGC
me-13	TGAGTCCAAACCGGAAG	em-23	GACTGC GTACGAATTATT
me-16	TGAGT CCTT TCCGGTAA	em-24	GACTGCGTACGAATTACG
me-23	CTGGCGAACTCCGGATG	em-25	GACTGCGTACGAATTATG
me-24	GGTGAACGCTCCGGAAG	em-26	GACTGCGTACGAATTCGG

2.4 PCR-SRAP data analysis

Reproducible DNA bands were scored as 1 (presence) or 0 (absence) for strains. Dice's similarity coefficients between strains were calculated using the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc, version 2.1) software package[18]. The SHAN program was applied to the distance matrices cluster analysis using UPGMA algorithm[19], and a dendrogram was produced based on simple matching matrix.

3 Results and discussions

3.1 Isolation and identification of the endophytic fungus, EFC I-13

About 50 strains of endophytic fungi were isolated from barks of *C. acuminata* grown in the Zhangjiajie, Hunan province, China, non-sporulating fungi(48.9%), *Alternaria* (12.6%), *Phomopsis* (6.9%), *Penicillium* (6.3%), *Mucor* (4.6%) and *Fusarium* (4.6%). Among these fungi, the metabolites of one strain, EFC I-13, had a spot with the same Rf value of reference CPT in TLC (Fig. 1), and the same retention time of reference CPT in HPLC (Fig. 2). This strain was considered to produce CPT and was identified as *Penicillium* according to its morphological features (Fig. 3).

White mycelium was produced after the strain was cultured one day on PDA at 25℃, and green spores appeared in the center of colonies on the second day. Then, apophysis showed up in the center and white aerial hyphae existed on the edge. Under the microscope, multi-

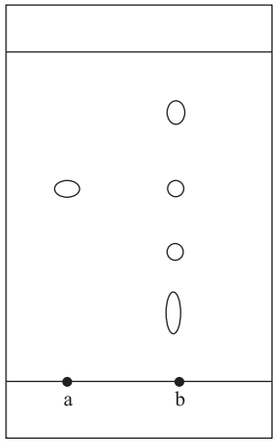

Fig. 1 Result of TLC

(a) Reference CPT (b) Extract from EFC I-13

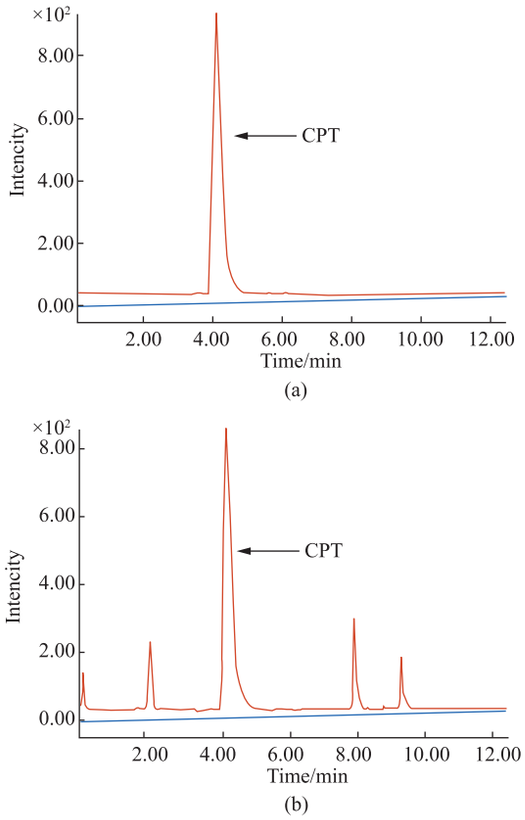

Fig. 2 Determination of CPT by HPLC chromatogram

(a) Reference CPT (b) Extract from EFC I-13

branched hyphae with diaphragm were observed. Conidiophores, produced by hyphostroma, were about 2.4 ~ 2.7μm with septum, and the top of which were not swelled, but generated multiple branches which shaped like broom. Conidia, generated by conidiophores through

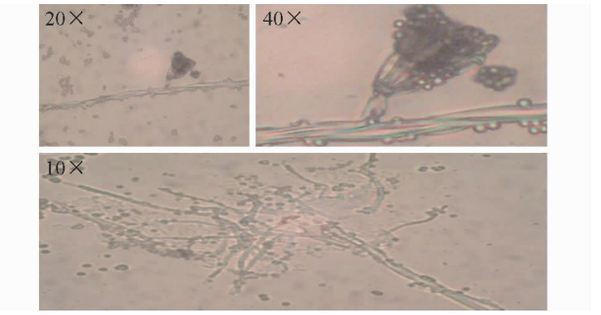

Fig. 3 Photos of microscopical morphological characters of EFC I-13

excision, were rough and appeared like a chain. Conidium was 1.2 ~ 1.4μm and shaped as spherical or ellipse without transversum.

The results showed the fungus, EFC I-13, is a species of *Penicillium*. This is the first time to find a species of *Penicillium* isolated from *C. acuminata* that could produce campothecin. The effect of various chemical factors on the CPT biosynthesis could be investigated in future work. To the non-producing CPT fungi, a lot of work could be done to isolate some other active components from these fungi.

3.2 PCR-SRAP analysis

According to different culture characteristics, 10 endophytic fungi were chosen to be amplified for SRAP analysis (Fig. 4). Thirty-six random SRAP primer combinations revealed polymorphisms among test strains. The similarity matrix of test strains used in SRAP analysis showed the similarity coefficient value was ranging from 0.41 to 0.77, indicated that the genetic relationships of 10 strains were different, which was consistent with the morphological method.

The phylogenetic dendrogram using cluster analysis based on SRAP (Fig. 4) showed that the 10 strains could be grouped into 3 main clusters at a similarity index value of 0.52. Cluster A comprised 7 genotyped (EFC Ⅰ-12, EFC Ⅰ-13 EFC Ⅰ-14 EFC Ⅰ-15, EFC Ⅰ-51, EFC Ⅲ-51 and EFC Ⅴ-12) that were divided into 4 sub-clusters at similarity index value of 0.65. Within Cluster A, strains EFC Ⅴ-12 and EFC Ⅰ-13 appeared to be the most closely related with a similarity index value of 0.77. Cluster B was made up of 2 genotyped (EFC Ⅰ-40, EFC Ⅰ-52) that

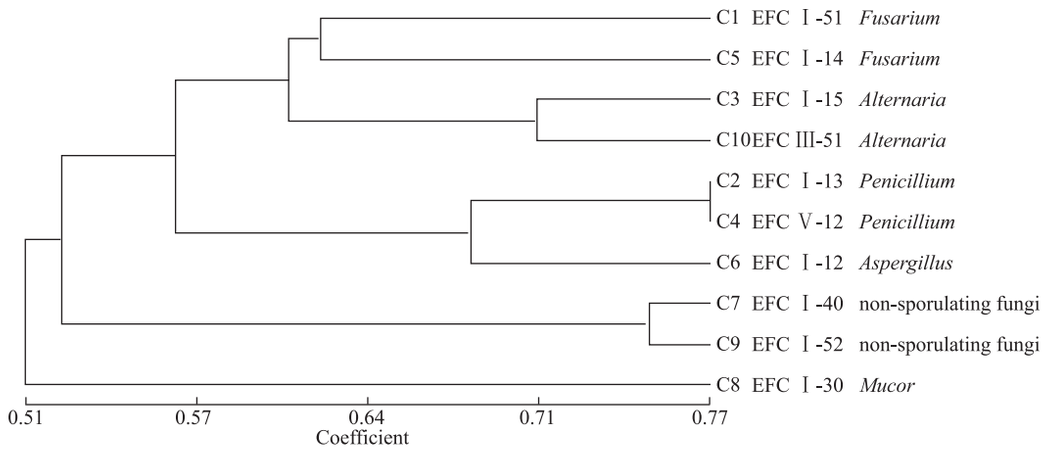

Fig. 4 UPGMA dendrogram of 10 strains constructed based on molecular profiles revealed by SRAP markers

could be separate at similarity index value of 0.75. EFC Ⅰ-30 was the only one in cluster C.

Though there are only 10 strains used in the study of SRAP analysis, it identified that the SRAP could isolate endophytic fungi of *C. acuminata* efficiently and manifested that it's feasible to discuss the genetic diversities of species using SRAP molecular technology by expanding the number of isolates.

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产喜树碱喜树内生真菌的筛选及喜树内生真菌的 SRAP 分析

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摘要 生活在宿主植物里的内生真菌是很重要的药用资源。喜树是中国的传统药用植物。从喜树植物中分离得到了大约 50 种菌株,其中一株产喜树碱的菌株通过形态学鉴定为青霉属,这是首次在喜树植物中发现产喜树碱的青霉属菌株。为研究简单序列重复相关序列扩增多态性(SRAP)方法在喜树内生真菌中应用的可行性,选择了十株喜树内生真菌进行 SRAP 多态性分析。SRAP 引物共扩增出 1 295条带,而这些菌株也被分为三大类。这些结果表明,SRAP 研究喜树内生真菌具有高效性,是讨论喜树内生真菌的遗传多样性的有效方法。

关键词 内生真菌 青霉属 分子标记 遗传多样性

中图分类号 Q75

收稿日期:2011-04-02 修回日期:2011-05-11

* 国家自然科学基金(30900913)、国家教育部重点项目(03126)资助项目

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