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Research Article

Systematic Analysis of the Anticancer Agent Taxol-Producing Capacity in *Colletotrichum* Species and Use of the Species for Taxol Production

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Abstract Paclitaxel (taxol) has long been used as a potent anticancer agent for the treatment of many cancers. Ever since the fungal species *Taxomyces andreanae* was first shown to produce taxol in 1993, many endophytic fungal species have been recognized as taxol accumulators. In this study, we analyzed the taxol-producing capacity of different *Colletotrichum* spp. to determine the distribution of a taxol biosynthetic gene within this genus. Distribution of the taxadiene synthase (*TS*) gene, which cyclizes geranylgeranyl diphosphate to produce taxadiene, was analyzed in 12 *Colletotrichum* spp., of which 8 were found to contain the unique skeletal core structure of paclitaxel. However, distribution of the gene was not limited to closely related species. The production of taxol by *Colletotrichum dematium*, which causes pepper anthracnose, depended on the method in which the fungus was stored, with the highest production being in samples stored under mineral oil. Based on its distribution among *Colletotrichum* spp., the *TS* gene was either integrated into or deleted from the bacterial genome in a species-specific manner. In addition to their taxol-producing capacity, the simple genome structure and easy gene manipulation of these endophytic fungal species make them valuable resources for identifying genes in the taxol biosynthetic pathway.

Keywords *Colletotrichum* spp., Genome, Horizontal gene transfer, Taxadiene synthase, Taxol

The natural anticancer agent taxol, originally isolated from the *Taxus* spp. of yew trees, has been used to treat various cancers, including refractory ovarian cancer, metastatic breast cancer, and lung cancer [1]. Because of its low yield from *Taxus* spp., intensive research has been conducted to produce taxol more effectively. The approaches used have included total chemical synthesis [2], semisynthetic methods [3], *Taxus* cell culture [4], hairy root culture [5], and fungal production. Naturally occurring endophytic fungi were shown to produce taxol, with the first identified organism being *Taxomyces andreanae* [6-11]. Although the taxol yield from these endophytic fungi is insufficient for any profitable commercial production of the agent, the relative simplicity of the fungal genome renders these fungi valuable resources for identifying the genes responsible for taxol biosynthesis.

The genus *Colletotrichum* (teleomorph: *Glomerella*) belongs to the ascomycetes, the species of which are responsible for the most common and devastating plant pathogenic diseases such as anthracnose spots and blight [12]. Almost every crop can serve as their hosts, including fruits, vegetables, and ornamentals grown throughout the world [12]. *Colletotrichum* species cause anthracnose in apple and grape (*C. gloeosporioides*) [13], pepper (*C. coccodes*, *C. gloeosporioides*, *C. capsici*, *C. dematium*, and *C. acutatum*) [14], cucurbits (*C. lagenarium*) [15], cucumber (*C. orbiculare*) [16], sugarcane red rot (*C. falcatum*) [17], maize (*C. graminicola*) [18], *Brassica* spp. (*C. higginsianum*) [19], and soybean (*C. truncatum*) [20]. There are two distinct types of crop diseases; a preharvest type affecting developing crops in the field, and a postharvest type that damages mature crops during storage.

Many pathogenic and non-pathogenic *Colletotrichum* species have been identified. However, systematic research using the genus *Colletotrichum* to identify the distribution

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of genes for taxol production and the species with taxol-producing capacity has not yet been conducted. In this study, 12 pathogenic *Colletotrichum* spp. were analyzed for their taxol-producing capability and the presence of a taxol biosynthetic gene.

MATERIALS AND METHODS

Colletotrichum spp. were obtained from the Korean Agricultural Culture Collection (KACC, Gene Resources Center, Rural Development Administration, Republic of Korea; http://www.genebank.go.kr) and Center for Fungal Genetic Resources (CFGR, Republic of Korea; http://genebank.snu.ac.kr). The 12 species tested were *C. coccodes* CBP1 (KACC 40009), *C. dematium* CBP2 (KACC 40013), *C. acutatum* CBP3 (KACC40042), *C. higginsianum* CBP4 (KACC 40806), *C. truncatum* CBP5 (KACC40810), *C. boninense* CBP7 (KACC 40893), *C. liliacerum* CBP8 (KACC 40981), *C. caudatum* CBP10 (KACC 41028), *C. lindemuthianum* CBP11 (KACC 42433), *C. musae* CBP18 (KACC 40947), and *C. orbiculare* CBP17 (KACC40809) from the KACC, and *C. falcatum* CBP42 (2007-112-00023) from the CFGR.

All 12 species were cultured on potato dextrose agar (PDA) medium at 25°C. Genomic DNA was isolated from 4-day-old mycelia cultured on PDA medium, using the standard quick method [21]. DNA analyses were conducted by sequencing the PCR products of the internal transcribed spacer (ITS) region of the 18S rRNA gene using the primer set ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′). Phylogenetic analysis was carried out by the neighbor-joining method using MEGA ver. 6.0 software (The Biodesign Institute, Tempe, AZ, USA) [22]. Bootstrap analysis of each dataset was conducted with 1,000 replicates. After testing several primer pairs for specificity for the taxadiene synthase (*TS*) gene, the candidate fungi for taxol production were screened by PCR amplification using the *TS* primer set TS-2F (5′-GTCGAATTGAGAAGCGTGGT-3′) and TS-2R (5′-TCCCATCTCTTTACTCCCTCA-3′).

Selected fungal strains were grown in 250 mL Erlenmeyer flasks containing 100 mL of potato dextrose broth for 2 wk at 24°C. The mycelia and culture fluid were homogenized at 3,000 rpm using a WiseTis homogenizer (Daihan Scientific Co., Seoul, Korea), mixed with an equal volume of ethyl acetate, and incubated overnight with gentle shaking. The mixture was then separated by centrifugation at 3,000 rpm and only the organic phase was collected. The organic solvent was removed using a rotary vacuum evaporator (Eyela, Tokyo, Japan) and the dry solid residue was dissolved in high-performance liquid chromatography (HPLC)-grade methanol to identify the taxol.

The presence and quantification of taxol were analyzed using a liquid chromatography-mass spectrometry (LC/MS) system composed of an HPLC apparatus (model 2695; Waters, Milford, MA, USA) equipped with a pentafluorophenyl column (Luna, 3 × 150 mm, 3 μm; Phenomenex, Torrance, CA, USA) and a mass spectrometer (model 3100; Waters). The HPLC conditions were as follows: injection volume = 2 μL, solvent A = 0.1% formic acid, solvent B = acetonitrile, and solvent program = 70 : 30 (A/B) to 30 : 70 (A/B) over 15 min at a flow rate of 0.4 mL/min. The mass spectrometry (MS) analysis conditions were as follows: desolvation gas (N_2) flow rate = 500 L/hr, cone gas (N_2) flow rate = 50 L/hr, desolvation temperature = 400°C, capillary voltage = 3.5 kV, cone voltage = 60 V, ionization mode = electrospray positive, and single ion recording m/z = 854. The crude methanol extract of each *Colletotrichum* species was purified by silica-gel purification. In brief, the crude methanol extract was reconstituted in 1 mL of dichloromethane and loaded onto a dichloromethane-activated Sep-Pak silica cartridge (700 mg; Waters). Taxol was then eluted from the cartridge with 15 mL of dichloromethane : ethyl acetate (70 : 30, v/v) after washing with 15 mL of dichloromethane:ethyl acetate (70 : 25, v/v). The eluate was dried and redissolved in 0.25 mL of methanol before being analyzed by LC/MS.

RESULTS AND DISCUSSION

Characterization of the morphological differences among 12 *Colletotrichum* spp. (11 from the KACC and 1 from the CFGR) was conducted by incubating each species on PDA and then evaluating the colony growth and shape over 1 week (Fig. 1A~L). Eight of the 12 species developed white colonies, but 3 of them soon developed orange conidial masses near the inoculum point (Fig. 1I, 1J and 1K). *C. coccodes* and *C. dematium* developed abundant sclerotia, whereas only *C. truncatum* grew gray-olive colonies. The mycelial growth rates were quite different, with *C. coccodes* and *C. caudatum* growing the fastest, and *C. lindemuthianum* and *C. orbiculare* growing slowly. The phylogenetic relationship among the species was identified by comparing their 18S rRNA sequence homology (Fig. 1M).

The taxol biosynthetic pathway is assumed to be composed of at least 20 different steps [23], the most critical of which is cyclization of geranylgeranyl pyrophosphate to taxa-4(5)-11(12)-diene (taxadiene, the unique taxane core skeleton), catalyzed by the *TS* enzyme [24]. After screening for the *TS* gene with several primer pairs, a *TS* primer set was selected to verify the presence of the gene in the 12 different *Colletotrichum* spp. The *TS* gene was widely distributed among the evaluated fungi, being present in 8 of the 12 species (gray-colored *Colletotrichum* in Fig. 1M). All PCR products were identified as a segment of the *TS* gene, showing 100% similarity with the gene from *Taxus brevifolia* (NCBI accession No. U48796). Distribution of the *TS* gene was not limited to closely related *Colletotrichum* spp. (as shown in *C. boninense*, *C. musae*, *C. higginsianum*, and *C. lindemuthianum*) (Fig. 1M), suggesting that the gene distribution follows a species-specific pattern with random integration/loss of the gene in the genomes of *Colletotrichum* species.

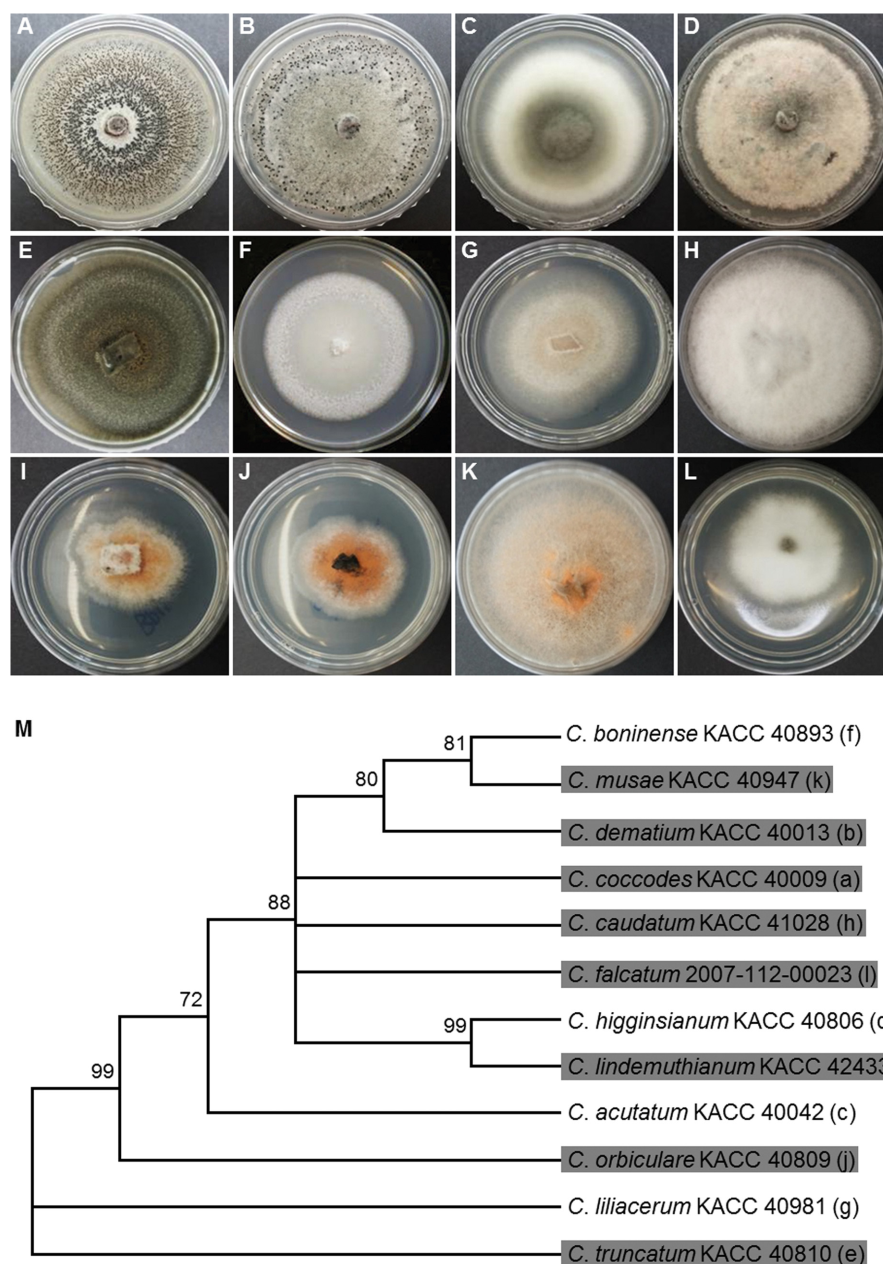

Fig. 1. Colony morphology (A~L) and phylogenetic analysis (M) of *Colletotrichum* species. A~L, Growth of *Colletotrichum* species on potato dextrose agar medium for 7 days at 25°C. A, *C. coccodes*; B, *C. dematium*; C, *C. acutatum*; D, *C. higginsianum*; E, *C. truncatum*; F, *C. boninense*; G, *C. liliacerum*; H, *C. caudatum*; I, *C. lindemuthianum*; J, *C. orbiculare*; K, *C. musae*; L, *C. falcatum*. M, Phylogenetic analysis of the 12 *Colletotrichum* species based on sequences of the 18S rRNA gene (internal transcribed spacer 1, 5.8S rRNA, and internal transcribed spacer 2). The tree was prepared using MEGA ver. 6.0, and bootstrap confidence values were tested by 1,000 repetitions. Bootstrap values are indicated on the branches. The gray box indicates that the fungi contain the taxadiene synthase gene.

Table 1. Characteristics of the taxane compounds analyzed by mass spectroscopy

No.	Taxane compound	m/z [M+Na$^+$]	Molecular formula	Mass [M]
1	10-Deacetylbaccatin III (5 ppm)	567.23	$C_{29}H_{36}O_{10}$	544.23
2	Baccatin III (0.5 ppm)	609.24	$C_{31}H_{38}O_{11}$	586.24
3	10-Deacetyltaxol C	828.37	$C_{44}H_{55}NO_{13}$	805.37
4	10-Deacetyltaxol	834.32	$C_{45}H_{49}NO_{13}$	811.32
5	Taxol (5 ppm)	876.33	$C_{47}H_{51}NO_{14}$	853.33

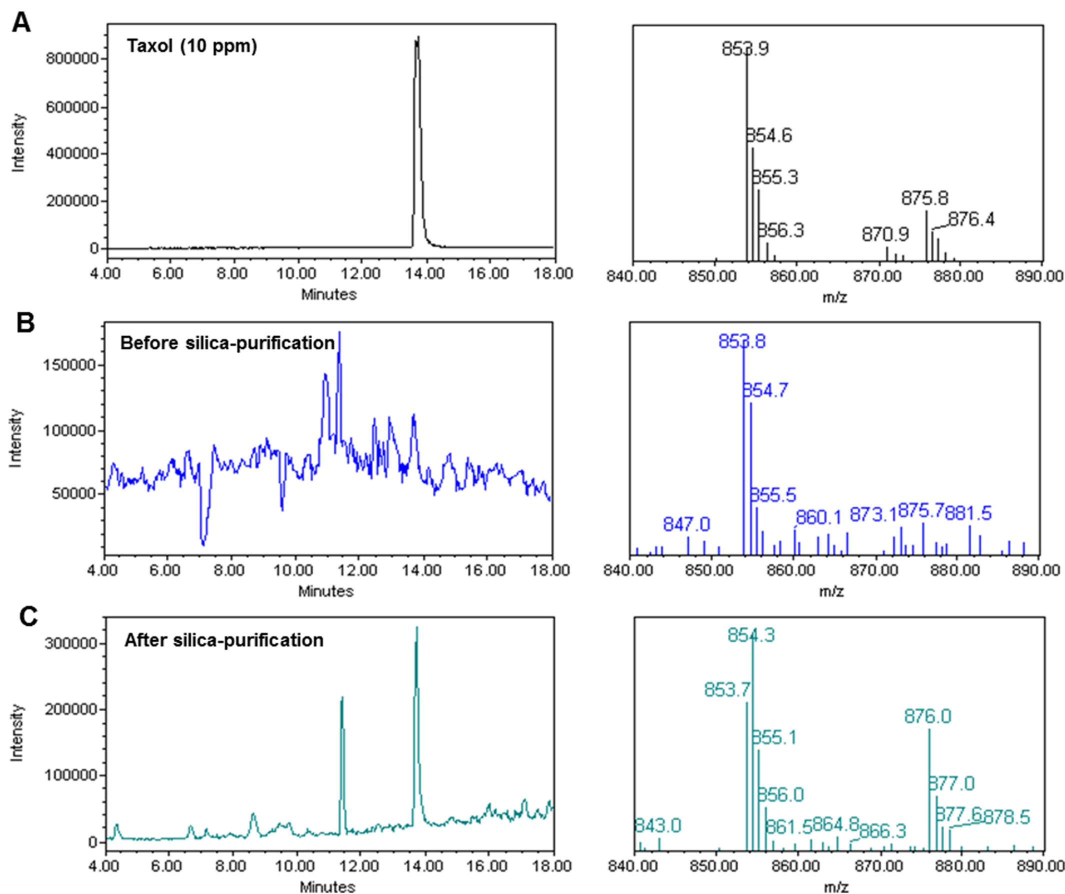

Fig. 2. Liquid chromatography-mass spectrometry analysis of taxol from *Colletotrichum dematium*. The left and right columns indicate high-performance liquid chromatography (HPLC) peaks and mass spectra, respectively. A, Authentic taxol; B, The HPLC peaks from the extract of *C. dematium* (left) and the mass spectrum of the HPLC peak at 13.8 min (right); C, The HPLC peaks from the extract of *C. dematium* after silica-gel purification (left) and the mass spectrum of the 13.8 min peak (right). The fungal extract exhibited a specific mass spectrum with peaks at molecular weights of 854 and 876, which were identical to those of authentic taxol.

The 8 species carrying the *TS* genes were subjected to ethyl acetate extraction for LC/MS identification of the presence of taxol and other taxane compounds. The characteristics of the taxane compounds are summarized in Table 1. Among the 8 species, *C. dematium* appeared to have the highest peak corresponding to the HPLC peak at 13.8 min for the 10 ppm authentic taxol used as a positive control (Fig. 2A). However, the background peaks surrounding the *C. dematium* taxol peak were relatively high (Fig. 2B), and therefore, further purification of the extract was carried out using solid-phase extraction. The resultant HPLC 13.8 min peak for the purified extract was shown to have the same MS spectrum as that of taxol (Fig. 2C), clearly identifying taxol production in this species. The low yield of taxol from the other 7 species (indicated by the small peak areas in the HPLC analysis) was problematic for their further identification by MS (data not shown).

We attempted to increase the taxol yield from *C. dematium* by changing the medium composition and pH and evaluating the effects of different incubation temperatures and periods (Supplementary Table 1). The culture media were also amended with various elicitors, such as soytone, serinol, and benzoic acid (Supplementary Table 1). Although more than 70 different incubation conditions were tested, we failed to increase the taxol production by *C. dematium*. During the repeated tests, we found that taxol production decreased after successive transfers from the parent culture. As detected by LC/MS, the first culture from the stock produced the highest amount of taxol, the second generation produced dramatically less, and the third generation did not produce any at all after 14 days in potato dextrose broth at 24°C.

As *C. dematium* is stored in liquid nitrogen, under mineral oil, or in a deep freezer at the KACC, we requested to test samples from each storage condition to compare the effects of storage method on taxol production. Although all the *C. dematium* samples could produce taxol, the one stored under mineral oil produced more than 10 times the amount of taxol than those stored under the other conditions (Fig. 3).

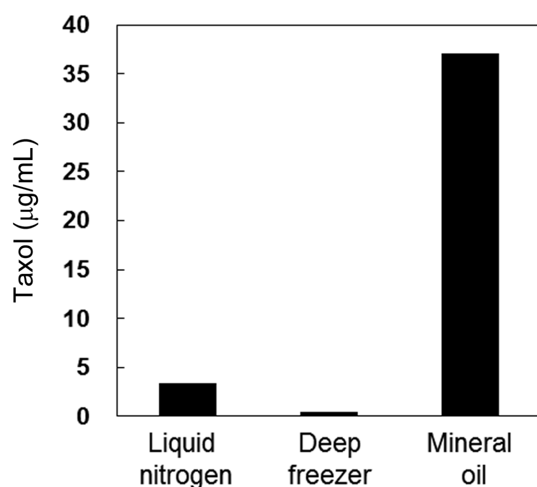

Fig. 3. Taxol production by *Colletotrichum dematium* cultures stored under different conditions. The production of taxol was measured from cultures of *C. dematium* that had been stored in liquid nitrogen, in a deep freezer, or under mineral oil.

Thus, mineral oil storage would be the best choice for maintaining the fungal capacity to produce secondary metabolites, especially taxol. The drawback of taxol production by endophytic fungi is that the yield is too low to be commercially feasible [25]. Additionally, successive transfer of the fungi reduces their production of taxol. Nevertheless, the data presented herein provide useful information that will help reveal the genes and regulatory factors involved in the taxol biosynthetic pathway. Specifically, a comparison of the gene expression profiles of *C. dematium* samples previously stored in a deep freezer or under mineral oil could enable the identification of these biosynthetic aspects.

The distribution of the *TS* gene among the *Colletotrichum* species clearly indicates that movement of the gene, which is essential for taxol production in an organism, was widespread in various *Colletotrichum* spp. To date, at least two *Colletotrichum* spp. have been shown to produce taxol; namely, *C. gloeosporioides* isolated from the leaves of *Justicia gendarussa* [10], *Plumeria acutifolia* [26], and *Tectona grandis* [27], and *C. capsici* isolated from diseased chili pepper fruits [28]. In this study, we identified 6 additional *TS*-bearing *Colletotrichum* spp. with the potential to produce taxol.

Among these 8 different *Colletotrichum* spp. carrying the *TS* gene, we identified *C. dematium* as a new taxol-producing fungal species (Fig. 2). *C. dematium* is an anthracnose-causing pathogen that invades the host stems and leaves [29]. The occurrence of *C. dematium* anthracnose is not limited to a single host plant or specific climatic region [30], and it can infect a wide variety of hosts, including cowpea [31], mulberry [32], onion [33], spinach [34], Japanese radish [35], pepper [36], and soybean [37]. Although the pathogenic *C. dematium* strain (KACC 40013) investigated in this study was isolated from pepper anthracnose, it can be a valuable resource for verifying the genes in the taxol biosynthetic pathway.

It is unclear why the majority of *Colletotrichum* spp. have the ability to produce taxol (based on *TS* gene integration) as a secondary metabolite. One possible explanation is that taxol can work as an antimicrobial agent. Indeed, we found that *TS* gene-transformed *Nicotiana benthamiana* showed increased resistance to *Botrytis cinerea* (data not shown). A previous study reported horizontal gene transfer between *Taxus* spp. and fungal endophytes, resulting in a widely diverse fungal group capable of producing taxol [38]. However, the wide distribution of taxol biosynthesis capability among various fungal species indicates that horizontal gene transfer may have occurred from a fungus to the *Taxus* spp., and that the relatively easy transfer of the genes among fungal species had led to its widespread distribution. We are suspicious of the *TS* gene origin, because the 100% homology among *TS* genes from the *Taxus* spp. and *Colletotrichum* spp. indicates that the gene from the *Taxus* spp. originated from taxol-producing endophytic fungi rather than plants.

In summary, we have developed a rapid and simple method to screen taxol-producing *Colletotrichum* species using the *TS* gene as a molecular marker. After screening several PCR primer pairs, we identified the most sensitive *TS*-gene-specific primer set as TS-2F and TS-2R. In addition to determining the taxonomic distance of 12 different *Colletotrichum* spp. using 18S rRNA ITS sequences, 8 of the species (distributed in different groups in the phylogenic analysis) were identified to contain the *TS* gene. Among these 8 species, *C. dematium* produced the highest amount of taxol, the production capacity of which was determined by the species' storage conditions. *Colletotrichum* spp. are the most devastating pathogens to plant species. Nevertheless, genomic studies of taxol-producing *Colletotrichum* spp. would be very valuable for identification of the taxol biosynthetic genes. Furthermore, the simplicity of the fungal genome structure, in addition to the adaptability of the genes to manipulation by molecular approaches, makes this species a valuable bioresource for improving taxol production and yield, which is important considering the wide spread of cancers among the growing and aging populations worldwide.

ELECTRONIC SUPPLEMENTARY MATERIAL

Supplementary data including one table can be found with this article online http://www.mycobiology.or.kr/src/sm/mb-44-105.s001.pdf.

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