Curr Microbiol (2010) 61:254–260
DOI 10.1007/s00284-010-9604-7

Protoplast Mutation and Genome Shuffling Induce the Endophytic Fungus *Tubercularia sp.* TF5 to Produce New Compounds

Mingzi Wang · Shaosong Liu · Yaoyao Li · Ren Xu · Chunhua Lu · Yuemao Shen

Received: 6 November 2009 / Accepted: 1 February 2010 / Published online: 16 March 2010

Abstract *Tubercularia* sp. TF5 is an endophytic fungal strain isolated from the medicinal plant *Taxus mairei*. Previously, taxol has been detected in the fermentation products of this strain. However, it lost the capability of producing taxol after long-term laboratory culture. Herein, we tried to reactivate the production of taxol by protoplast mutations and genome shuffling. The protoplasts of *Tub.* sp. TF5 were prepared from its mycelia, and mutated by UV and NTG. The mutant strains regenerated from the mutated protoplasts were selected and classified into four groups on the basis of their phenotypes, the profile of their metabolites analyzed by TLC, MS, and bioassay data. Then, genome shuffling was subsequently carried out with eight mutant strains, with two representatives from each protoplast mutant group, and genome shuffling mutant strains were obtained and screened using the same screening procedure. Although taxol has not been detected in any mutant, two important mutants, M-741 and G-444 were selected for metabolites isolation and determination due to their phenotypes, and differences in TLC analysis result from TF5 and other mutants. Three new sesquiterpenoids, namely tubercullariols A–C (**1**–**3**), and a known dihydroisocoumarin (**4**) were obtained from M-741. Eighteen novel compounds were isolated from G-444, including five new sesquiterpenoids (**5**-**9**), two new dihydroisocoumarins (**10**, **11**), one new tetralone (**12**), together with 10 known compounds (**13**–**20**, **1**, and **2**). The compounds isolated from the M-741 and G-444 were different in structure types and substitutions from those of TF5 (**15**, **21**–**29**). The results showed, for the first time, that protoplast mutations and genome shuffling are efficient approaches to mining natural products from endophytic fungi. Understanding the mechanisms of unlocking the biosynthesis of new metabolites will facilitate the manipulation of the secondary metabolism in fungi.

Electronic supplementary material The online version of this article (doi:10.1007/s00284-010-9604-7) contains supplementary material, which is available to authorized users.

M. Wang · S. Liu · Y. Li · R. Xu · C. Lu · Y. Shen (✉)
Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, Xiamen Engineering Research Center of Marine Microbial Drug Discovery, Fujian Laboratory of Pharmaceutical Engineering, School of Life Sciences, Xiamen University, 422 South Siming Road, Xiamen, Fujian 361005, People's Republic of China
e-mail: yshen@xmu.edu.cn

M. Wang
Engineering Research Center of Industrial Microbiology, Ministry of Education, School of Life Sciences, Fujian Normal University, Fuzhou, Fujian 350108, People's Republic of China

Introduction

Endophytic fungi that live inside the tissues or in the intracellular spaces of plants have been known to be rich in novel and bioactive natural products [1–3]. The fungal strain *Tubercularia* sp. TF5 was isolated from the inner bark of *Taxus mairei* collected from Fujian Province, southeast China. Previously, taxol was detected in the metabolites from *Tub.* sp. TF5 by bioassays and MS analysis [4]. However, after long-term laboratory culture, the production of taxol in this strain is not detectable due to strain degeneration. Meanwhile, dozens of non-taxol diterpenoids and cytochalasins were isolated from it [5].

Classic microbial breeding can improve the production of the target compounds through the feedback loop of random mutations and rational selections for accumulating beneficial mutation in a very slow way [6, 7]. Cross-breeding based on hybridizing parents can rapidly change

the characters of offspring, and has been successful in the breed improvement of poultry, livestock, and crops. Due to the lack of sexual hybridization ability, many valuable microorganisms cannot be improved by crossbreeding; however, the genome shuffling technique can simulate sexual hybridization by the combining of random mutations in the whole-genome via the fusion of multi-parent protoplasts, and has been successful in improving antibiotic production in bacteria [8]. Here, we applied the protoplast mutation and genome shuffling breeding technique on *Tub.* sp. TF5, a fungus, for the first time. And expected to restore the production of taxol and activate new secondary metabolism in general.

Materials and Methods

Strains and Culture Medium

The fungal strain *Tubercularia* sp. TF5 was isolated from *Taxus mairei*, and was maintained in 20% glycerol at −70°C in the Laboratory of Microbial Pharmaceutics, School of Life Sciences, Xiamen University. The potato-dextrose-agar (PDA) was used as the culture medium. The taxol-sensitive yeast (*Saccharomyces cerevisiae* AD1-8-tax) was kindly provided by Prof. Himes at the University of Kansas, and cultivated on the YPD medium (yeast extract, peptone, dextrose) as described by Foland [9].

Protoplasts Preparation, Mutagensis, and Mutant Isolation

Protoplasts preparation, mutagensis, and mutant screening were performed by our previous method [10]. The mycelia were collected by filtration and suspended in 10 ml lytic enzyme solution containing 1% lywallzyme (Guangdong Institute of Microbiology, Guangzhou, China), 0.5% cellulase (Sinopharm Chemical Reagent CO., LTD., Shanghai, China) and 0.5 M NaCl in the 0.05 M potassium phosphate buffer, pH 6.0. After incubation for 3–6 h with gentle shaking (50 rpm) at 30°C, protoplasts were produced and collected by filtration through a small mass of cotton under centrifugation for 5 min at 3500 rpm. The protoplast pellets were washed with and resuspended in stabilizer solution (0.5 M NaCl). The purified protoplast suspension was irradiated for 30 min by UV (15 W) at 20 cm in the dark, and followed by treating with 40 μg/ml NTG for 60 min, which was then gradiently diluted 10^4 times with stabilizer, and spreaded 0.1 ml of the diluted solution on PDA regeneration plate containing 0.5 M NaCl as stabilizer. After cultivation for about 6 days at 28°C, 860 colonies were picked up, and transferred onto new slants, respectively. After cultivation for 21 days at 25°C, these strains (M-1–M-860) were screened by colony morphology and TLC analysis of their methanol extracts.

Protoplast Fusion and Genome Shuffling

Genome shuffling by recursive protoplast fusions was carried out using the method modified from the literature [8, 11–13]. The initial strains, M-92, M-99, M-156, M-741, M-749, M-763, M-844, and M-845, from the protoplast mutagenesis library were used for the preparation of protoplasts by the procedure above. Each type of parent protoplast was mixed at equal ratio, and collected by centrifugation (4000 rpm, 5 min). The protoplast pellet was resuspended by tapping in the left solution, and was supplemented with 1 ml 40% PEG (MW 6000) dissolved with stabilizer solution containing 25 mM $CaCl_2$. The protoplast suspension was immediately stirred by pipetting rapidly once in and out of a Pasteur pipette. After being kept still for 15 min at room temperature, the protoplast suspension was gradiently diluted with stabilizer solution to 10^{-4}, 0.1 ml of the suspension solution was inoculated onto one individual PDA regeneration plate containing 0.3 M mannitol. After incubation for 21 days at 25°C, the regenerated strains (G-1–G-1100) were screened by colony morphology and TLC analysis of the methanol extracts.

Screening

All of the colonies from protoplast regeneration plates were transferred onto new slants one by one, and cultivated for 7 days. Then, they were inoculated in a 6-well plate, each well containing 5 ml PDA, and incubated for 21 days at 25°C. After fermentation, the cultivated agar was chopped, diced, and extracted with 5 ml methanol.

All extract solutions were loaded onto thin layer chromatography (TLC) plates precoated with silica gel 60 F254, and developed by chloroform: methanol (20:1, v/v). The developed TLC plates were stained by spraying 5% vanillin and 5% H_2SO_4 in anhydrous ethanol. The methanol extract solution was filtrated with a syringe filter unit (0.22 μm) and analyzed by electrospray ionization/tandem mass spectrometry (ESI–MS/MS).

The screening method for the taxol-producing strain was modified from Foland [9]. The assay was performed in liquid YPD medium in aseptic 96-well plates. Each well contained 100 μl YPD medium supplemented with 100 U penicillin and 100 μg streptomycin. The taxol-sensitive yeast (*Saccharomyces cerevisiae* AD1-8-tax) was inoculated into each well by adding 10 μl broth cultivated for 48 h in YPD medium, and then 10 μl methanol extract solution was added. The plates were incubated for 48 h at 30°C with gentle shaking (100 rpm). After incubation, the optical density was read at 610 nm in a 96-well plate

reader. Taxol isolated in our laboratory was used as a positive control and methanol as a blank control.

Product Isolation

The strains TF5, M-741 and G-444 were cultivated on PDA plates for 21 days at 25°C. The cultures were extracted three times with equal volume of AcOEt–MeOH–AcOH (80:15:5, v/v/v) at room temperature. After the removal of solvents under vacuum, the extracts were purified by repeated column chromatography (*RP-18*, *Sephadex LH-20*, and silica gel) as described [5, 10], respectively. The structure of the isolated compounds was determined by high-resolution fast atom bombardment MS and by 1D (^{1}H, ^{13}C, DEPT) and 2D (HSQC, HMBC, ^{1}H-^{1}H COSY, NOESY) NMR spectroscopy. The structure elucidation of compounds **5**–**20** and **29** will be reported in forthcoming papers.

Results

The Preparation and Regeneration of Protoplasts

The hyphae of *Tub.* sp.TF5 contained many cells with dense and complex cell wall. Outside of the hyphae is a sheath composed of complex polysaccharide and protein. This firm sheath made digestion of the hyphae difficult by any kind of cell wall hydrolytic enzymes, so it was difficult to obtain highly active protoplasts of *Tub.* sp.TF5 in large quantities. But protoplasts with high quantity, quality, purity, and activity are the most important factor for successful protoplast breeding including mutagenesis, fusion, regeneration, as well as genome shuffling. The preparation of desired protoplasts from the mycelia of *Tub.* sp. TF5 is difficult by general procedure [14] due to the roughness of mycelia and the denseness of the cell walls. There are many factors affecting protoplast formation, e.g., mycelium age, cell wall hydrolytic enzymes, stabilizer, hydrolytic time, buffer compositions, and other conditions including temperature, pH, rotation, and air. According to the results of pre-trial experiments (data not shown), the most significant factors that affect the preparation of protoplasts from the mycelia of *Tub.* sp. TF5 are lywallzyme (A), cellulase (B), snail enzyme (C), and hydrolytic time (D). Therefore, these four factors were selected for orthogonal design and optimization at three levels. The orthogonal design was shown as: A1, 2, 3 represent containing lywallzyme 0, 0.25, 0.5%, respectively; B1, 2, 3 represent containing cellulase 0, 0.5, 1.0%, respectively; C1, 2, 3 represent containing snail enzyme 0, 0.25, 0.5%; D1, 2, 3 represent hydrolytic time 3, 6, 9 h, respectively. (Table S1 of supplementary material). The relative importance of the each factor above affecting protoplast preparation according to the Range (R) value is in the order R_A (23.5×10^4) > R_B (18.5×10^4) > R_C (11.7×10^4) > R_D(11.3×10^4). R value analysis revealed that factor A(lywallzyme) had the greatest impact on the protoplast preparation, therefore, it is the dominant factor. K value analysis revealed optimal level of each factor and the results showed that the optimized combination was $A_3B_2C_1D_3$, that is 0.5% lywallzyme + 0.5% cellulase + 0% snail enzyme + hydrolytic time 9 h, and showed that the factor A (lywallzyme) affected most efficiently, indicating that increasing the concentration of lywallzyme (A) could improve the yield of protoplast further. Similarly, increasing hydrolytic time (D) could increase the yield of protoplast as well (Table S1 of supplementary material). But considering protoplast activity and the efficiency of protoplast regeneration, hydrolytic time cannot be too long because protoplast broke and the activity reduced rapidly due to the poisoning effects of buffer.

The orthogonal design for optimization of TF5 protoplast regeneration was shown as: A1, 2, 3 represent lywallzyme percentage 0, 0.5, 1.0%, respectively; B1, 2, 3 represent cellulase percentage 0, 0.25, 0.5%, respectively; C1, 2, 3 represent hydrolytic time 3, 6, 9 h, respectively (Table S2 of supplementary material). The results showed that although factor B(cellulase) is favored in TF5 protoplast preparation, it reduced the regeneration rate. The analysis of R values indicated that these three factors affected the protoplast regeneration in the order, R_B (7.3%) > R_A(5.4%) > R_D (2.3%), the concentration of cellulase (B) has the most significant effect, and that the optimized combination is $A_3B_2D_2$, that is, 1.0% lywallzyme + 0.25% cellulase + hydrolytic time 6 h (Table S2 of supplementary material).

Under the optimal conditions, the mycelia of TF5 lysed and began to release the protoplasts at 2-h post-treatment (Fig. 1A, a). After 6 h, nearly all mycelia were converted into protoplasts (Fig. 1A, b). The protoplasts swelled and underwent breakdown in water or common medium and could not generate colonies. NaCl (0.5 M) can stabilize the protoplasts to regenerate a complete cell wall, thus forming renewable colonies (Fig. 1A, c).

In addition to the preparation of highly active protoplasts, there are many other factors that affect on the protoplast regeneration rate, such as regeneration medium, hypertonic solution, cell membrane stabilizer, and regeneration conditions (temperature and pH). Regeneration medium with hypertonic NaCl solution is able to maintain the solution environment, and its concentration showed a great influence on protoplast regeneration. Concentrations below 0.2 M or above 0.5 M inhibited the regeneration rate (Fig. 1b). Hypertonic solutions supplemented with different additives have significant impacts on protoplast regeneration. At the same concentration 0.3 M, mannitol was the best compared to sodium chloride, potassium

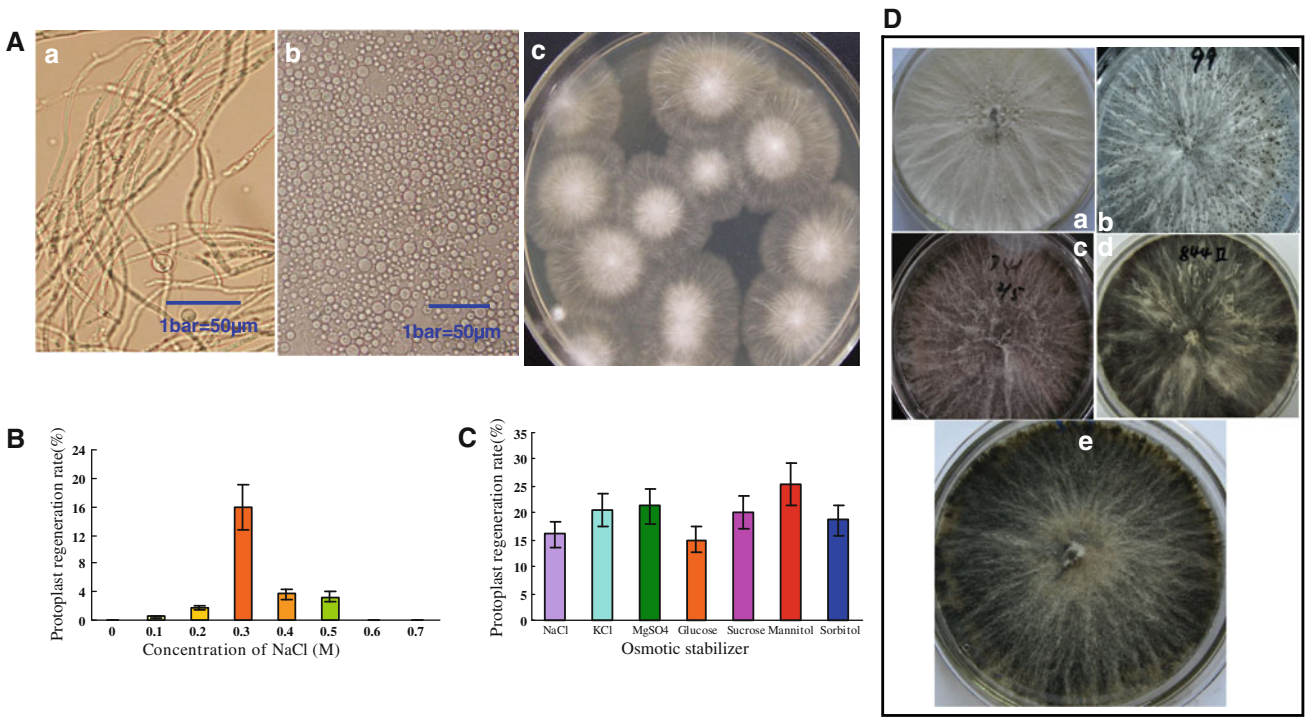

Fig. 1 The results of protoplasts preparation, regeneration, mutagensis, and genome shuffling of the *Tubercularia* sp. TF5 strain. **A** The representative mycelia (*a*), protoplasts (*b*), and the regenerated colonies (*c*). **B** The protoplast regeneration rates of the strain TF5 in different concentrations of NaCl as stabilizer. **C** The protoplast regeneration rates of the strain TF5 in different stabilizers at 0.3 M. **D** The typical colonies of the mutants obtained from protoplast mutation and genome shuffling of the strain TF5. *a* initial stain (TF5), *b* M-99, *c* M-741, *d* M-844, *e* G-444

chloride, magnesium sulfate, glucose, sucrose, and sorbitol, respectively (Fig. 1c).

Protoplast Mutation and Mutant Screening

The protoplast mutations by UV and NTG produced 860 mutagenesis strains (M-1–M-866). They were divided into four groups according to their colony morphology: (a) white mycelia and colony without stroma (Fig. 1D, a), the same as TF5; (b) white mycelia and colony with plenty of small stroma (Fig. 1D, b); (c) black and slim mycelia and colony without stroma (Fig. 1D, c); (d) black and coarse mycelia and colony with black stripe (Fig. 1D, d). Representatives of each group were screened by bioassays and by analysis of TLC and MS. Colonies of both mutant strains M-741 and M-844 were black, no stroma and non-spore-producing. Taxol was not detected in the strains TF5, M-741 and M-844 (data not showed). However, TLC and MS analysis of the strains M-741 and M-844 showed a great variation between their metabolites.

Genome Shuffling and Mutant Screening

The genome shuffling of eight parent strains M-92, M-99, M-156, M-741, M-749, M-763, M-844, and M-845 produced 1100 strains. TLC analysis with authentic taxol as a positive control indicated that the strains G-372, G-442, G-444, G-776, G-803, G-810, G-818, and G-827 produced compounds that had the same R_f value as taxol, but all of them were excluded by bioassay and MS analysis (data not shown).

In biosynthetic pathways, structural genes, resistance genes, and regulatory genes are usually tandemly clustered, so the resistance (sensitivity) of different strains to a metabolic product may represent a different production. Therefore, we examined and compared the taxol resistance of strains TF5 and G-444 to different taxol concentrations in liquid PDA medium. Both of them can endure doses of taxol less than 30 μg/ml and significant effect of the concentration gradient is observed. When the taxol concentration was higher than 40 μg/ml, the growth of both strains was significantly inhibited with the same level of resistance on taxol. Similar results were obtained from other genome shuffling mutants, indicating that resistance cannot be used for screening taxol-producing strains.

However, the strain G-444 was different from TF5 in TLC analysis and morphology, since it was black, without stroma and was a non-spore colony (Fig. 1D, e); therefore it was selected for metabolites isolation.

Metabolites of the Strains M-741, G-444, and TF5

Both colonies of mutant strains M-741 and G-444 are black, and they do not form a stroma or spores. Taxol was

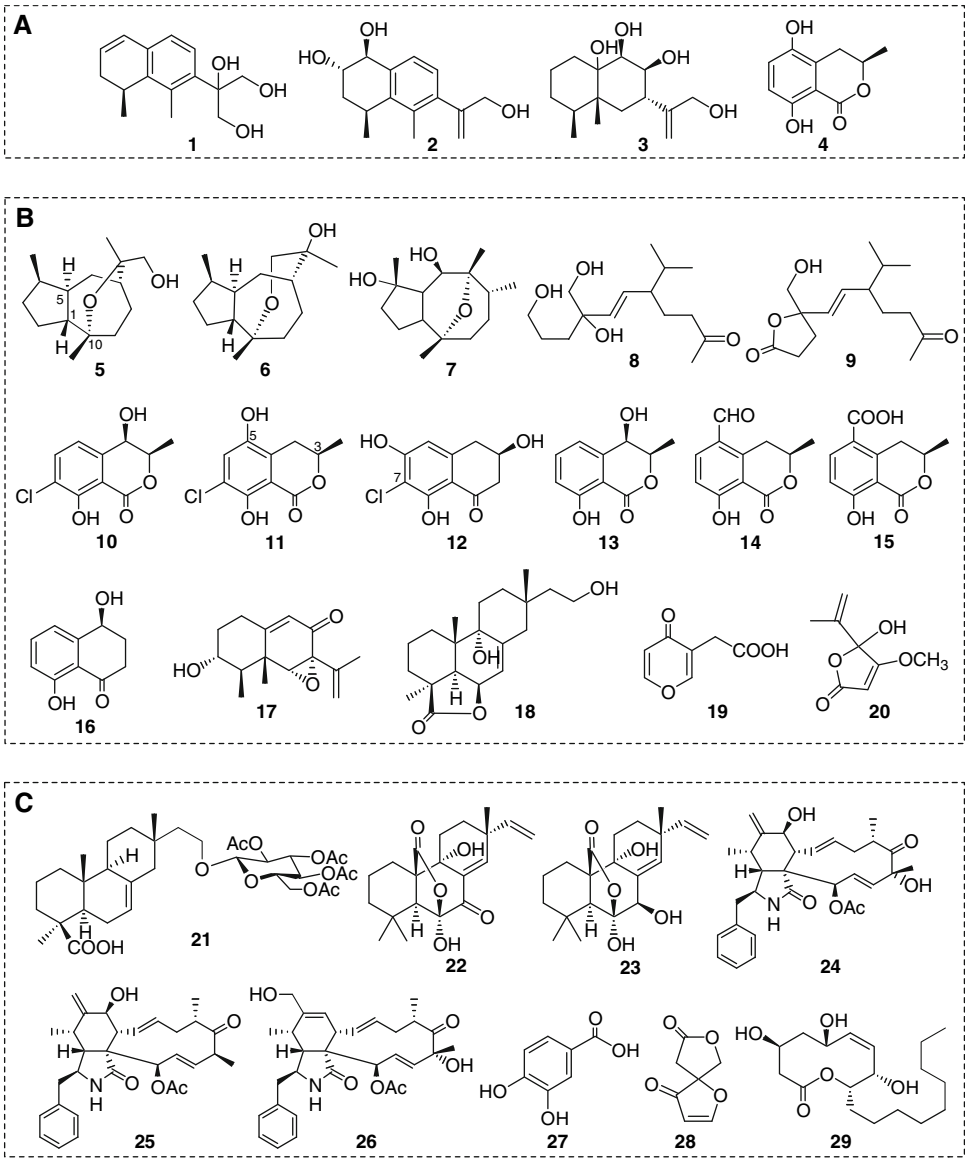

Fig. 2 The structures of metabolites from the strains M-741 (**a**), G-444 (**b**), and TF5 (**c**)

not detected in the strains TF5, M-741, and G-444 by bioassay, TLC and MS analysis. However, TLC, and MS analysis showed great differences in the metabolites of the strains M-741 and G-444 from TF5.

Accordingly, through fermentation and metabolite isolation, three new sesquiterpenoids belonging to eremophilane-type, tubercullariols A–C (**1**–**3**) [10], and a known dihydroisocoumarin, 5-hydroxymellein (**4**) were isolated from the strain M-741 (Fig. 2a). Eighteen compounds were isolated from the strain G-444 (Fig. 2b), including five new sesquiterpenoids, namely 10,11-epoxy-12-guaianol (**5**), 10,12-epoxy-11-guaianol (**6**), a new backbone sesquiterpene rearranged from guaiane (**7**), two 1,5/1,10-diseco- guaianes (**8**, **9**), two new dihydroisocoumarins, 7-chloro-4-hydroxymellein (**10**), and 7-chloro-5-hydroxymellein (**11**), and one new tetralone, 7-chloroscytalone (**12**), together with 10 known compounds, namely 4-hydroxymellein (**13**), 5-formylmellein (**14**), 5-carboxymellein (**15**), (4*S*)-4-Hydroxy-α-tetralone (**16**), sporogen-AO1 (**17**), hymatoxin E (**18**), 4-oxo-4H-pyran-3-acetic acid (**19**), penicillic acid (**20**) (Fig. 2b), and **1** and **2** as well. In contrast, one new diterpene glycoside (**21**) named 16-tetra-*O*-acetyl-D-glucopyranosyl- hymatoxin C, along with eight known glycosides, including two diterpenoids (**22**, **23**), three cytochalasins (**24**, **25**, **26**), a dihydroisocoumarin (**15**), two C7-tetraketides (**27**, **28**) [5], and one new 10-membered macrolide (**29**) (Xu et al., *Chem Nat Prod*, in press) were isolated from *Tub.* sp. TF5 (Fig. 2c).

Discussion

The production of secondary metabolites in filamentous fungi is usually associated with the advent of sporulation

and development [15, 16]. The process of secondary metabolites biosynthesis is strictly regulated to ensure that the production of secondary metabolites is beneficial [17]. There are two types of regulatory elements for secondary metabolisms: global regulators and specific regulators. The global regulators can uncouple sporulation and secondary metabolism. They are extremely desirable because they have broad specificity for the activation or repression of entire families of secondary metabolite gene clusters. Activation of such regulatory elements would increase the production of many secondary metabolites [18, 19]. The pathway specific regulators only control a single pathway or a few specific metabolic pathways and the production of corresponding metabolites [20, 21].

Both of the mutant strains M-741 and G-444 showed black colonies and this is different from the initial strain *Tub.* sp. TF5. The analysis of TLC and MS, together with the metabolites isolated showed that the products of the strains M-741 and G-444 were different from the strain TF5 (Fig. 2). Although dihydroisocoumarins were isolated from all three strains, tetralones (**12**, **16**) [22] that are involved in the biosynthesis of melanin were isolated only from G-444, and this is consistent with the black appearance of the colonies. Moreover, the structure types of compounds isolated from G-444 are more diverse than TF5. These results revealed that certain global regulators could be activated by protoplast mutation and genome shuffling. Indeed, the similar phenomenon was observed in the variation of susceptive fungus *Monosporascus cannonballus*. This fungus often undergoes hypovirulence degenerative mutations associated with pigmentation and metabolites shifting due to dsRNA infection [23]. Additionally, the differences between the metabolites of these mutants and TF5 persist not only in the structure types, but also in substitutions. Three chlorine-containing compounds (**10**, **11**, **12**) of extreme interest due to the rarity of halogenated natural products in terrestrial microorganisms were also isolated from G-444 (Fig. 2b).

Overall, our results indicated that, besides its capability to improve the yield of target products against convention, protoplast mutation, and genome shuffling are also efficient in activating secondary metabolisms in general. This microbial breeding method could be a new strategy for natural product mining. In parallel, understanding the activation mechanisms evoked by genome shuffling would help rationally manipulate secondary metabolism since their regulatory networks are quite complicated and not readily disentangled by conventional approaches in fungi [16, 17, 24].

Acknowledgments We are grateful to Prof. Himes at the University of Kansas for generosity in donating the taxol-sensitive yeast strain (*Saccharomyces cerevisiae* AD1-8-tax). This work was supported by 863 Programs (No. 2007AA091503).

References

1. Ting L, Xiang L, Chunhua L, Zhiyu H, Wenyu H et al (2009) Secondary metabolites of *Phomopsis* sp. XZ-26, an endophytic fungus from *Camptotheca acuminate*. Eur J Org Chem 2009:2975–2982
2. Kumaran RS, Hur BK (2009) Screening of species of the endophytic fungus Phomopsis for the production of the anticancer drug taxol. Biotechnol Appl Biochem 54:21–30
3. Zhang H-W, Song Y-C, Tan R-X (2006) Biology and chemistry of endophytes. Nat Prod Rep 23:753–771
4. Wang J, Li G, Lu H, Zheng Z, Huang Y et al (2000) Taxol from Tubercularia sp. strain TF5, an endophytic fungus of *Taxus mairei*. FEMS Microbiol Lett 193:249–253
5. Li Y, Lu C, Hu Z, Huang Y, Shen Y (2009) Secondary metabolites of Tubercularia sp. TF5, an endophytic fungal strain of *Taxus mairei*. Nat Prod Res 23:70–76
6. Patnaik R (2008) Engineering complex phenotypes in industrial strains. Biotechnol Prog 24:38–47
7. Parekh S, Vinci VA, Strobel RJ (2000) Improvement of microbial strains and fermentation processes. Appl Microbiol Biotechnol 54:287–301
8. Zhang YX, Perry K, Vinci VA, Powell K, Stemmer WP et al (2002) Genome shuffling leads to rapid phenotypic improvement in bacteria. Nature 415:644–646
9. Foland TB, Dentler WL, Suprenant KA, Gupta ML Jr, Himes RH (2005) Paclitaxel-induced microtubule stabilization causes mitotic block and apoptotic-like cell death in a paclitaxel-sensitive strain of *Saccharomyces cerevisiae*. Yeast 22:971–978
10. Ren X, Ming-Zi W, Chun-Hua L, Zhong-Hui Z, Yue-Mao S (2009) Tubercularіols A–C, new sesquiterpenes from the mutant strain M-741 of *Tubercularia* sp. TF5. Helv Chim Acta 92:1514–1519
11. Patnaik R, Louie S, Gavrilovic V, Perry K, Stemmer WP et al (2002) Genome shuffling of Lactobacillus for improved acid tolerance. Nat Biotechnol 20:707–712
12. Dai M, Copley SD (2004) Genome shuffling improves degradation of the anthropogenic pesticide pentachlorophenol by *Sphingobium chlorophenolicum* ATCC 39723. Appl Environ Microbiol 70:2391–2397
13. Hatvani L, Manczinger L, Kredics L, Szekeres A, Antal Z et al (2006) Production of Trichoderma strains with pesticide-polyresistance by mutagenesis and protoplast fusion. Antonie Van Leeuwenhoek 89:387–393
14. Kevei F, Peberdy JF (1984) Further studies on protoplast fusion and interspecific hybridization within the *Aspergillus nidulans* group. J Gen Microbiol 130:2229–2236
15. Wei GH, Liu DP, Liang CC (2004) Charting gene regulatory networks: strategies, challenges and perspectives. Biochem J 381:1–12
16. Bok JW, Keller NP (2004) LaeA, a regulator of secondary metabolism in Aspergillus spp. Eukaryot Cell 3:527–535
17. Schardl CL (2006) A global view of metabolites. Chem Biol 13:5–6
18. Kosalkova K, Garcia-Estrada C, Ullan RV, Godio RP, Feltrer R et al (2009) The global regulator LaeA controls penicillin biosynthesis, pigmentation and sporulation, but not roquefortine C synthesis in *Penicillium chrysogenum*. Biochimie 91:214–225
19. Bouhired S, Weber M, Kempf-Sontag A, Keller NP, Hoffmeister D (2007) Accurate prediction of the *Aspergillus nidulans*

terrequinone gene cluster boundaries using the transcriptional regulator LaeA. Fungal Genet Biol 44:1134–1145
20. Brakhage AA, Schuemann J, Bergmann S, Scherlach K, Schroeckh V et al (2008) Activation of fungal silent gene clusters: a new avenue to drug discovery. Prog Drug Res 66(1):3–12
21. Smith CA, Woloshuk CP, Robertson D, Payne GA (2007) Silencing of the aflatoxin gene cluster in a diploid strain of *Aspergillus flavus* is suppressed by ectopic aflR expression. Genetics 176:2077–2086
22. Wheeler MH, Abramczyk D, Puckhaber LS, Naruse M, Ebizuka Y et al (2008) New biosynthetic step in the melanin pathway of Wangiella (Exophiala) dermatitidis: evidence for 2-Acetyl-1, 3, 6, 8-Tetrahydroxynaphthalene as a novel precursor. Eukaryot Cell 7:1699–1711
23. Wheeler MH, Bruton BD, Puckhaber LS, Zhang J, Stipanovic RD (2004) Identification of 1, 8-dihydroxynaphthalene melanin in *Monosporascus cannonballus* and the analysis of hexaketide and pentaketide compounds produced by wild-type and pigmented isolates of the fungus. J Agric Food Chem 52:4113–4120
24. Bok JW, Balajee SA, Marr KA, Andes D, Nielsen KF et al (2005) LaeA, a regulator of morphogenetic fungal virulence factors. Eukaryot Cell 4:1574–1582