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Optimization of *Agrobacterium tumefaciens*-mediated transformation method of oleaginous filamentous fungus *Mortierella alpina* on co-cultivation materials choice

Shunxian Wang[a,b], Haiqin Chen[a,b,*], Yuzhuo Wang[a,b], Xin Tang[a,b], Hao Zhang[a,b], Wei Chen[a,b,c,d], Yong Q. Chen[a,b,c,e]

[a] State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, China
[b] School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, China.
[c] National Engineering Research Center for Functional Food, Jiangnan University, Wuxi, Jiangsu 214122, China
[d] Beijing Innovation Centre of Food Nutrition and Human Health, Beijing Technology and Business University (BTBU), Beijing 100048, China
[e] Department of Cancer Biology, Wake Forest School of Medicine, Winston-Salem, NC 27157, USA

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ABSTRACT

The *Agrobacterium tumefaciens*–mediated transformation (ATMT) method is commonly applied in the oleaginous filamentous fungus *Mortierella alpina*. During the ATMT process, the spores of *M. alpina* have traditionally been used as a co-cultivation material, but their long spore-producing cycle and low sporulation rate make the transformation process tedious. This study explores the use of germinating spores, mycelium and single solid colonies of uracil auxotrophic *M. alpina* CCFM501 as a co-cultivation material with *A. tumefaciens* AGL1. The results show that *A. tumefaciens* AGL1 can successfully transform the germinating spores, mycelium and single solid colonies of *M. alpina*. In addition, the transformation rate of the germinating spores was 50% higher than that of the fresh spores. Due to its concise preparation process, the mycelium was chosen as a co-cultivation material for two plasmids of different lengths and proven to be an efficient co-cultivation material for *M. alpina*.

1. Introduction

The oleaginous filamentous fungus *Mortierella alpina* can produce a high content of polyunsaturated fatty acids and was certified in 2006 as 'generally regarded as safe' (GRN No.94) by the United States Food and Drug Administration for the production of arachidonic acids. A proper DNA transformation system is needed to manipulate *M. alpina* to produce various fatty acids. The published transformation methods of *M.alpina* include particle bombardment (Takeno et al., 2004), protoplast transformation (Mackenzie et al., 2000) and the *Agrobacterium tumefaciens*–mediated transformation (ATMT) method (Ando et al., 2009). The process of preparing materials for particle bombardment is tedious and expensive. And the protoplasts of *M.alpina* are difficult to obtain. Besides, the basic principle of the two methods is transforming plasmids with a selection marker, whether reserving in the cytoplasm or being integrated into the genome via homologous recombination. The selection pressure for non-integrating plasmids and the low homologous recombination rate of fungal cells both result in unstable and inefficient transformation. Thus, the ATMT method is currently more often used in *M. alpina*.

The accurate principle of the ATMT method still remains unclear. Gram-negative *A. tumefaciens* contains a virulence plasmid and a binary plasmid. In the presence of acetosyringone, *vir* genes on the virulence plasmid are activated to cut, protect, move and integrate T-DNA – the sequence between the left and right borders of binary plasmid – into the host's genome (de Groot et al., 1998). The ATMT method for *M. alpina* was first established in 2009 by Shimizu et al. (Ando et al., 2009), who used *A. tumefaciens* C58C1 to transform the spores of *M. alpina* 1S-4 for the production of eicosapentaenoic acid. In 2014, a new *M. alpina* species, *M. alpina* ATCC32222, was also transformed with the same *A. tumefaciens* (Hao et al., 2014). Both teams used *A. tumefaciens* C58C1, and the co-cultivation material of *M. alpina* was fresh spores. In the co-cultivation phase of the original case with *A. tumefaciens* C58C1 transforming fresh spores of *M. alpina,* we found that the growth of fresh spores hardly established its competence over *A. tumefaciens* C58C1 even after adjusting for the high ratios between spores and *A.*

* Corresponding author at: School of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, China.
E-mail addresses: 7140112027@vip.jiangnan.edu.cn (S. Wang), haiqinchen@jiangnan.edu.cn (H. Chen), 61701120284@stu.jiangnan.edu.cn (Y. Wang), xintang@jiangnan.edu.cn (X. Tang), zhanghao@jiangnan.edu.cn (H. Zhang), weichen@jiangnan.edu.cn (W. Chen), yqchen@jiangnan.edu.cn (Y.Q. Chen).

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Table 1
Filamentous fungi with different co-cultivation materials that has been successfully transformed with *A.tumefaciens*.

Fungal species	Selection marker	Starting material	References
Ascomycetes			
Aspergilus awamori	*hph, BLE,* Aa *pyrG*	Protoplasts, conidia	(de Groot et al., 1998, Gouka et al., 1999, Michielse et al., 2004a, 2004b)
A. giganteus	*hph*	Conidia, germinated conidia	(Meyer et al., 2003)
Beauveria dermatiditis	*hph, Hc ura5*	Yeast-like cells, germinated conidia	(Brandhorst et al., 2002, Sullivan et al., 2002)
Coccidiodes immitis	*hph*	Protoplasts, germinated conidia	(Abuodeh et al., 2000)
Fusarium venenatum	*hph*	Conidia, freeze-dried mycelium	(de Groot et al., 1998)
Histoplasma capsulatum	*hph, Hc ura5*	Yeast-like cells, germinated conidia	(Sullivan et al., 2002)
Mycosphaerella graminicola	*hph*	Protoplasts, yeast-like cells	(Zwiers and De Waard, 2001)
Basidiomycetes			
Agaricus bisporus	*hph*	Germinating conidia, mycelium, fruiting body tissue	(Chen et al., 2000, de Groot et al., 1998 and Mikosch et al., 2001)
Oomycetes			
Phytophthora palmivora	*nptII, hph*	Germinated conidia, mycelium	(Vijn and Govers, 2003)

Adapted from Michielse et al., 2005.

tumefaciens. This resulted in a short attachment time and low transformation efficiency. To overcome this problem, we selected an *A. tumefaciens* strain with a higher virulence to improve *M. alpina's* growth advantage by selecting its more vegetative cells.

A. tumefaciens species that have been successfully applied in the transformation of filamentous fungi include *A. tumeaciens* LBA4404 (Kong et al., 2002), LBA1100 (de Groot et al., 1998), EHA105 (Zhang et al., 2013), GV3101 (Zhao and Li, 2006), C58C1 (Ando et al., 2009) and AGL1 (Staats et al., 2007). AGL1 stands out for its strong virulence. When transforming the mycelium of *Sporothrix schenckii*, AGL1 achieved the best result, with a four-fold increase in transformation efficiency of EHA105 and a ten-fold increase of LBA4404 (Zhang et al., 2011). In some plants, AGL1 also transform more host cells than other species (Chabaud et al., 2003), which indicates that AGL1 possesses some advantage in transforming different types of recipient cell.

In previous studies, *A. tumefaciens* showed the ability to transform spores and germinating conidia, protoplast, mycelium and single solid colonies of other filamentous fungi (Table 1) (Michielse et al., 2005; Pardo et al., 2002). However, transformation efficiency of different recipient cell types does not show the same consistency. The transformation efficiency of *Agaricus bisporus* is greatly determined by the choice of co-cultivation materials. The transformation efficiency of vegetative and fruiting body mycelia is much higher than that of germinated conidia (Chen et al., 2000; de Groot et al., 1998; Mikosch et al., 2001). In *Coccidioides immitis*, prolonged germination is necessary for transformation (Abuodeh et al., 2000), whilst in *Rhizopus oryzae*, only the protoplast can be successfully transformed (Michielse et al., 2004a,b). Traditional parameters that could affect the transformation rate, including the concentration of acetosyringone, the co-cultivation temperature, the co-cultivation time and the concentration of spores, were explored by the above two teams. Whether other forms of co-cultivation materials, such as germinating spores, mycelium and single-solid colonies other than fresh spores, could have positive effects on the transformation efficiency of *M. alpina* is unknown.

This article also describes for the first time the use of *A. tumefaciens* AGL1 to transform *M. alpina.* The use of germinating spores, hyphae and single solid colonies of *M. alpina* as co-cultivation materials for ATMT are first explored, and their transformation efficiencies are compared to those of fresh spores. The process of preparing different co-cultivation materials is also explored.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

A. tumefaciens AGL1 was previously reserved in our laboratory (Culture Collection of Food Microorganism of Jiangnan University, Wuxi, China). The uracil auxotrophic strain *M. alpina* ATCC3222 was previously isolated and named MAU1 (CGMCC No. 8414). The binary plasmid pBIG2-ura5-Its (Chinese patent CN103571762A) with ura5 gene was previously isolated.

YEP medium (10 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCL) was used to cultivate *A. tumefaciens* AGL1, and 100 μg/mL kanamycin and 100 μg/mL rifampicin were added when needed. Broth medium (20 g/L glucose, 5 g/L yeast extract, 1 g/L KH_2PO_4, 0.25 g/L $MgSO_4$, 10 g/L KNO_3) was used to cultivate *M. alpina*, and 0.1 g/L uracil was added when needed. GY medium, MM medium and IM medium were the same as in previous studies. YPG medium (3 g/L yeast extract, 10 g/L peptone, 20 g/L glucose) (Bartnicki and Nickerson, 1962) was used for spore germination.

2.2. Agrobacterium-mediated transformation of different co-cultivation materials

The process of *Agrobacterium*-mediated transformation of *M. alpina* was shown in Fig. 1. Streak conserved AGL1 on YEP solid medium with 100 μg/ml of kanamycin and 100 μg/ml of rifampicin. After 2 to 4 days cultivation at 28 °C, a single colony was put in liquid YEP medium with 100 μg/ml of kanamycin and 100 μg/ml of rifampicin and cultivated at 200 rpm for 2 or 3 days at 28 °C. 1% of this medium was transferred into MM medium for 48 h. The OD600 value was then measured, and the calculated amount of the MM medium was added into the IM medium to adjust the OD600 value to 0.3. Cultivation in the same conditions was continued for 8 to 12 h, until the OD600 value to 0.8–1.2. Then, 100 μl diluted AGL1 with different concentrations (OD600 value 0.2, 0.4, 0.6) was mixed with the prepared co-cultivation materials (with exception of mycelium and single solid colonies) of the host cells for further use. The specific processes for the preparation of different forms of the host cells are as follows.

2.3. Fresh spores collection

The steps to produce spores and ATMT with spores are as follows, with slight adjustment from previous studies (Hao et al., 2014). The spores were collected with 15 ml of liquid MM medium from GY slants (uracil added) after 14 days of cultivation at 28 °C and 14 days of cultivation at 4 °C. The spores were centrifuged at 12,000 g for 20 min and suspended with a specific volume of MM medium (uracil added) to 10^7/ml. *A. tumefaciens* (100 μl) and 100 μl of fresh spores were mixed together and transferred into IM solid medium with a cellophane membrane on the surface. The IM solid medium was cultivated at 23 °C for 2 to 4 days, and the cellophane membrane was transferred into the SC medium. When single colonies appeared on the selection medium, they were transferred to new SC medium.

2.4. Germinating spores collection

With the exception of the germination steps, the steps for producing