Agrobacterium-MEDIATED TRANSFORMATION OF *Trichoderma reesei* OVEREXPRESSING THE *FAD2* GENE

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ABSTRACT

*Fatty acids are the main groups of components of plant membrane lipid and seed storage lipid, it is also the major source of energy in plant. According to bioinformation analysis of the cDNA sequence, the specific fragment of fatty acid desaturase gene (FAD2) from immature maize embryos was isolated by RT-PCR (*Reverse transcription polymerase chain reaction)*. Results of sequence analysis indicate that FAD2 fragment contains the open reading frame of 1,236 bp coding for the 46 kD polypeptide. Transgenic Trichoderma reesei Rut-C30 strains, over-expressing the FAD2 gene from maize were generated by Agrobacterium tumefaciens-mediated transformation. The presence of hph and FAD2 gene in the transformants were confirmed by polymerase chain reaction (PCR) analysis. The expression of the FAD2 gene of the transgenes from Trichoderma reesei and E. coli BL21 were demonstrated by SDS-PAGE.*

Keywords: *Agrobacterium tumefaciens, FAD2* gene; HPLC; *Trichoderma reesei*

INTRODUCTION

Trichoderma reesei is a biotechnically essential filamentous fungus known as an efficient producer of enzymes and proteins. Because of its ability to secrete enzymes in high yields, *T. reesei* has been exploited as an industrial host for homologous and heterologous protein production (0 1998;0). Though large amounts of homologous proteins can be found from this organism, the production of heterologous proteins is often rather low (Conesa *et al*., 2001; Punt *et al*., 2002). Consequently, isolation of the genes consisted in protein secretion and the characterization of their role and pathways are crucial for fungal molecular biology, which may be approached by genetic mutagenesis analysis. Besides, the establishment of high throughput methods for discovering gene function in this production organism is becoming an essential requirement. Among diverse strategies for functional genomics in fungi, insertional mutagenesis has proven to be assigning a stimulating approach. A function to regulate the expression/secretion of enzymes will be a fundamental highlight in the search for new therapeutic strategies in the revelation of new drug targets, and therefore, the microorganisms that are harmful to human being will be controlled. More recently, insertional mutagenesis via *Agrobacterium*-mediated transformation (AMT) has been successful in natural plant hosts as well as in yeast (Bundock *et al*., 1995), *Trichoderma reesei* (Yaohua *et al*., 2001) and even human cells (Kunik *et al*., 2001). AMT has been tested by simple and reproducible filamentous fungal transformation method in many presentations (Michielse *et al*., 2005). Furthermore, the response of *Trichoderma* to its host are also explained to include stress response, nitrogen shortage response, cross pathway control, lipid metabolism, and signaling proceeding (Seidl *et al*., 2009b).

synthesized by Sangon (Shanghai, China). γ-, α- tocopherols, S-Adenosylmethionine (SAM) was purchased from Sigma (Deisenhofen, Germany). Chromatographic materials and columns were obtained from Agilent Technologies, USA. All other chemical reagents were of analytical purity.

Primer design

The following sequences are designed by Primer Premier 5.0 software (Table 1).

Table 1. Primers used in *FAD2* gene

Primers	Sequences (5'-3')
P1ZmFAD2	TATTATATGGGTGCCGGCGGCAGGATGACCGAG
P2ZmFAD2	GCGCGCTAGAACTTCTTGTTGTACCAGAAGACG
P3ZmFAD2	TTAATT(*Pac*I)AAG**ATG**GGTGCCGGCGGCAGG
P4ZmFAD2	GATATC(*Eco*RV)**TTA**CTAGAACTTCTTGTTGTACCAGAAGACG
P5ZmFAD2	GGATCC(*Bam*HI) G**ATG**GGTGCCGGCGGCAGG
P6ZmFAD2	GTCGAC(*Sal*I)**TTACTA**GAACTTCTTGTTGTACCAGAAGACG
P1hph	TGGATATGTCCTGCGGGTAAATAG
P2hph	ATTTGTGTACGCCCGACAGTCC

RNA isolation and reverse transcription PCR

Total RNA was isolated from high oil maize (*Zea mays*) inbred line 4K261 was used for 1st strand cDNA synthesis reaction by using PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. RNA samples (0.5-1g) were vacuum dried and used for RT (reverse transcriptase) reactions. 10 µL of PCR mixture containing 5 µL of Premix Taq Version 2.0 (TaKaRa), 1.0 µL of dNTP (10 mmol/L), 20 pmol of forward primer P1ZmFAD2, 20 pmol of reverse primer P2ZmFAD2 (Table 1) and 5 ng of template DNA were used. The experimental conditions of RT-PCR were 95°C for 5 min, then 30 cycles of 95°C 30 s, 60°C 30 s, 72°C 1.5 min, followed by a final extension at 72°C for another 10 min.

Transformation plasmid into *Escherichia coli* -DH5a strain

Amplification products were fractionated on 1% agarose gel from which the selected band was purified. The amplified DNA was inserted into the pMD19-T vector (TaKaRa) and transformed into *E. coli* DH5α. One hundred microlitres of bacteria stock solution were mixed with 100ng plasmid, chilled on ice for 30 min and put in 42°C water bath for 2 min. The mixture was put immediately on ice again for about 2 min, incubated in LB broth in shaking incubator (160 rpm) for 60 min at 37°C and streaked onto different LB agar plates containing ampicillin (100 µg/ml). The plates were incubated overnight at 37°C and analysed. The positive plaques were identified by PCR and then sequenced by Jieli Biotech (Shanghai, China). The negative control would be untransformed *E. coli* strain. The bacteria plate was sub-cultured once every week to make sure that the bacteria were fresh before every plasmid extraction process.

Vector construction

FAD2 gene was isolated from total RNA of *maize* seed was described in detail previously. The full coding region of *FAD2* gene was amplified by PCR using pFAD2 plasmid as template. The upstream primer P3ZmFAD2 was underlined is *Pac*I site and a translational start codon and the downstream primer was P4ZmFAD2 underlined is *Eco*RV site and the stop codon (Table 1). The PCR product was

cloned into pMD19-T vector to generate pFAD2. The pFAD2 plasmid was digested with *Pac*I and *Eco*RV. The fragments of product were purified and cloned into the vector pWE32F00 to obtain the express vector pPK5-FAD2 (Fig. 2). The plasmids were then transformed into *T. reesei* Rut-C30 by *Agrobacterium*-mediated transformation.

The full-length cDNA of *FAD2* was amplified by PCR with forward primer P5ZmFAD2 and the reverse primer P6ZmFAD2 (Table 1). The fragments were digested by the *Bam*HI and *Sal*I enzymes and then ligated into the pET28a vector, generating the plasmid pET-FAD2 (Fig. 3)

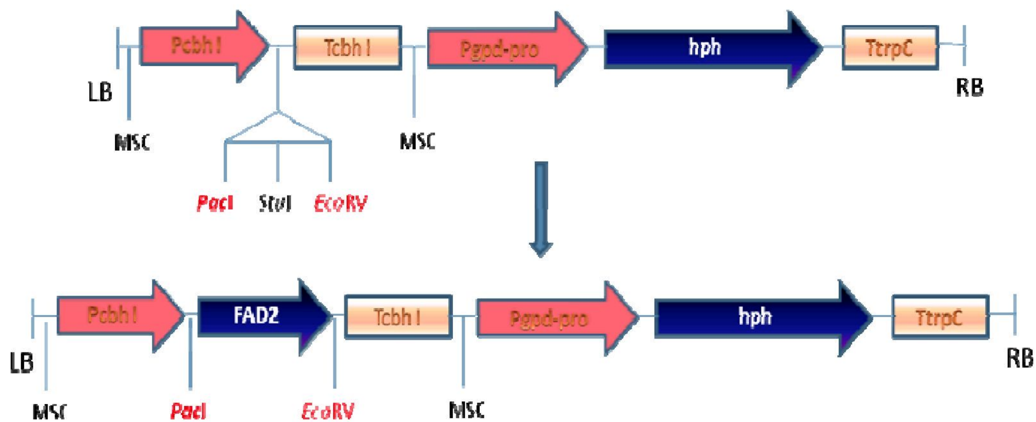

Figure 2. Schematic diagram of the binary vectors pPK5-FAD2 (15 kb) will use for fungal transformation, which was constructed by ligation of *FAD2* sequencing at the *Pac*I and *Eco*RV site of vector pWE32F00. The binary vector pPK5-FAD2 was containing *FAD2*gene and *hph* gene. The *FAD2* gene was driven by the constitutive Pcbh I promoter. The *hph* gene was under control Pgpd promoter of the *Aspergillus nidulans* glyceraldehydes-3-phosphate dehydrogenase gene. RB right border, LB left border.

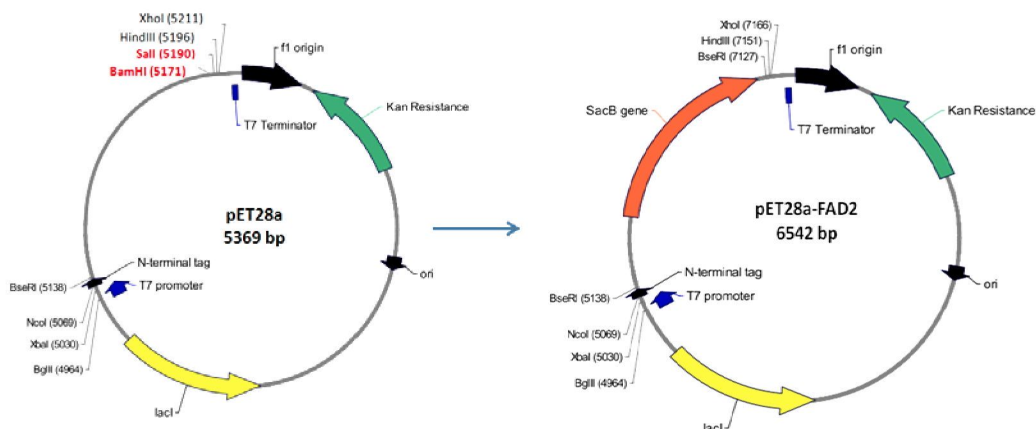

Figure 3. Schematic diagram of the binary vectors pET-FAD2

***Agrobacterium tumefaciens*-mediated fungal transformation**

The transformation protocol was a modification of the method developed by (De Groot *et al*., 1998). Protoplasts of *T. reesei* Rut-C30 were prepared as described by

(Covert *et al*., 2001) with modifications. *T. reesei* Rut-C30 conidia were obtained by growing the strain on PDA plate for 5 days and washing the plate gently with a sterile physiologic salt solution. *A. tumefaciens* strain GV1301, containing the binary vector pPK5-FAD2, was grown at 28°C for 18h in LB media supplemented with kanamycin (50 µg/ml) and gentamycin (50 µg/ml). The *A. tumefaciens* cells were diluted to (optical density) OD 660 =0.15 in the induction medium (IM) with the presence or absence of 200 µM acetosyringone (AS). The cells were grown under the same conditions until an OD660 of 0.4–0.8 was reached before mixing them with an equal volume of a conidial suspension of the *Trichoderma reesei* Rut-C30. This mix (200 µl per plate) was plated on a 90-mm diameter cellophane sheet and placed on cocultivation medium (same as the induction medium except that it contains 20 g agar per liter) in the presence or absence of 200 µM AS. After incubation at 28°C for 48 h, the membranes were transferred to M-100 plates that contained hygromycin B (600 µg/ml) as the selection agent for fungal transformants and Cephalosporins (300 µg/ml) to inhibit growth of *A. tumefaciens* cells. Putative transformants (visible 7 days later), were transferred to M-100 + 600 µg/ml of hygromycin B and plates were incubated as previously. The obtained transformants were further subcultured for 4 generations, and then examined the expression of *FAD2* gene.

Molecular analysis of transformants

DNA was isolated from T. reesei Rut-C30 transformants and wild-type strain using the method of Pattee with modifications (Pattee *et al*., 2002). Approximately 2 g of filtered, frozen mycelia from a liquid culture was ground to a powder and lysed using lysis buffer (50 mM Tris–HCl, pH 7.4, 50 mM EDTA, 3% SDS, 10 mM β-mercaptoethanol) at 65°C for 1 h. The lysate was extracted once using one volume of Tris-buffered phenol followed by two extractions with one volume of chloroform/isoamyl alcohol (24:1). RNA was removed by treating with RNase (10 lg/ml) for 1 h at 37°C. DNA was precipitated and suspended in Tris-HCl (10 mM). After adding TE buffer, samples were vortex for 30s, and centrifuged at 13,000 rpm for 5 min. The supernatant containing DNA was transferred to a new tube and stored at -20°C until use in PCR. For FAD2 gene, 10 µL of PCR mixture containing 5 µL of Premix Taq Version 2.0 (TaKaRa), 5 ng of template DNA, 20 pmol of forward primer P1ZmFAD2 and 20 pmol of reverse primer P2ZmFAD2 (Table 1) were used. For hph gene, 10 µL of PCR mixture containing 5 µL of Premix Taq Version 2.0 (TaKaRa), 5 ng of template DNA, 20 pmol of forward primer P1hph and 20 pmol of reverse primer P2hph (Table 2) were used. The experimental conditions of PCR were 95°C for 5 min, then 30 cycles of 95°C 30 s, 55°C 30 s, 72°C 1.5 min, followed by a final extension at 72°C for another 10 min.

Expression FAD2 gene in *E. coli* and *Trichoderma reesei*

The *T. reesei* Rut-C30 harboring plasmid pPK5-FAD2 was cultured in Glucose yeast medium ($NH_4H_2PO_4$ 0.1% (w/v), KCl 0.02% (w/v), $MgS0_4.7H_2O$ 0.02% (w/v), glucose 1% (w/v), 0.1% (v/v) 0.5% aqueous $CuSO_4.5H_2O$, 0.1% (v/v) 1% aqueous $ZnS0_4.7H_2O$) on a rotary shaker for 60-72 h (28°C, 180 rpm). Total protein was isolated from *T. reesei* Rut-C30 was used for SDS-PAGE by using RNA/DNA/Protein Isolation Kit (Omega Bio-Tek) according to the manufacturer's instructions.

The *E. coli* BL21 transformants harboring plasmid pET-FAD2 were cultured at 37°C in LB medium until OD_{600nm} reached 0.5-0.7. IPTG was added to final concentration of 0.5 mmol/L and the cultivation was continued for another 4 hours at 37°C. The cell was harvested by centrifugation.

The protein concentration was measured by Bio-Rad Protein Assay. Total protein from *E.coli* BL21 and *T. reesei* Rut-C30 transformant was run on 12% SDS-PAGE. Gels were stained with Coomassie Blue R250 and the quantity of the expressed protein was estimated by comparing the intensity of the protein bands.