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Highly efficient transformation of a (hemi-)cellulases-producing fungus *Eupenicillium parvum* 4–14 by *Agrobacterium tumefaciens*

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ABSTRACT

The mesophilic fungus *Eupenicillium parvum* 4–14 is an important producer of thermotolerant hemicellulolytic and cellulolytic enzymes. The aim of this study was to establish a method for genetic manipulation of the fungus by *Agrobacterium tumefaciens*. The promotor PgpdA of a glyceraldehyde-3-phosphate dehydrogenase gene was isolated from *E. parvum* 4–14. To transform the fungus, an expression plasmid containing a superfolder green fluorescent protein (sfGFP) gene under the control of PgpdA promotor was constructed using the plasmid pAg1-H3 as a parental plasmid. Using the fungal ascospores as receptor and hygromycin B resistance as a selection marker, the recombinant plasmid was successfully introduced into the fungal cells by *A. tumefaciens*-mediated transformation (ATMT) method. Acetosyringone (AS) was essential to the successful transformation. The transformation frequency was significantly affected by the co-culture temperature and time, the quantity of fungal spores and the AS concentration. The highest transformation frequency was up to 373 transformants per 10^5 fungal spores, which was higher than those of other fungal species. The fungal transformants were genetically stable after five subcultures in the absence of antibiotic. GFP protein was strongly expressed in the hypha of fungal transformants. In conclusion, the ATMT is a highly efficient method for genetic manipulation of *E. parvum* 4–14, and will improve the molecular researches on the fungus.

1. Introduction

The newly reported fungal strain 4–14 of *Eupenicillium parvum* possesses high ability upon production of thermotolerant hemicellulolytic and cellulolytic enzymes including endoglucanase, β-glucosidase, xylanase and feruloyl esterases (Long et al., 2016). The high efficient extraction of ferulic acid from wheat bran by the crude enzymes of *E. parvum* 4–14 showed an important potential of the fungus in industrial application (Long et al., 2016). Well understanding the functional genomics of *E. parvum* 4–14 by genetic engineering is significant to reveal the characterization of enzyme-production or the biodegradation mechanism by the fungus. Other isolates of *E. parvum* exhibited diverse values in agriculture and pharmaceutical industries for the produce of phytase, mycophenolic derivatives and new compounds (Vyas et al., 2007; Habib et al., 2008; Fugthong et al., 2010; Leon et al., 2013). Up to date, there is lacking of reports about the genetic manipulation of *E. parvum*.

Agrobacterium tumefaciens-mediated transformation (ATMT) method is initially used in genetic modification of plant cells, and then developed for filamentous fungi and yeast (Pacurar et al., 2011; Wang et al., 2014; Dai et al., 2017). By expression of several virulence genes in helper plasmid, *A. tumefaciens* could transfer the transferred DNA (T-DNA) in donor plasmid into the genome of a host to produce mutants (Wang et al., 2017). Unlike electroporation or PEG-mediated transformation systems typically depend on protoplasts, *A. tumefaciens*-mediated transformation (ATMT) works well with various fungal materials including spores, mycelia and gill tissues of mushroom (Chen et al., 2000; Mullins et al., 2001; Park et al., 2013). With this advantage, ATMT method is more suitable for the fungal species that are difficult to form protoplasts. Meanwhile, fungal protoplasts were excellent receptors for obtaining high quantity of mutants by ATMT method (Zhong et al., 2007). The advantages of ATMT method include simple operation, higher transformation frequency, stable transformants with a single T-DNA copy and various available receptors (Fan et al., 2016). ATMT is a powerful tool for fungal genetic manipulation involved in construction of insertional mutagenesis, target gene knockout or knockdown, gene expression or molecular breeding (Michielse et al., 2005; Idnurm et al., 2017).

ATMT technology has been successfully used in many lignocellulose-degrading fungi, such as *Trichoderma reesei, Humicola*

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insolens Penicillium expansum, *Phanerochaete chrysosporium* and so on (Zhong et al., 2007; Sharma and Kuhad, 2010; Schubert et al., 2013; Zhang et al., 2013). Fungal conidia are usually used as start materials in ATMT transformation, and the transformation efficiency is depended on rate of fungal and bacterial cells, co-culture temperature and time and concentration of acetosyringone (Zhong et al., 2007; Xu et al., 2016). *E. parvum* 4–14 mainly formed ascospores by sexual propagation and hardly formed conidia on standard media (Long et al., 2016). Herein, we developed a high-efficiency transformation system of *E. parvum* 4–14 mediated by *A. tumefaciens* using the fungal ascospores as receptor. With this transformation method, a foreign gene encoding green fluorescent protein was successfully expressed in the fungal cells.

2. Materials and methods

2.1. Strain, plasmid and media

The mesophilic fungus *Eupenicillin parvum* 4–14 (CCTCC M2015404) producing thermotolerant hemicellulolytic (also cellulolytic) enzymes was preserved in our lab (Long et al., 2016). *Agrobacterium tumefaciens* AGL-1 was used for fungal transformation. The vector pAg1-H3 (Zhang et al., 2003) was employed to construct fungal expression plasmid. PDA medium (per liter, potato 200 g, glucose 20 g, agar 15 g) and Mandels medium (Mandels and Andreotti, 1978) were used to induce fungal sporulation and/or mycelial growth, respectively. MM, IM and CM media which used for *A. tumefaciens*-mediated transformation (ATMT) were prepared according to documentary description (Wang, 2006).

2.2. Observation of E. parvum ascospores by SEM

E. parvum 4–14 was grown on PDA plate at 37 °C until ascocarps formed. The newly formed ascocarps were transferred into a 1.5-mL centrifuge tube containing one milliliter of distilled water and crushed with a mini grinding rod. After filtering with two layers of lens tissue, pure ascospores were collected by centrifuged at 5000 rpm for 10 min. After treatment with gradient dehydration and critical point drying, the ascospores were observed under a field emission scanning electron microscope (SEM) (JEOL, model JSM-7600F, Japan).

2.3. Test of fungal sensitivity to hygromycin B

E. parvum 4–14 was pre-cultured on PDA plate at 37 °C for one week. Fungal cultures (about 1–2 mm^2) were picked up by an inoculation shovel and transferred to PDA plates containing final concentrations of hygromycin B from 0 to 200 μg/mL, respectively. The inoculated plates were incubated at 28 °C or 37 °C for 4 days, and the fungal growth was monitored every day.

2.4. Cloning of a gpdA promotor from E. parvum

A glyceraldehyde-3-phosphate dehydrogenase encoding gene (named as *gpd*A) was found in the genome of *E. parvum* 4–14 by analysis of the transcriptome data of this fungus (unpublished data). The self-formed adaptor PCR (SEFA PCR) (Wang et al., 2007) was employed to isolate the promotor of *gpd*A gene. All of the primers were designed based on the *gpd*A gene sequences and were listed in Table 1. The genomic DNA of *E. parvum* 4–14 was isolated with a Plant Genomic DNA Isolation kit (TransGen, Beijing, China). The first round PCR (SEFA PCR) amplification was conducted with primers Sp3 and Sp1 using the isolated fungal DNA as template. The second round PCR was run with a single primer Sp2 and appropriate product of SEFA PCR as template. The detailed PCR parameters were designed according to the documentary description (Wang et al., 2007). The target DNA fragment (named as f-Sp2) was purified with a DNA Gel Purified kit (TransGen, Beijing, China) and cloned into pEASY-Blunt vector (TransGen, Beijing, China). A 2.2 kb DNA fragment containing the promoter and partial coding sequence of *gpd*A gene was amplified from the fragment f-Sp2 with primers Pgpd-f1 and Sp2. And the fragment was ligated into pEASY-Blunt vector for complete sequence analysis. DNA sequencing was accomplished by the BioSune Biotechnologies Co. Lid (Shanghai, China).

2.5. Construction of a GFP expression plasmid

A superfolder green fluorescent protein (sfGFP) encoding gene (containing sites *Eco*RI and *Apa*I at the 5′ or 3′, respectively; accession number: MG252999) was synthesized by GENEWIZ Company (Suzhou, China) with optimized codons according to the codon preference of *Aspergillus niger*. The *gpd*A promoter (1214 bp) was amplified with primers Pgpd-f2 and Pgpd-r2b using the corresponding plasmid as template. The product was subcloned into the *Xba*I-*Eco*RI sites of pBluescript II KS(+) after termination with the same restriction enzymes, yielding pB-PgpdA. The sfGFP gene was digested with *Eco*RI-*Apa*I and inserted into the same sites of pB-PgpdA, resulting in pB-sfGFP. A length 1088 bp *cbh*1 gene terminator (T*cbh*1) was amplified from *Trichoderma reesei* D-86271 (VTT, Finland) with primers Tcbh1_f and Tcbh1_r. The DNA fragment was inserted into the *Xho*I-*Apa*I sites of pAg1-H3 after termination by the same enzymes, yielding pAg-Tcbh1. A 2.0 kb of DNA fragment containing *gpd*A promoter and sfGFP gene was amplified with M13-T7 h/M13-Rh from plasmid pB-sfGFP. The obtained product was ligated into plasmid pAg-Tcbh1 (terminated by *Kpn*I-*Xho*I) by Hieff Clone™ One Step Pcr Cloning Kit (Yeasen, Shanghai) to yield expression plasmid pAg-sfGFP.

2.6. Fungal transformation with ATMT method

Plasmids pAg1-H3 and pAg-sfGFP were introduced into *A. tumefaciens* cells by heat shock transformation (Wang, 2006), respectively. A single colony of *A. tumefaciens* transformant was incubated in MM medium at 28 °C and 200 rpm for two days. And the bacterial suspension was diluted to OD_{600} = 0.15 in IM medium and inductive culture was incubated for six hours (OD_{600} reached to 0.6) at 28 °C and 200 rpm. Fungal spores were collected from pre-cultured PDA plates and suspended in sterile distilled water at different concentrations from 10^4 to 10^6 per milliliter. One hundred microliter of fungal spores were mixed with the same volume of *Agrobacterium* cells, and the mixture was spread on a CM plate (covered with a sheet of cellophane paper) for co-culture under different temperatures (22–28 °C), culture time (24–48 h) or acetosyringone (AS) concentrations (0–0.4 mM), respectively. Then the co-cultures were transferred to selective PDA plates containing 100 μg/mL of hygromycin and 400 μg/mL of cefotaxime sodium, and incubated at 37 °C for 3 to 5 days until transformants appearance. The fungal tranformants were transferred to new selective plates and further identification was conducted by PCR amplification with primers hph_f and hph_r.

2.7. Determination of T-DNA insertion sites by SEFA PCR

T-DNA insertion sites of randomly selected fungal transformants were determined by SEFA PCR method (Wang et al., 2007). Primers RB_Sp1, RB_Sp3 and RB_Sp3 were designed according to the up stream sequences of the T-DNA right border (RB). Genomic DNA extraction from transformants and SEFA PCR amplifications were conducted as described above. Target DNA fragments were cloned into pEASY-Blunt vector for sequences analysis. The insertion sites of T-DNA were determined by analysis of target sequences with Basic Local Alignment Search Tool (BLAST).

2.8. Mitotic stability of fungal transformation

Four randomly selected fungal transformants were inoculated on PDA plates without hygromycin B, and incubated at 37 °C for six days.