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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.

Table 1
Primers used in this study.

Primer	Sequence (5′ to 3′)	Source or reference
Sp1	TTGGTGGTGCACGAGGCGTTGGAGATGACG	This study
Sp2	CTCGGTGGTGGTGAAGACACCGGTGGACTC	This study
Sp3	GTGCCCTTGAACTGGCCNNNNNNNNNGTCGTA	This study
Pgpd-f1	CCACCAACATGGACTACGACAAGG	This study
Pgpd-f2	CTAGTCTAGATAGAGGTTCGACTTTCATGGG	This study
Pgpd-r2b	CCGGAATTCAGATAGAGAGGATGGGATAG	This study
M13-T7 h	AGTGAATTCGAGCTCGGTACCGTAATACGACTCACTATAGGGC	This study
M13-Rh	CTTTCGCACGGAGCTCTCGAGGGAAACAGCTATGACCATGA	This study
Tcbh1_f	GACTCGAGAGCTCCGTGCGAAAGCCTGACGCA	This study
Tcbh1_r	CTGGGCCCATCGTAACCGAGAATCCAGAGCTG	This study
hph_f	AAGTTCGACAGCGTCTCC	Long et al. (2013)
hph_r	TTCCACTATCGGCGAGTA	Long et al. (2013)
RB_Sp1	CGCTTGTTTCGGCGTGGGTATGGTGGCAGG	This study
RB_Sp2	GCTCAACGGCCTCAACCTACTACTGGGCTGC	This study
RB_Sp3	GACTCCCTTAATTCTCCNNNNNNNNNCAGATT	This study

Note: the restriction enzyme sites were underlined.

The new-grown mycelia were transferred to fresh PDA medium and grown for another six days. After subculture for 5-times, about 10^5 fungal spores (conidia) from each transformant were collected and suspended in 1 mL sterilized ddH_2O. Aliquots of three 10^2-fold or 10^3-fold dilutions of each spore suspension were spread on PDA plates with or without hygromycin B (200 μg/mL), and the plates were incubated at 37 °C for colony formation.

2.9. Fluorescence microscopy and image analysis

Randomly selected fungal transformants (containing pAg-sfGFP) were pre-cultured on PDA plates for 5 days. A piece of culture was inoculated into a tube containing 5 mL Mandels medium (containing 1% glucose), and incubated at 37 °C and 200 rpm for 3 days. The fresh fungal hypha were picked up and used for fluorescence detection. The green fluorescence emission from sfGFP in the fungal cells was detected under a Confocal Laser Scanning Microscopy (CarlZeiss LSM710, Germany) at an excitation wavelength of 450–490 nm (EGFP model). Images were taken by a Zeiss AxioCam MR camera with Axiovision Release 4.8.2 software.

3. Results and discussion

3.1. Morphological observation of E. parvum ascospores

When grown on PDA plates, *E. parvum* 4–14 formed many ascocarps and ascospores. By observation under a SEM, the ascospores were round and in cake-like shapes with two wrinkled and uplifted surfaces (Fig. 1). The size of these ascospores ranged from 2.4 to 2.6 μm in diameter. The morphology of *E. parvum* 4–14 ascospores was similar to that of the ascospores from some *Eupenicillum* species such as *E. shearii* and *E. tropicum* (Wang and Zhuang, 2009).

3.2. Construction of a GFP expression plasmid for ATMT transformation

The growth of *E. parvum* 4–14 on PDA medium was completely suppressed by 100 μg/mL of hygromycin B (see the Supplementary material Fig. S1). The vector pAg-H3 containing a hygromycin B phosphotransferase (*hph*) gene could be used to construct a gene expression plasmid for this fungus. To clone a constitutive promoter, about 3 kb upstream fragment flanking *gpd*A gene was amplified from *E. parvum* 4–14 by SEFA PCR (see the Supplementary material Fig. S2). Based on the sequence analysis, a shorter DNA fragment (2231 bp, accession number MG253000) containing the *gpd*A promoter was successfully isolated from the fungus. Constitutive expression plasmid pAg-sfGFP that contained a sfGFP gene under the control of P*gpd*A promoter and T*cbh*1 terminator was successfully constructed by homologous recombination method.

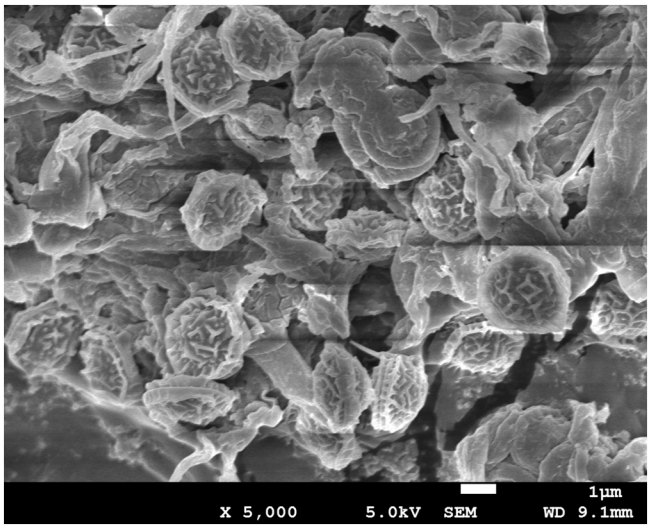

Fig. 1. Scanning electron microscopy (SEM) of *E. parvum* 4–14 ascospores.

3.3. Transformation of E. parvum 4–14 with A. tumefaciens

The ascospores of *E. parvum* 4–14 were used as starting materials for ATMT transformation. By co-culture of the ascospores with *A. tumefaciens* cells harboring plasmid pAg1-H3 or pAg-sfGFP for 48 h, the hygromycin-resistant transformants appeared after incubation for 3 to 4 days on selective plates. Most of these transformants were grown after retransferred to a new selective plate. The resistant gene was detected in the transformants by specific amplification (see the Supplementary material Fig. S3). Meanwhile, strong green fluorescence was observed in the fungal transformants (containing plasmid pAg-sfGFP) but not in the parental strain under a fluorescence microscopy (Fig. 2). It was suggested that the exogenous sfGFP gene could be successfully expressed in the fungal cells. Fungal asexual spores (especially conidiospores) were common receptors for the genetic transformation by ATMT method (Mullins et al., 2001; Zhong et al., 2007; Zhang et al., 2013). This study indicated that the fungal ascospores of *E. parvum* were efficient materials for ATMT transformation.

3.4. Optimization of transformation conditions

Four co-culture parameters were optimized for transformation.

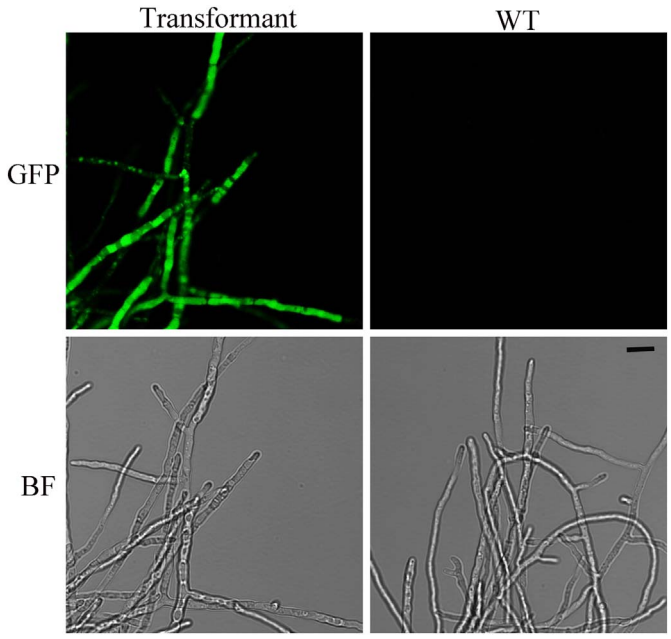

Fig. 2. Detection of green fluorescence protein in the hypha of *E. parvum* 4–14 transformant. The fungal transformant (transformed by plasmid pAg-sfGFP) grew in liquid Mandels medium for 3 days. The fungal hypha were detected under a confocal laser scanning microscopy. WT, wild type; GFP: fluorescence image; BF, bright-field image; bar, 5 μm.

Acetosyringone, as an inducer of the virulence genes in *A. tumefaciens*, played a key role for T-DNA transfer by the bacteria (Michielse et al., 2005). There were no *E. parvum* transformant on selective plates without AS in co-culture stage (see the Supplementary material Fig. S4), suggesting AS was requisite for the fungal transformation. Total 239 fungal transformants appeared from 10^5 fungal spores when a low concentration of AS (200 mM) was added into the co-culture plates. A higher concentration of AS (400 mM) led to 38.4% increment of the transformation frequency (Fig. 3A). To some fungal species, the addition of AS was not required for successful ATMT transformation, suggesting that these fungus could produce inducers (phenolic compounds) of the bacterial virulence genes (Sharma and Kuhad, 2010).

The optimum temperature of co-culture for the fungal transformation was similar to those in many documentary reports (Zhong et al., 2007; Zhang et al., 2013; Lu et al., 2017). Co-culture at 24 °C led to the highest levels of transformation frequency (Fig. 3B). The transformation rate had a slight decrease when the co-culture temperature was increased to 26 °C. When the temperature decreased to 22 °C or increased to 28 °C, the quantity of transformants had a significant decrement (Fig. 3B). The spores concentration up to 1.6×10^5 per milliliter was optimum for the fungal transformation. Higher or lower spore concentrations led to a low transformation rate or even no transformation (Fig. 3C). The time of co-culture also influenced the transformation. No transformant occurred when co-cultured for 24 h. The extension of co-culture to 36 h made 89 transformants per 10^5 fungal spores. The co-culture for 48 h sharply increased the transformation frequency (Fig. 3D).

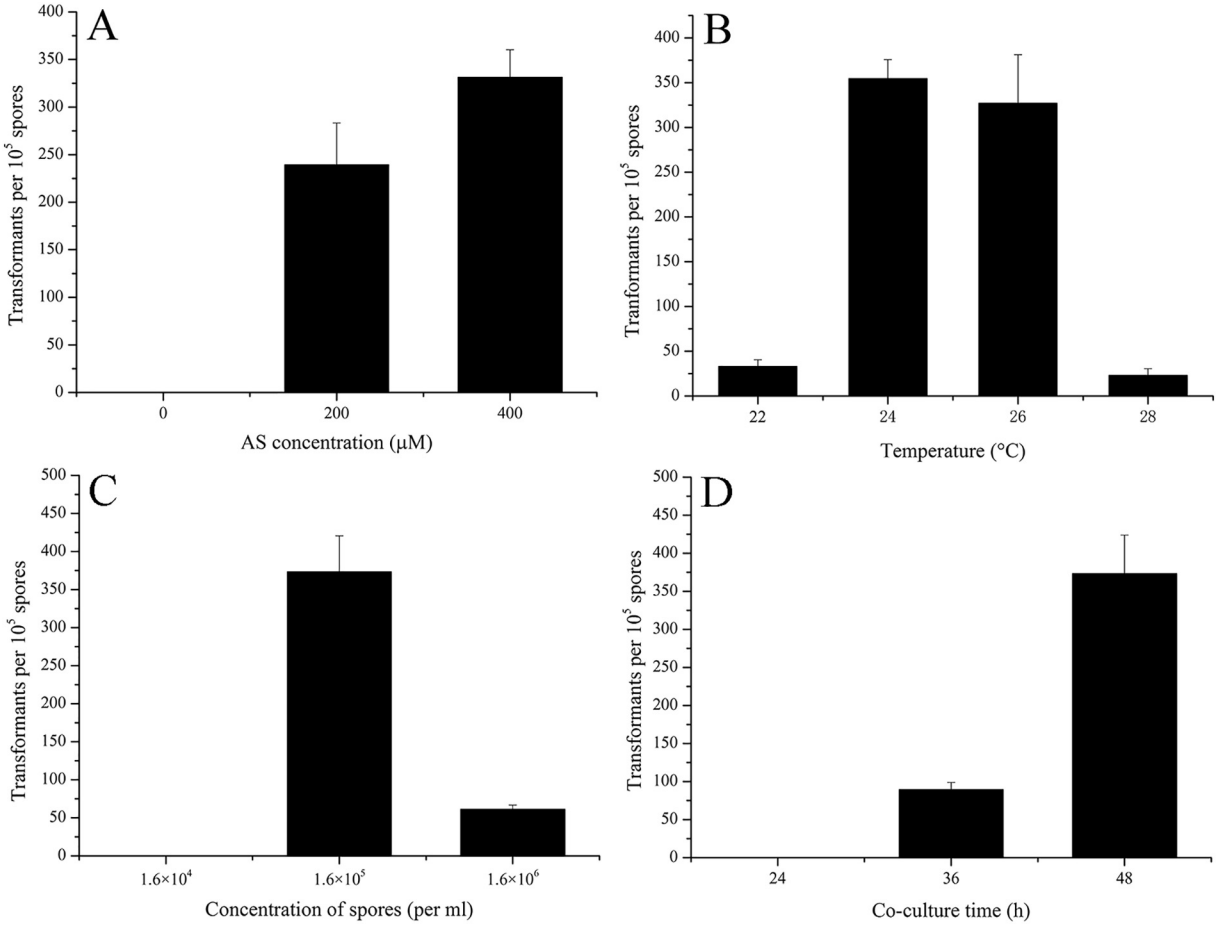

Fig. 3. Transformation frequency of *E. parvum* 4–14 by ATMT method under different conditions. (A) Effect of acetosyringone (AS) on the fungal transformation. The co-culture at 24 °C for 48 h and 1.6×10^5 fungal spores per milliliter were used in the experiment. (B) Effect of co-culture temperature on the fungal transformation. The 400 μM of AS, 1.6×10^5 fungal spores per milliliter and co-culture for 48 h were used in the experiment. (C) Effect of fungal spore concentration on the fungal transformation. The co-culture at 24 °C for 48 h and 400 μM of AS were used in the experiment. (D) Effect of co-culture time on the fungal transformation. The 400 μM of AS, 1.6×10^5 fungal spores per milliliter and co-culture at 24 °C were used in the experiment. The colonies grown on selective plates were considered as resistant transformants. Error bars represent standard deviations from three independent experiments.

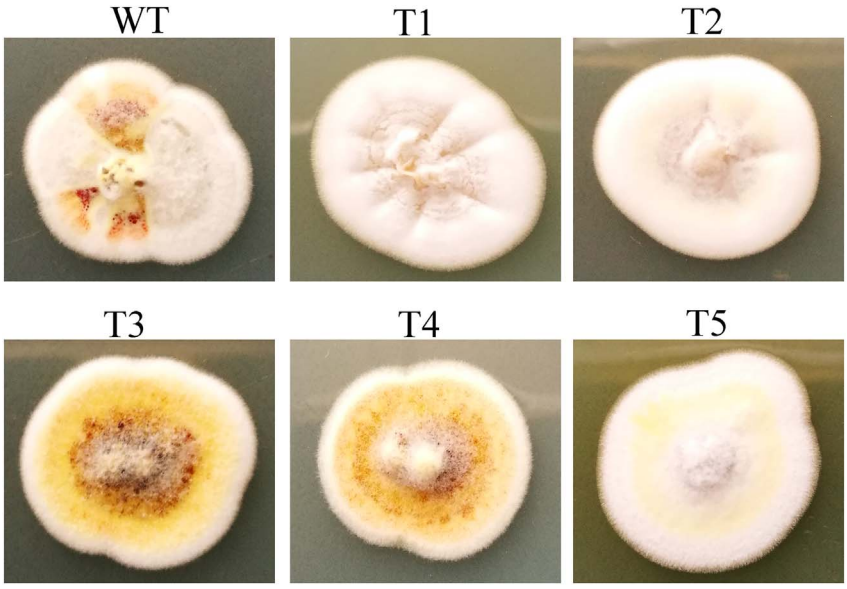

Fig. 4. Colonial morphologies of the parental strain and the transformants of *E. parvum* 4–14. All of the fungal strains grew on PDA plates at 37 °C for four days. The fungal transformants were transformed with the plasmid pAg-sfGFP. WT, wild type; T1-T5, different transformants.

Table 2
Resistant stability of *E. parvum* 4–14 transformants after five generations of subculture on PDA medium without antibiotic.

Transformants	Colony formation on different plates (CFU per plate)[a]	
	PDA	PDA with hygromycin B[b]
Ta	15.3 ± 2.5	14.3 ± 2.1
Tb	27.8 ± 3.3	28.2 ± 4.3
Tm	88.3 ± 9.7	90.7 ± 7.1
Tn	62.2 ± 6.0	61.3 ± 6.7

[a] In each plate, 50 μL spores suspension (10^3 spores per milliliter) was used.
[b] The final concentration was 200 μg per milliliter.

Enough mutants are required for genetic manipulation of fungus. In this study, up to 373 resistant transformants appeared from 10^5 fungal spores (Fig. 3C). The transformation efficiency is far higher than those in many reported fungal species (such as *Penicillium expansum*, *T. reesei*, *Aspergillus terreus* and *Lecanicillium lecanii*) with ATMT system (see the Supplementary material Table S1). Genetic transformation of *E. parvum* 4–14 by *A. tumefaciens* was a strong tool for further studies of the fungus at molecular level.

3.5. Morphological observation and mutant sites analysis of transformants

The fungal transformants could be divided into two different strain types (A and B) according to the colonial morphology on PDA medium (Fig. 4, and the Supplementary material Fig. S5). In Fig. 4, the transformants T1, T2 and T3 are type A strain (thick and white colony without brown exudation), the tranformants T3 and T4 belong to type B strain (formed ascocarps-like structures and brown exudation), and the wild type strain displayed a mixed colonies of types A and B. The quantity ratio of the two strain types was about 1:1 in every transformation. It was speculated that each transformant was formed from one parental ascospore. The single transformant could not form ascospores, suggesting that the fungus is a heterokaryon.

The T-DNA insertion sites of five fungal transformants (T1-T5) were determined by SEFA PCR. Finally, the 655 bp, 695 bp and 923 bp franking sequences of inserted T-DNA (see the Supplementary material Table S2) were amplified from transformants T1, T2 and T4, respectively. BLAST analysis indicated that the T-DNAs of transformants T1 and T2 were located in different unknown functional regions. The franking sequences of transformant T4 shares 74% sequence identity with the N-terminal of Choline kinase (PEX2_039750) from *P. expansum*. The T-DNA fragments were randomly inserted into the genome of *E. parvum* 4–14.

3.6. Transgenic stability of fungal transformation

Four randomly selected transformants were continuously cultured for five generations on PDA medium without antibiotic addition. The fungal offspring normally grew on selective medium and remained the resistance ability. And the amounts of colony formed on PDA medium with antibiotic were similar to those on PDA medium without antibiotic (Table 2). It was demonstrated that the integrated gene in the transformants was genetically stable. ATMT is an effective method for the genetic manipulation of *E. parvum* 4–14.

4. Conclusion

A. tumefaciens-mediated genetic transformation (ATMT) of *E. parvum* 4–14 was established using the fungal ascospores as receptor. The hygromycin B resistance gene and sfGFP gene were successfully introduced into the fungal cells by co-culture of *A. tumefaciens* cells and fungal spores. Acetosyringone, co-culture temperature and time, and the concentration of fungal spores significantly affected the transformation efficiency. The highest transformation efficiency was 373 transformants per 10^5 fungal spores, and the transformants were genetically stable. The sfGFP protein was expressed in the hypha of fungal transformants. The high efficient ATMT method was important for research of the fungus at molecular or genetics level.

Abbreviations

ATMT	*Agrobacterium tumefaciens*-mediated transformation
AS	acetosyringone
sfGFP	superfolder green fluorescent protein
SEFA PCR	self-formed adaptor PCR
hph	phosphotransferase gene
RB	right border

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2018.01.013.

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