

# An improved method for *Beauveria bassiana* transformation using phosphinothricin acetyltransferase and green fluorescent protein fusion gene as a selectable and visible marker

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**Abstract** An improved transformation method for the biocontrol agent, *Beauveria bassiana*, was developed. For convenience of transformation selection and detection, the coding regions of the genes for phosphinothricin acetyltransferase and green fluorescent protein were fused and an expression vector, pBFT, carrying this fusion was constructed. Under optimum conditions, over 60 transformants  $\mu\text{g}^{-1}$  plasmid DNA were obtained. *B. bassiana* conidia frozen 1 month at  $-80^{\circ}\text{C}$  were fully competent for transformation. The method was significantly less laborious and more rapid than current methods for *B. bassiana*. The *bar::egfp* provides a selectable and visible marker which may expedite future genetic engineering of this fungus.

**Keywords** *Beauveria bassiana* ·  
Fusion gene · Green fluorescent protein ·  
Phosphinothricin resistance · Transformation

## Introduction

The entomopathogenic fungus, *Beauveria bassiana*, is an important biological control agent for a variety of insect pests, including both agricultural pests and vectors of human pathogens. In recent years, *B. bassiana* research has received greater attention and efforts to determine the molecular basis of its development, virulence and pathogenicity have expanded (Cho et al. 2006a, b; Fan et al. 2007; Fang et al. 2005). To produce transgenic *B. bassiana*, several genetic transformation methods including protoplast-based, blastospore-based and *Agrobacterium*-mediated methods, have been developed. Protoplast-based methods (Daboussi et al. 1989; Pfeifer and Khachatourians 1992) are time-consuming and have low efficiency. The blastospore-based method is also time-consuming, requiring 3 d to prepare blastospores (Jiang et al. 2007; Ying and Feng 2006). The higher efficiency of the *Agrobacterium*-mediated transformation method is attractive; however, target gene cassettes must be constructed in a binary vector and mobilized into *Agrobacterium* strains prior to *B. bassiana* transformation and co-cultivation of the fungus and bacterium requires 48 h (Fang et al. 2004). Fungal conidia have been successfully employed as competent cells in the electroporation transformation of several filamentous fungi (Robinson and Sharon 1999; Sánchez and Aguirre 1996). Germinated conidia have proved to be most competent for receiving foreign DNA before

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nuclear division and septa formation in *Colletotrichum gloeosporioides* (Robinson and Sharon 1999) and *Aspergillus nidulans* (Koukaki et al. 2003).

Here we describe an improved method for genetically transforming *B. bassiana* with pBFT, a new expression vector that we constructed. The vector contains the marker genes for phosphinothricin acetyltransferase and green fluorescence protein as a fusion sequence, *bar::egfp*. We demonstrate that the method is simple, reliable, and highly efficient, and thus potentially very useful for genetic research with *B. bassiana*.

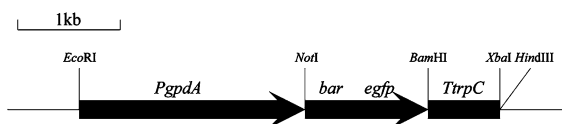
## Materials and methods

### Strains and culture conditions

*Beauveria bassiana* strain Bb0062 single spore isolate and cultivation were previously described (Fang et al. 2005).

### Construction of the vector of fusion gene *bar::egfp*

For convenience of transformation selection and detection, a plasmid containing the fusion gene *bar::egfp* was constructed (Fig. 1). The *bar* and *egfp* were amplified from PCB200 (Fang et al. 2004) and pEGFP-C1 (Clontech) using primers B-F/B-R and G-F/G-R (Table 1), respectively. All the amplified fragments were cloned into pUCm-T (Sangon), and sequenced. The *bar* was excised from the cloning vector by *NotI* and *XbaI*, and fused upstream to the *egfp* to form pUC-*bar::egfp*. Then, the fusion gene was linked with *PgpdA* promoter and *trpC* terminator from *Aspergillus nidulans* in plasmid pUC-PT to form the final plasmid, designated as pBFT.



**Fig. 1** Design of the transformation plasmid pBFT. The fusion gene *bar::egfp* was under the control of the *A. nidulans* *gpd* promoter (*PgpDA*); efficient termination was facilitated by the *A. nidulans* *trpC* terminator. The cleavage sites for the endonucleases *EcoRI*, *NotI*, *BamHI*, *XbaI* and *HindIII* were indicated

**Table 1** Primers used in this work

Name	Sequence (5'–3') <sup>a</sup>	Restriction enzyme site
B-F	<u>GCGGCCGCGGATCTACCATGA</u> GCCCAG	<i>NotI</i>
B-R	TCTAGAGATTTCGGTGACGG <u>GCAG</u>	<i>XbaI</i>
G-F	<u>ACTAGTGTGCCACCATGGTG</u> AGCAA	<i>SpeI</i>
G-R	<u>GGATCCTTAGGACTTGTACAG</u> CTCG	<i>BamHI</i>

<sup>a</sup> Underlined sequences are restriction sites

### Preparation of competent cells

The conidia were cultivated in SDY at 26°C on a rotary shaker at 180 rpm. To determine the germination stage of conidia, the cultures were observed at 0, 8, 12, and 16 h after inoculation. To confirm the nucleus number in the germinated conidia, the conidia were stained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) and imaged in the same microscopic field of light and fluorescence.

To prepare competent cells, 500 ml SDY medium was inoculated with approximately  $5 \times 10^9$  conidia and incubated at 26°C on a rotary shaker at 180 rpm. Aliquots of 50 ml (about  $5 \times 10^8$  conidia) were rapidly removed at 0, 8, 12, and 16 h after inoculation. Conidia were harvested and washed twice in 50 ml ice-cold sterile water, resuspended in 25 ml ice-cold YED (1% yeast extract, 1% glucose, w/v) plus 20 mM HEPES (adjusted to pH 8.0 with 100 mM Tris/HCl) and incubated at 26°C for 1 h on a rotary shaker at 100 rpm. Conidia were collected with centrifugation and resuspended in 2.5 ml (about  $2 \times 10^8$  conidia ml<sup>-1</sup>) of ice-cold electroporation buffer (10 mM Tris/HCl, 200 mM sucrose, 1 mM lithium acetate, pH 7.5). Subsequently, 500 µl sterile glycerol was added into tube and mixed well. The resultant suspension in 50 µl aliquots was stored at –80°C and used as competent cells for electroporation transformation.

### Electroporation transformation

One µg of pBFT DNA was linearized by *HindIII*, mixed with 50 µl competent cells in a total volume of 60 µl, and kept on ice for 15 min. The ice-cold suspension was transferred to 0.2 cm electroporation

cuvette and electroporated at various settings with Gene Pulser apparatus (BioRad). After pulse, 1 ml ice-cold YED was immediately added into the electroporation cuvette, and the suspended cells were transferred to a 10 ml pre-cooled sterile tube and kept on ice for 15 min. The sample was then incubated at 26°C for 45 min on a rotary shaker at 100 rpm. The conidia were harvested by centrifugation and resuspended in 1 ml sterile deionized water. One hundred  $\mu\text{l}$  of suspension was spread on each Czapek's plate containing 60  $\mu\text{g}$  phosphinothricin  $\text{ml}^{-1}$  and incubated at 26°C. About 5 d later, the resistant colonies were transferred to a fresh Czapek's plate with 60  $\mu\text{g}$  phosphinothricin  $\text{ml}^{-1}$  for further analysis.

#### Southern blotting analysis

Genomic DNA of *B. bassiana* was extracted as previously described (Reader and Broda 1985). Southern blotting analysis was performed according to the method (Sambrook et al. 1989).

#### Mitotic stability analysis of transformants

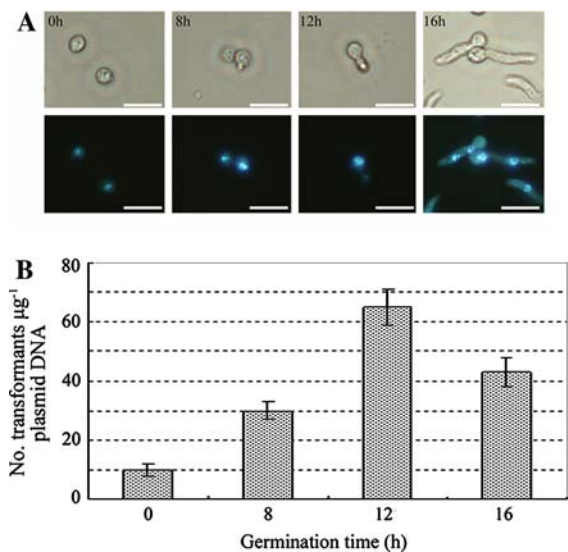
Mitotic stability analysis was performed as previously described (Fang et al. 2006).

## Results

Transformation frequencies were dependent on the germination stage of conidia

To determine the optimum germination time of conidia for transformation, the conidia morphology and nucleus number were observed at different germination stages. The conidia began to swell at 8 h after inoculation. At 12 h after incubation, the germ tube was formed at one end of the conidia and all germinated cells observed were found with single nucleus. After 16 h of incubation, the germinated cells had two or more nuclei (Fig. 2a).

To further confirm the optimum culture time for transformation, competent cells were harvested in the various germination stages of conidia and treated with 1  $\mu\text{g}$  linear pBFT plasmid DNA. The highest transformation frequency (about 60 transformants  $\mu\text{g}^{-1}$  plasmid DNA) was achieved with conidia harvested at 12 h after inoculation (Fig. 2b). Based



**Fig. 2** Transformation frequencies were dependent on the germination stage of conidia. (a) The conidia of *B. bassiana* at 0, 8, 12, and 16 h after inoculation were stained with DAPI and viewed using a Leica D filter cube with peak transmission for excitation at 355–425 nm and suppression at above 470 nm. Bar = 10  $\mu\text{m}$ . (b) The highest frequency was obtained with the field strength value of 5.5  $\text{kV cm}^{-1}$  and the pulse duration of 4 ms. Capacitance and resistance was kept fixed at 25  $\mu\text{F}$  and 400 ohms, respectively. Experiments were carried out in three independent experiments

on the results, the germinated conidia cultivated for 12 h were employed to prepare competent cells for transformation.

#### Optimization of transformation conditions

To achieve high transformation efficiency, the field strength values (from 3.5 to 6.5  $\text{kV cm}^{-1}$ ) and pulse duration (from 1 to 4 ms) were tested. The highest frequency ( $65 \pm 7$  transformants  $\mu\text{g}^{-1}$  plasmid DNA) was obtained with the field strength value of 5.5  $\text{kV cm}^{-1}$  and the pulse duration of 4 ms (Table 2).

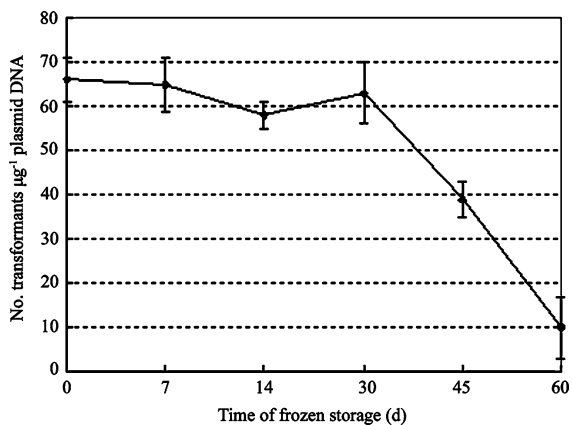
#### Effect of frozen storage on transformation frequency

The competent cells, containing glycerol stored at  $-80^\circ\text{C}$ , were used daily to perform the transformation. No obvious difference in transformation frequency (about 60 transformants  $\mu\text{g}^{-1}$  plasmid DNA) during 1 month of storage was found (Fig. 3). However, 2 month storage led the transformation frequency

**Table 2** Transformation frequency on varying electric field strength and pulse time

Field strength value (kV cm <sup>-1</sup> )	Time constant (ms)	No. of transformants µg <sup>-1</sup> plasmid DNA ± SE
3.5	1	5 ± 2
4.5	2	24 ± 3
5.5	3	40 ± 6
5.5	4	65 ± 7
6.5	4	38 ± 7

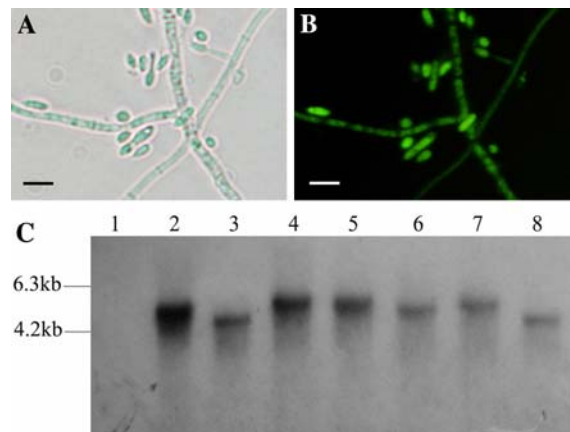
In these experiments, receptive cells were the *B. bassiana* conidia incubated for 12 h as described in “Materials and methods”. Experiments were carried out in three independent experiments

**Fig. 3** Effect of frozen storage on transformation frequency. The competent cells stored at  $-80^{\circ}\text{C}$  were used to perform the transformation at several-day intervals

dramatically decreasing (about 10 transformants µg<sup>-1</sup> plasmid DNA).

#### Validation of transformants

Fluorescent observation showed that transformants selected by phosphinothricin resistance had GFP activity (Fig. 4a, b), indicating that BAR::EGFP fusion protein had both GFP and phosphinothricin resistant activity. Thus, the fusion gene *bar::egfp* could be used as selectable and visible marker. Seven transgenic lines were selected randomly for Southern blotting analysis. Strong hybridization bands appeared in transgenic lines and these seven transgenic lines contained a single copy insert of the transgene (Fig. 4c). To monitor mitotic stability of

**Fig. 4** Validation of *B. bassiana* transformed with vector pBFT. Conidia and mycelia of transformants were imaged in the same microscopic field of light (a) and fluorescence (b). Bar = 10 µm. Southern blotting analysis of the transformants (c). About 5 µg genomic DNA of the wild-type strain Bb0062 (lane 1) and the transformants (lanes 2–8) was digested with *DraI*, fractionated on a 1% (w/v) agarose gel, transferred to a nylon membrane and hybridized with  $\alpha$ -<sup>32</sup>P labeled *bar*. Molecular weight markers were indicated in the left margin.

transformants, 30 randomly selected transformants were used. After five successive subcultures on nonselective media, most conidia and mycelia still displayed intensive green cytoplasmic fluorescence. Statistically, about 90% of the transformants were mitotically stable.

#### Discussion

The results presented here showed that *B. bassiana* could be efficiently transformed by electroporation of germinated conidia using the fusion gene *bar::egfp* as selectable and visible marker. Compared to other transformation methods, the approach for *B. bassiana* transformation provided several advantages. First, it was significantly timesaving. It took about 18 h from initial inoculation to final plating on selective media, while other previous methods for *B. bassiana* needed at least 48 h of incubation (Daboussi et al. 1989; Fang et al. 2004; Pfeifer and Khachatourians 1992; Ying and Feng 2006). Second, it was simple and convenient. Like bacteria or yeast, after frozen at  $-80^{\circ}\text{C}$  in 1 month, the germinated conidial cells were still fully competent for transformation, and the operation could be finished within 1.5 h. Third, it was highly efficient. In contrast with previously described

methods such as polyethylene glycol (PEG)-mediated protoplast transformation (Daboussi et al. 1989; Pfeifer and Khachatourians 1992) and blastospore-based transformation (Jiang et al. 2007; Ying and Feng 2006), in which transformation frequencies were about 5–50 transformants  $\mu\text{g}^{-1}$  plasmid DNA, this method was able to obtain over 60 transformants  $\mu\text{g}^{-1}$  plasmid DNA. Finally, the fusion protein, BAR::EGFP, provided us a convenient maker for resistant selection and visible validation. Moreover, the engineered fungi could be monitored during various development or infection stages using this fusion gene.

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