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An Efficient and Stable Method for the Transformation of Heterogeneous Genes into *Cephalosporium acremonium* Mediated by *Agrobacterium tumefaciens*

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Abstract A transformation system mediated by *Agrobacterium tumefaciens* is routinely used for the genetic engineering of plants. Here, we report an efficient and stable method for transformation of heterogeneous genes into an industrial *Cephalosporium acremonium* by using a similar transformation system established in plants. Both the phleomycin-resistant gene and *vgb* gene were used as screening markers to confirm the success of transformation by either Southern hybridization or PCR amplification. It was found that acetosyringone (AS) was necessary only for protoplast transformation and the heterogeneous genes transferred were integrated into the genome of *C. acremonium*. The transformation efficiency obtained with this system was much higher than the conventional techniques used for transformation of *C. acremonium*.

Key words: *Agrobacterium tumefaciens*, *Cephalosporium acremonium*, protoplast, Ti plasmid, transformation, triparental mating, *vgb*

Cephalosporium acremonium belongs to filamentous fungi and is a producer of β-lactam antibiotic cephalosporin C, which can be chemically modified to produce many clinically significant β-lactam antibiotics. Recombinant DNA technology has been developed [14] and applied to the engineering of this strain so as to obtain some production-improved strains. However, lack of an efficient transformation system represents a major impediment to carrying out the study of genetic engineering in this filamentous fungus.

A method for genetic transformation mediated by *Agrobacterium tumefaciens* has been most widely used in gene cloning of plants [7, 16]. Besides its natural plant hosts, *Agrobacterium* has been shown to be able to transfer DNA to other microorganisms such as yeast [10] and several species of fungi [5]. However, whether *Agrobacterium* can be used to transform genes into *C. acremonium* remains unknown. In this work, we reported an efficient and stable method for introduction of genes into an industrial *C. acremonium* mediated by *A. tumefaciens*.

Materials and Methods

Strains and Plasmids

Strains and plasmids used in this work are shown in Table 1. *Escherichia coli* DH5α was grown on LB medium [13]. *A. tumefaciens* (with pYG306) was grown in induction broth as previously described [9] except that the pH for induction broth was maintained at 5.3 with 62.5 mM potassium phosphate. *C. acremonium* was grown at 28°C in YPS medium [8] and used as the recipient in cocultivation with *A. tumefaciens.*

Construction of Plasmid pYG306

A 3-kb *SacI*/*Hin*dIII fragment containing the β-glucuronidase (Gus) gene was deleted from the binary vector pBI121. The remaining fragment of 10 kb containing the T-DNA sequence was blunted and ligated itself, and the resultant plasmid was designated as pYG305. The binary vector pYG306 was constructed by insertion of a 6-kb *Eco*RI fragment containing both the phleomycin-resistance gene and the *vgb* structural gene from pYG715/*vgb* [12] into the *Eco*RI site of the binary vector pYG305 (Fig. 1).

Triparental Mating

A. tumefaciens LBA4404 (with Rif and Sm resistance) was cultured in LB medium supplemented with rifampin (Rif, 50 μg/ml) and streptomycin (Sm, 30 μg/ml) at 28°C with shaking, while *E. coli* HB101 bearing plasmid pRK2013 was incubated in LB medium containing kanamycin (Km, 50 μg/ml) at 37°C, and *E. coli* DH5α bearing plasmid pYG306 was incubated in LB medium containing ampicillin

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Table 1. Strains and plasmids used in this work.

Strains or plasmids	Relevant characteristics	Source
Cephalosporium acremonium-JM-2002	Host	SIPI
E. coli DH5α, HB101	Host	SIPI
Agrobacterium tumefaciens helper strain LBA4404	Sm^r, Rif^r, *vir*	SIPI
Plasmid pYG715/*vgb*	PtrpC, $phleo^r$, Ap^r, *vgb*	SIPI
Plasmid pBI121 (13 kb)	Km^r	SIPI
Plasmid pYG306 (16 kb)	Ap^r, Km^r	This work

SIPI, Shanghai Institute of Pharmaceutical Industry; Ap^r, ampicillin resistance; $phleo^r$, phleomycin resistance; Sm^r, streptomycin resistance; Km^r, kanamycin resistance; Rif^r, rifampin resistance; PtrpC, *trpC* gene promoter from *Aspergillus nidulans*; *vgb*, *Vitreoscilla* hemoglobin gene.

(Amp, 100 μg/ml) and kanamycin (Km, 50 μg/ml) at 37°C. After incubation for approximately 12 h to late-exponential phase, the three bacteria were mixed with the same volume, transferred onto an LB plate, and incubated overnight at 28°C. Then, they were scraped with LB liquid medium and plated on LB medium plus Rif, Sm, Amp, and Km at 28°C for 2–3 days. Thereafter, transconjugates of *A. tumefaciens* were selected.

Bacterial Clones

A summary of each donor *Agrobacterium* clone and its source is shown in Table 2. Donor *Agrobacterium* clones bearing plasmid pYG306 were confirmed by DNA minipreps, as described by Beven [3].

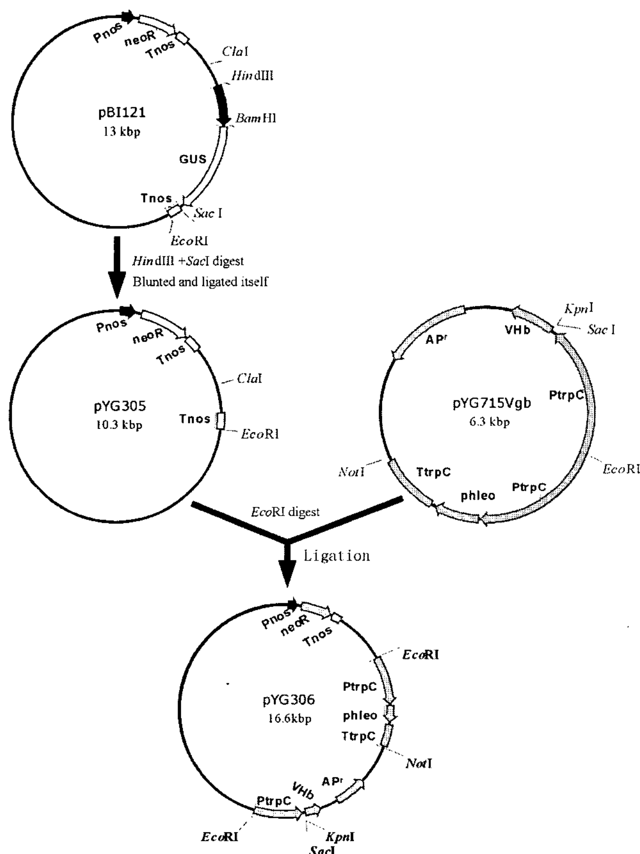

Fig. 1. Construction of binary vector pYG306.

Polymerase Chain Reaction (PCR)

For PCR analysis, a pair of primers specific for the amplification of the *vgb* gene were designed as follows:

Sense Primer:
5'-CCGGAATTCCGG TCGATCCCCCTAGAAAGCGG-3'
*Eco*RI
Antisense Primer:
5'-CCCAAGCTTGGG CGAGCTCGGTACCCTATGTT-3'
*Hin*dIII

PCR was carried out in a total 20 μl comprising 30 ng DNA, 2 μl 10×PCR buffer, 0.25 mM volume dNTP, 2.1 μM of each primer, 1.5 U of Taq DNA polymerase, and sterile distilled water, and was run as follows: denaturation for 2 min at 94°C followed by 28 cycles of amplification (94°C, 30 sec; 60°C, 1 min; 72°C, 1 min) and a final extension at 72°C for 5 min. The products of PCR were analyzed by agarose gel electrophoresis.

Transformation Protocol

Protoplasts of *C. acremonium* were prepared as described by Isogai *et al.* [8]. Cocultivation between donor *A. tumefaciens* and *C. acremonium* was performed as follows: For transformation of protoplasts, 100 μl of protoplasts at a concentration of 2×10^7–3×10^8 protoplasts/ml were mixed with 100 μl of the *Agrobacterium* culture. When mycelia were transformed, 100 mg wet weight mycelia were used. Subsequently, these mixtures and dilutions thereof were plated on nitrocellulose filters placed on induction medium (IM) plates [4] containing 10 mM glucose with or without AS. The plates were incubated for 4 days at 28°C. The filters were transferred to *C. acremonium* selective medium plates [2] containing 200 μM cefotaxime to kill the *Agrobacterium* cells and 5 μg/ml of bleomycin to select for transformants.

Analysis of Transgenes by Using Southern Blots

Genomic DNA from *C. acremonium* was extracted as described by Raeder and Broda [11]. DNA (20 μg) was

Table 2. *Agrobacterium tumefaciens* LBA4404 clones (bearing pYG306) used in this study.

Clone	Source
2002	Triparental mating among LBA4404, *E. coli* HB101/pRK2013, and *E. coli* DH5α/pYG306
2002A	Competent cells of LBA4404 transformed directly with pYG306 by electroporation

digested with the appropriate restriction enzymes and was subjected to electrophoresis (0.7% agarose gels, TAE buffer, 10 V/cm for 4 h). DNA in the gels was blotted onto nylon membranes and hybridized to digoxigenin (DIG) high prime-labeled DNA probes (Roche Molecular Biochemicals, Mannheim, Germany). A positive band was detected using 484 bp of the *vgb* gene as a probe, which was cut from plasmid pYG306 and isolated by a gel-purified method, according to the manufacturer's protocol.

Carbon Monoxide Binding Assay

This assay is based on the method of Webster and Liu [15]. Whole cells sonicates (10 ml) from *C. acremonium* transformants and untransformed control cells were reduced with 20 mg of sodium sulfite. The sample was divided into two aliquots, one of which was exposed to CO for 15 min, the other to air. The samples were then scanned on a spectrophotometer (Ultrospec 2100 pro, Amersham Pharmacia Biotech, Cambridge CB4 0FJ, England) and the difference spectrum was plotted after normalization to total protein content.

RESULTS

Construction of the Binary Vector

The binary vector pYG306 was constructed as described in Materials and Methods (Fig. 1) and used for transfer of both phleomycin-resistance and *vgb* genes from *A. tumefaciens* to *C. acremonium*.

PCR for *Agrobacterium* Strains

DNA samples were extracted from each of the transformants after triparental mating or electroporation. For confirming the presence of the *vgb* gene in *A. tumefaciens* LBA4404, a PCR assay was carried out by amplification of the *vgb* gene from genomic DNA extracted from the *Agrobacterium* strains. After electrophoresis, a PCR-amplified DNA fragment of ~500 bp was clearly observed on agarose gel. This showed that the *vgb* gene had been co-integrated into the DNA of *A. tumefaciens* LBA4404 (Fig. 2).

T-DNA Transfer from *A. tumefaciens* to *C. acremonium*

In order to establish whether *A. tumefaciens* could transfer T-DNA to the filamentous fungus *C. acremonium*, we constructed the binary vector pYG306. This plasmid contains both a fragment coding for a phleomycin-resistance gene and a *Vitreoscilla* hemoglobin gene located downstream of the *trpC* gene promoter from *Aspergillus nidulans* between the left border and right border repeats of the binary vector pBI121. Plasmid pYG306 was then introduced by either electroporation or triparental mating into the *A. tumefaciens* LBA4404, which carries the *vir* gene that codes for the T-DNA transfer system.

Bleomycin-resistant colonies could be obtained by incubation of *C. acremonium* protoplasts with *A. tumefaciens* (bearing pYG306) if the cocultivation was done on medium containing AS (Table 3).

The negative controls have never given rise to resistant colonies. This result shows that induction of the *vir* gene is essential for transfer of the resistance trait to the *C. acremonium* cells, which suggests that *A. tumefaciens* can use its virulence system for the introduction of related plasmid into *C. acremonium*. The transformation frequency varied from approximately 80 to 140 cfu/10^7 protoplasts of *C. acremonium*.

Bleomycin-resistant colonies could also be obtained by incubation of mycelium of *C. acremonium* with *A. tumefaciens* (bearing pYG306) on medium with or without AS, the former having more transformants (Table 4).

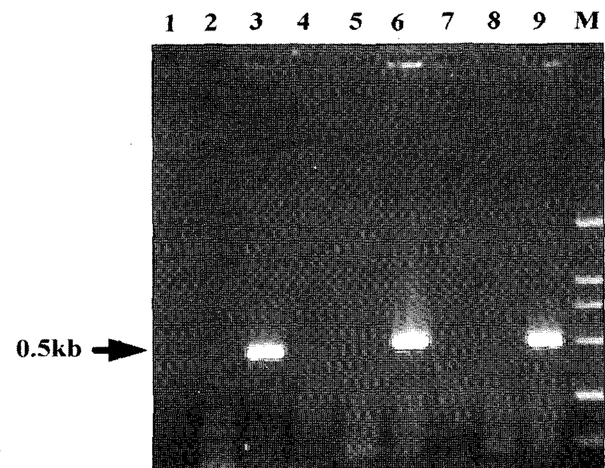

Fig. 2. Electrophoresis analyses of PCR-amplified *vgb*.
Lane: M, DNA Marker DL2,000 (TaKaRa Biotech); 1, Negative control without *Taq* polymerase (LBA4404 by Triparental mating); 2, Negative control without template DNA (LBA4404 by Triparental mating); 3, *vgb* amplified from a chromosome DNA sample isolated from a transformant (LBA4404 by Triparental mating); 4, Negative control without *Taq* polymerase (LBA4404 by electroporation); 5, Negative control without template DNA (LBA4404 by electroporation); 6, *vgb* amplified from a DNA sample isolated from a transformant (LBA4404 by electroporation); 7, Negative control without *Taq* polymerase (*E. coli* DH5α); 8, Negative control without template DNA (*E. coli* DH5α); 9, pYG306 (*E. coli* DH5α).

Table 3. Transformation of *C. acremonium* protoplasts using *A. tumefaciens*.

Experiments	Medium	Number of protoplasts	Number of bleomycin-resistant transformants	Number of bleomycin-resistant transformants per 10^7 recipients
1	With AS	2.85×10^6	40	140
	Without AS	2.85×10^6	0	0
2	With AS	3.20×10^6	35	109
	Without AS	3.20×10^6	0	0
3	With AS	7.0×10^6	56	80
	Without AS	7.0×10^6	0	0

AS: acetosyringone.

Stability of the bleomycin-resistant phenotype was confirmed by transfer of transformants to fresh plates without bleomycin and the transfer was carried out until the fifth generation. It was found that those transformants tested could still grow well when they were transferred to fresh plates containing 5 μg/ml bleomycin. The results showed that the bleomycin resistant phenotype of transformants was stable (results not shown here).

Comparison of Transformation Efficiency Between Two Different Transformation Methods

The efficiency for the transformation of genes into the protoplasts of *C. acremonium* mediated by *A. tumefaciens* was up to 15 times higher than the values obtained by PEG-$CaCl_2$ mediated transformation of protoplasts (Table 5).

Evidence for Integration of Heterogeneous Genes into the Genome of *C. acremonium*

Genomic DNA was isolated from the transformants and used for confirming the presence of the *vgb* gene in the transformants of *C. acremonium* by Southern blotting (Fig. 3) and PCR assay (Fig. 4).

Southern Hybridization. When the probe was hybridized to digested DNA (*Cla*I fragments), the hybridization bands could be clearly observed on the x-ray film, which coincided with the band of the *vgb* gene shown on agarose gel, confirming that the *vgb* gene was integrated into chromosomal DNA of the transformed *C. acremonium*.

PCR for Genomic DNA of *C. acremonium*. An assay for the presence of the *vgb* gene in transformants of *C. acremonium* was performed by PCR amplification of the *vgb* gene band from chromosomal DNA extracted from the *C. acremonium* transformants. A band for DNA fragment of ~500 bp amplified by PCR was clearly observed on agarose gel. This showed that the *vgb* gene was integrated into *C. acremonium* chromosomal DNA (Fig. 4).

Expression of Active VHb

Transformants resistant to bleomycin were isolated and tested for VHb expression by a carbon monoxide (CO)-binding difference spectrum. It demonstrated the presence of active VHb in *C. acremonium* (Fig. 5). The peak at 420 nm results from a shift in absorbance due to CO binding to the active VHb expressed in the transformant.

Discussion

In this report, we established for the first time an efficient and stable method for the transformation of heterogeneous genes into *C. acremonium* mediated by *A. tumefaciens*. Transfer occurred via an active process mediated by the *A. tumefaciens vir* system, which leads to processing of the T-region (formation of T-strands by VirD1/VirD2 proteins) and establishment of a VirB pilus/pore structure that can mediate transfer of T-strands to *C. acremonium*. Since the structure of the T-DNA in fungi is similar to that in plants and yeast [4], we considered that T-DNA transfer to *C. acremonium* and plants occurs via a common mechanism.

Agrobacterium can mediate transformation not only for protoplasts, but also for fungal mycelium. Our results showed that the direct transformation of the mycelium of

Table 4. Transformation of *C. acremonium* mycelium mediated by *A. tumefaciens*.

Experiment	Medium	Wet weight of mycelium (g)	Number of bleomycin-resistant transformants	Numbers of bleomycin-resistant transformants (per 100 mg of wet weight mycelium)
1	With AS	0.12	116	97
	Without AS	0.10	60	60
2	With AS	0.09	103	114
	Without AS	0.12	35	29
3	With AS	0.11	129	117
	Without AS	0.11	50	45

Table 5. Comparison of transformation efficiency between *A. tumefaciens*-mediated and PEG-$CaCl_2$-mediated transformation of *C. acremonium*.

Transformation methods	Number of protoplasts	Number of bleomycin-resistant transformants	Number of bleomycin-resistant transformants per10^7 recipients
	2.8×10^7	18	6
by PEG-$CaCl_2$	2.9×10^7	32	11
	7.4×10^7	58	8
	2.85×10^7	384	135
by *A. tumefaciens*	7.0×10^7	897	128
	3.7×10^7	359	97

C. acremonium makes *A. tumefaciens* T-DNA transfer a powerful and easy-to-use tool for genetic transformation of *C. acremonium* and it may be extended to the application in other filamentous fungi. The transformation frequency reached in this research work could be from 80 to 140 cfu/ 10^7 protoplasts of *C. acremonium*, which is at least 15 times higher than that obtained by conventional PEG-mediated protoplast transformation. On the other hand, the frequency of transformation in *C. acremonium* is still lower than that in other filamentous fungi and yeast, as compared with the data published [5]. The reasons for this are unclear. Nonetheless, the high transformation frequency and the successful cloning of *vgb* and other heterogeneous genes in *C. acremonium* mediated by *A. tumefaciens* T-DNA transfer allow this technology to be applied further in improving the industrial cephalosporin C-producing strains and decreasing energy consumption in industrial fermentation as well.

Phleomycin is a member of the bleomycin family so-called glycopeptide antibiotics [1]. The mechanism of phleomycin activity against some fungi is similar to that of its structural analog, bleomycin, even though phleomycin was substantially more cytotoxic. Based on this reason and its availability in the market, we adopted bleomycin instead of phleomycin as a selective pressure. Our results demonstrated that the minimal inhibitory concentration (MIC) of bleomycin to untransformed *C. acremonium* is 0.5 μg/ml, indicating that bleomycin is an alternative to phleomycin as the selective agent.

Finally, the system mediated with *A. tumefaciens* T-DNA transfer offers the potential to transfer intact high molecular weight DNA into fungal chromosome. *A. tumefaciens* can transfer at least 150 kb of foreign DNA to plant cells [6]. In our work, *A. tumefaciens* could transfer approx. 16 kb plasmid to *C. acremonium*, which was almost impossible by other transformation methods.

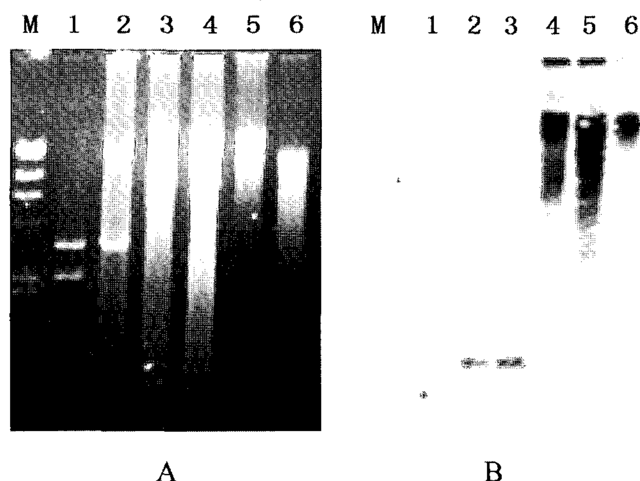

Fig. 3. Southern blot analysis of *C. acremonium* transformants.
A. Electrophoretogram of agarose gel. B. Southern blot. Lanes: M, λDNA-*Hin*dIII digest Marker (TaKaRa Biotech); 1, pYG715 (no *vgb* gene) digested by *Sca*I and *Eco*RI; 2, pYG306 (digested by *Cla*I); 3, chromosomal DNA from a transformant of *C. acremonium* digested by *Cla*I; 4, chromosomal DNA from a transformant of *C. acremonium*; 5, Control, chromosomal DNA from *C. acremonium*; 6, Control, chromosomal DNA from *C. acremonium* digested by *Cla*I.

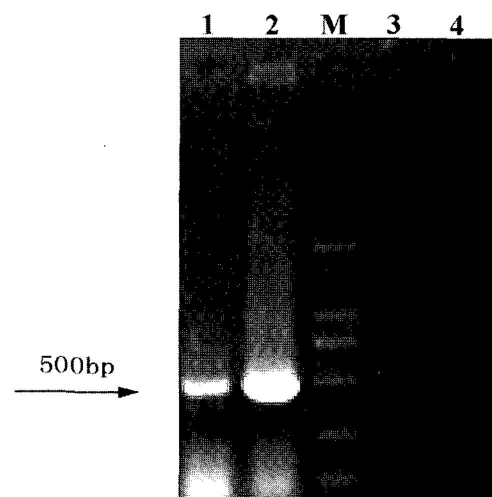

Fig. 4. PCR analysis for the presence of *vgb* in a transformant of *C. acremonium*.
Lanes: M, Marker DL2,000 (TaKaRa Biotech); 1, Transformant-p; 2, Transformant-m; 3, Transformant-p without *Taq* polymerase; 4, Transformant-m without *Taq* polymerase. Transformant-m: obtained by cocultivation with mycelia of *C. acremonium*; Transformant-p: obtained by cocultivation with protoplasts of *C. acremonium*.

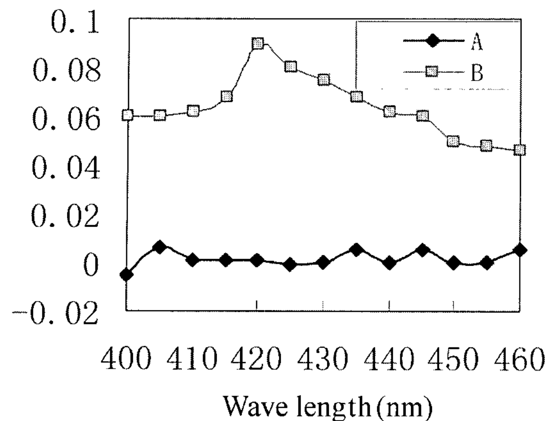

Fig. 5. Carbon monoxide-binding difference spectrum of cell extracts from a transformant of *C. acremonium*.
A. Control (without *vgb* gene) (◆); B. transformant (with *vgb* gene) (■).

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