

Efficient transformation of *Penicillium chrysogenum* mediated by *Agrobacterium tumefaciens* LBA4404 for cloning of *Vitreoscilla* hemoglobin gene

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The *vgb* and bleomycin resistance genes could be efficiently transferred into the filamentous fungus *Penicillium chrysogenum* under help of T-DNA of *Agrobacterium tumefaciens* LBA4404 and the transferred genes were integrated at a chromosomal locus of *P. chrysogenum*. Transformation using *A. tumefaciens* LBA4404 could be enhanced in the presence of acetosyringone (AS) when being carried out in the conidia and mycelium. The efficiencies of transformation could be improved up to 10 folds compared with conventional techniques for transformation of *P. chrysogenum* protoplasts.

Penicillins are β -lactam antibiotics which have broad clinical utility. Penicillins are produced by a number of microorganisms, including the several fungal species and several streptomycete species. *Penicillium chrysogenum* is an important industrial organism due to its capacity to produce penicillin which is still one of the main commercial antibiotics (Brakhage, 1998).

For decades, penicillin yields have been increased through development of better production strains by classical mutagenesis procedures and optimization of the growth conditions (Vanek and Hostalek, 1986; Fernandez-Canon et al. 1989; Keshavarz et al. 1989). Currently, together with

molecular techniques, this facilitated a thorough analysis of the genetic regulation of metabolic pathways. The great progress in elucidation of the molecular regulation of biosyntheses of penicillin has been made in the penicillin producer *P. chrysogenum*. Since the biochemistry of penicillin biosynthesis is rather well understood and recombinant techniques have been developed for some filamentous fungi, the overexpression of biosynthesis genes will lead to higher yields of penicillin in the respective production strains. Therefore, recombinant DNA technology of filamentous fungus will be an important tool for future strain improvement strategies with *P. chrysogenum* (Carr et al. 1986; Smith et al. 1990; Fernandez-Canon and Penalva, 1995).

Penicillin biosynthesis is regulated by environmental factors such as the phosphate, carbon, nitrogen and oxygen content of the medium (Feng et al. 1994). The overall rate of penicillin synthesis is severely reduced under conditions of low oxygen. Reduction of oxygen supply leads to accumulation of penicillin N, a precursor of penicillin. The mechanism of oxygen control over penicillin synthesis is not well understood. Possibly, low oxygen levels directly affect the biosynthetic pathway of penicillin, which includes several oxidation reactions. It is also possible that a more efficient overall metabolism provided by higher

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oxygen levels indirectly results in higher penicillin yields. Regardless of the mechanism, technologies that improve aerobic metabolism in these organisms should have a positive effect on penicillin production. *Vitreoscilla* hemoglobin (VHb) is so far the only one found in prokaryote. The expression of VHb is controlled by oxygen, and the amount of VHb expression increases greatly in lower oxygen environment. Possible mechanisms for VHb action include increasing the flux of oxygen to the respiratory apparatus, providing higher internal oxygen concentrations, altering the internal redox state, or functioning as an efficient terminal oxidase. VHb plays a major role in improving the conditions of bacterial growth, increasing the synthesis of protein and secondary metabolites, especially antibiotics, as well as promoting expression of heterogenous genes. The characteristic of VHb binding oxygen provides for us a possibility to enhance both the oxygen transfer and the efficiency of oxygen absorption in cells. Intracellular expression of a bacterial heme-binding protein (*Vitreoscilla* hemoglobin, or VHb) has resulted in higher productivity of a number of industrial cell types. For example, the expression of VHb in the filamentous bacterium *Streptomyces coelicolor* resulted in tenfold higher yields of the polyketide actinorhodin in bench scale batch fermentations run under reduced aeration (Magnolo et al. 1991). Also, the efficiency of cloned protein synthesis by oxygen-limited *Escherichia coli* cultures was increased in VHb-expressing strains (Khosla et al. 1990). Several VHb-expressing transformants of *Acremonium chrysogenum* produced significantly higher yields of cephalosporin C than control strains in batch culture experiments (Demodena et al. 1993). Therefore, intracellular expression of VHb in *P. chrysogenum* by recombinant DNA technology could provide a possibility that a productivity of penicillin was increased. Thus a basic requirement to establish a recombinant DNA system for the cloning of *vgb* gene (coding VHb) in *P. chrysogenum* is a gene transfer system. This includes a reproducible transformation procedure and proper selection markers. Several transformation techniques are available for filamentous fungi. Most protocols involve the transformation of protoplasts using electroporation or by a combination of CaCl_2 and polyethylene glycol (PEG). Filamentous fungi were transformed using two dominant selection markers, namely the bacterial resistance gene and complement gene of auxotrophic (Sanchez et al. 1987; Kolar et al. 1988). Intact fungal cells have also been transformed by the transformation of protoplasts, but usually with a lower transformation frequency. *Agrobacterium tumefaciens* can transfer part of its Ti plasmid, the T-DNA, into plant cells during tumorigenesis (Winans, 1992; Hooykaas and Beijersbergen, 1994; Ishida et al. 1996). It is routinely used for the genetic modification of a wide range of plant species. For years, the transformation using *A. tumefaciens* has been applied to the yeast *Saccharomyces cerevisiae* and filamentous fungi, including *Aspergillus niger*, *Fusarium venenatum*, *Trichoderma reesei*, *Colletotrichum gloeosporioides*, *Neurospora crassa*, and the mushroom *Agaricus bisporus*

(Bundock et al. 1995; Bundock and Hooykaas, 1996; de Groot et al. 1998).

Here we report that *Agrobacterium tumefaciens* LBA4404 transfers part of its Ti plasmid pBI121v vgb^+ , the T-DNA with *vgb* and bleomycin resistance gene, to *P. chrysogenum* cells. The T-DNA then integrates into the *P. chrysogenum* nuclear genome at a random position.

Materials and Methods

Strains

Fungal strain *P. chrysogenum* was obtained from biology department of Shanghai institute of pharmaceutical industry. *Escherichia coli* strain DH5 α was used as recipient hosts for all DNA work. The *A. tumefaciens* strain LBA4404 was used for the transformation of the *P. chrysogenum*.

Plasmid construction

pBI121v vgb^+ was constructed as follows. A 3kb *SacI/Hind* fragment containing Gus gene was deleted from the binary vector pBI121, the remaining other 10kb large fragment containing T-DNA sequence was blunted and ringwise ligated itself. The *EcoR* I site was between left and right border of TDNA. Meantime a 6.3 kb *EcoR* I fragment was obtained from pYG715/*vgb*, which containing the *vgb* structural gene and bleomycin resistance gene, both were fused to the transcriptional promoter region of the *trpC* gene of *A. nidulans* (*P_{trpC}*). Then the 6.3kb *EcoR* I fragment was ligated into the *EcoR* I sites between left and right border of TDNA of 10kb large fragment from the binary vector pBI121, resulting in pBI121v vgb^+ and pBI121v vgb^- .

Media and conditions

For the penicillin production experiments, seed cultures were prepared as follows. Spores from *P. chrysogenum* clones were grown on LCS Agar {lactose, 1.5%(w/v); corn-steep liquor, 0.25%(v/v); peptone, 0.5%(w/v); sodium chloride, 0.4%(w/v); magnesium sulfate, 0.05%(w/v); potassium dihydrogen phosphate, 0.06%(w/v); ferric chloride, 0.0005%(w/v); cupric sulfate, 0.0002%(w/v); agar 3.0%(w/v)}, harvested, and inoculated into 25 ml defined seed media {glucose, 3.0%(w/v); pharmamedia, 1.0%(w/v); corn steep liquor, 3.0%(v/v); ammonium sulfate, 0.2%(w/v); calcium carbonate, 0.5%(w/v); potassium dihydrogen phosphate, 0.05%(w/v); lactose, 1.0%(w/v); primary dry yeast, 1.0%(w/v)} in a 250 ml glass flask. The seed cultures were incubated at 25°C and 220 rpm for 48 hours in an orbital incubator. 5 ml of the seed culture was inoculated into 25 ml of minimal production media {lactose, 12.0%(w/v); pharmamedia, 2.5%(w/v); ammonium sulfate, 1.05%(w/v); calcium carbonate, 1.0%(w/v); calcium sulfate, 0.5%(w/v); sodium sulfate,

0.4%(w/v); lard oil, 1.0%(w/v); potassium dihydrogen, 0.05%(w/v); potassium sulfate, 0.5%(w/v); phenoxyacetic acid, 1.25%(w/v)} in 250 ml glass flasks. The cultures were incubated at 28°C and 220rpm for 240 hours (Vanek and Hostalek, 1986b).

***P. chrysogenum* transformation**

P. chrysogenum conidia were obtained by growing the strain on *LCS* Agar plate for 5 days and washing the conidia with a physiologic salt solution. Cocultivations between *A. tumefaciens* and *P. Chrysogenum* was performed as follows. For transformation of conidia, 100 ml conidia at a concentration of 10^6 - 10^8 conidia/ml were mixed with 100 ml of the *A. tumefaciens* culture prepared as described. The *A. tumefaciens* containing the binary vector pBI121vgb+ were obtained by growing the strain on a *LB* plate for 23 days and washing the strain with a physiologic salt solution. When mycelia were transformed, 100 ml mycelia at a concentration of 1 gram/ml were used. Subsequently, these mixtures incubated into 50 ml defied seed media with or without acetosyringone (AS) at 25°C and 220rpm for 48 hours. Then these mixtures cultures were transferred to *P. chrysogenum* minimal medium plates containing 10 mg/ml cefotaxime to kill the *A. tumefaciens* cells and 20 mg/ml bleomycin to select for transformants. Transformation of *P. chrysogenum* was performed using the polyethylene glycol method as described previously (Cantoral et al. 1987).

Isolation of DNA and Southern hybridization

The vegetative mycelial growth from a 48 hour culture prepared as described above was collected by filtration through cheesecloth, frozen in liquid nitrogen and lyophilized overnight. The dried mycelia were ground with sand in a mortar and pestle and resuspended in 25 ml of 100 mmol/L LiCl, 50 mmol/L EDTA, 10 mmol/L Tris pH8.0, 4% SDS. After heating the suspension to 55°C in a 60°C water bath, the mixture was extracted first with 1 mol/L Tris (pH 8.0) saturated phenol, followed by Tris-saturated phenol:chloroform (1:1, v:v) and then chloroform RNA was precipitated from the aqueous phase by the addition of an equal volume of cold 6mol/L LiCl and then the mixture was remained at -20°C for two to three hours. After centrifugation at 12000 g for 20 minutes at 4°C, the supernatant was made 66% (v/v) ethanol and cooled to -20°C for 15 minutes to precipitate the DNA. After centrifugation as described above, the DNA pellet was washed with 70% ethanol, dried and resuspended in TE Buffer (10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA) (Conder, 2000). The DNA concentration was estimated by comparison to known DNA standards when stained with ethidium bromide in agarose gel electrophoresis. 5 mg of the DNA samples digested by *Cla* I were run on a 0.7% agarose denaturing gel and blotted to nitrocellulose filter. The filter was hybridized to probes with the *vgb* gene probe. The blotting and hybridizations were carried out under standard conditions.

Results

Construction of the binary vector

The binary vector was constructed for transfer T-DNA from *A. tumefaciens* to *P. chrysogenum*. The vectors, pBI121vgb+ or pBI121vgb- (Figure 1), were constructed based on the bidirectional integration of the vector pYG715vgb and the vector pBI121. The *vgb* gene and the bleomycin resistance gene fused downstream of the transcriptional promoter region of the *trpC* gene from *Aspergillus nidulans* (*PtpC*) and were between left and right border of T-DNA of the binary vector.

Effect of the acetosyringone concentration and the mycelia collected at different growth time on transformation of *P. chrysogenum* mediated by *A. tumefaciens*

In order to establish a system in which *A. tumefaciens* could transfer T-DNA to the filamentous fungus *P. chrysogenum*, we constructed the binary vector pBI121vgb+. This plasmid contains the *vgb* and the bleomycin resistance gene between the left border and right border repeats of the binary vector pBI121. Plasmid pBI121vgb+ was then electroporated into the *A. tumefaciens* LBA4404 helper strain, which carries the *vir* genes that code for the T-DNA transfer system.

Incubation of *P. chrysogenum* cells with *A. tumefaciens* could lead to the formation of bleomycin-resistant colonies, and a greater number of bleomycin-resistant colonies were found if the cocultivation was done on medium containing AS (Table 1). The negative controls never gave rise to resistant colonies. This result shows that induction of the *vir* genes is essential for transfer of the resistance trait to the mold cells and wherefore suggests that *A. tumefaciens* can use its virulence system to transform the filamentous fungus *P. chrysogenum*. The transformation frequency varied from approximately 50 to 2700 transformants per 100 mg conidia or mycelia.

Comparison of transformation using *A. tumefaciens* with the transformation of protoplasts

The transformation frequency using *A. tumefaciens* is up to 10 times higher than the values obtained for PEG transformation of protoplasts (Table 2). These transformants obtained by both transformation methods showed different growth in the medium containing different KCl concentration (Table 3). Transformants-p get a halophilism, and transformants-a show same growth with control strain in the medium containing different salt concentration. We presumed that the DNA transfer mechanism is different in the two transformation methods.

The stability of bleomycin resistance in *P. chrysogenum* transformants

The stability of the bleomycin resistant phenotype was confirmed. First, transformants obtained by transformation

of protoplasts and by *A. tumefaciens* were transferred to fresh plates without bleomycin and the transfer were carried out from one generation to the next until the fifth generations. Then transformants F1-F5 were transferred to fresh plates containing 20 mg/ml bleomycin. The results showed that the bleomycin resistant phenotype of transformants obtained by both transformation methods was heritable and stable (Table 4).

Then genomic DNA of the transformants F5 resistant to bleomycin were isolated and tested by Southern blotting analysis with *vgb* probe for *vgb* cloning (Figure 2). Therefore the *vgb* and bleomycin resistance genes were integrated into *P. chrysogenum* chromosome genome.

Discussion

The results demonstrated that the *Agrobacterium tumefaciens* LBA4404 could transfer its T-DNA into *Penicillium chrysogenum*. The transfer could depend on the induction of the bacterial virulence genes, which expression could mediate transfer of T-DNA into *P. chrysogenum*. *A. tumefaciens* can transform not only the conidia of *P. chrysogenum*, but also hyphal tissue. Transformation using *A. tumefaciens* LBA4404 could be enhanced in the presence of acetosyringone when being carried out in the conidia and mycelia. However, acetosyringone is not necessary for transformation of DNA in mycelia. This paper showed that the efficiencies of transformation using *A. tumefaciens* could be improved up to 10 folds compared with the transformation of *P. chrysogenum* protoplasts combined of CaCl₂ and polyethylene glycol. Transformants of *P. chrysogenum* obtained by transformation of protoplast got a halophilism, they could grow at presence of high concentration of KCl salt, but transformants obtained by *A. tumefaciens* couldn't. We presumed that the DNA transfer mechanism was different between two transformation methods. Because of *A. tumefaciens* LBA4404 can transfer a foreign DNA into conidia and mycelia of *P. chrysogenum* directly, this is a powerful and simple tool for genetic transformation of industrial *P. chrysogenum*.

Finally, the successful cloning of *vgb* in *P. chrysogenum* by *A. tumefaciens* T-DNA transfer permits this technology to be applied in improving of industrial penicillin-producing strains as well.

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APPENDIX

Figures

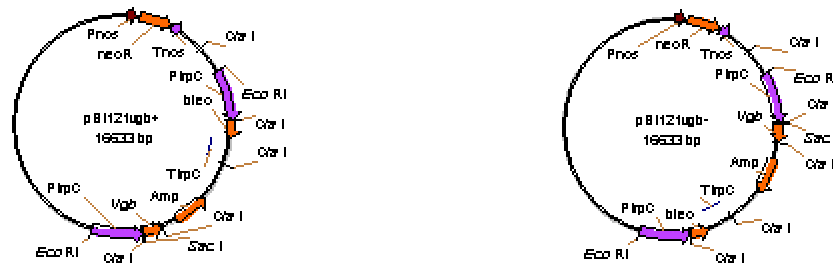


Figure 1. Construction of the binary vector pBI121vgb+ and pBI121vgb- with *vgb* and bleomycin resistance gene between left and right border of T-DNA.

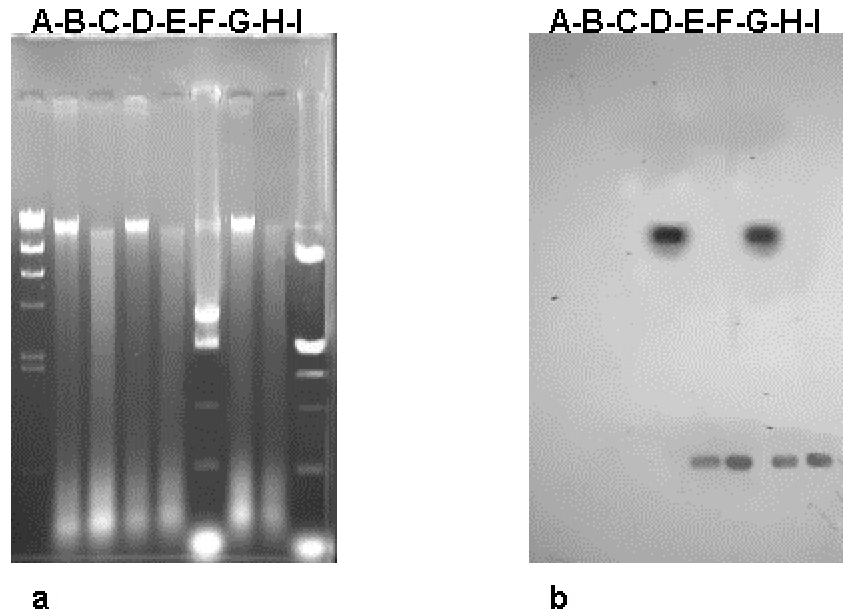


Figure 2.

a. Electrophoretogram of agarose gel

b. Southern blot

A: marker;

B: control *P. chrysogenum* genomic DNA;

C: digestion of control *P. chrysogenum* genomic DNA by *Cla* I;

D: transformant-p F5 genomic DNA;

E: digestion of transformant-p F5 genomic DNA by *Cla* I;

F: digestion of pYG715vgb by *Cla* I;

G: transformant-a F5 genomic DNA;

H: digestion of transformant-a F5 genomic DNA by *Cla* I;

I: digestion of pBI121vgb+ by *Cla* I;

transformant-p: *P. chrysogenum* transformant by PEG transformation of protoplasts;

transformant-a: *P. chrysogenum* transformant using *A. tumefaciens*.

Tables

Table 1. Numbers of bleomycin^R transformants per 100 mg conidia or mycelia when transformation of *P. chrysogenum* mediated by *A. tumefaciens*.

The concentration of AS(mg/ml)	Conidia	The growth time of mycelia (hours)			
		24	36	48	60
0	-	55	54	67	55
10	130	507	615	398	275
25	579	1395	1097	965	649
50	547	2535	2659	2640	843
100	841	2554	2570	2577	874
200	802	2536	2599	2565	580

Table 2. Effects of protoplasts prepared from per 100 mg mycelia collected at different growth time on the efficiencies of transformation.

The concentration of protoplasts	The growth time of mycelia for the preparation of protoplasts (hours)				
	0	24	36	48	60
1×10 ⁶ /ml	-	29	117	238	209
1×10 ⁷ /ml	-	25	145	255	213
1×10 ⁸ /ml	-	27	108	220	217

Table 3. Effect of KCl concentration on the growth of *P. chrysogenum* transformants obtained by two methods.

The average diameter of colonies (mm)	The concentration of KCl (mol/L)										
	0	0.5	0.9	1.3	1.7	2.1	2.5	2.9	3.3	3.7	4.1
Control	>7.5	>7.5	5-7.5	<5	-	-	-	-	-	-	-
Transformants-p	-	-	<5	5-7.5	>7.5	>7.5	>7.5	>7.5	>7.5	>7.5	>7.5
Transformants-a	>7.5	>7.5	5-7.5	<5	-	-	-	-	-	-	-

Note: the diameter of colony >7.5mm, good growth; 7.5mm>the diameter of colony<5mm, poor growth; -, no growth; Transformants-p: *P. chrysogenum* transformants by transformation of protoplasts with PEG; Transformants-a: *P. chrysogenum* transformants using *A. tumefaciens*; twenty strains were selected from control, transformants-p, transformants-a, respectively.

Table 4. Stability of bleomycin resistance in *P. chrysogenum* transformants.

	F1	F2	F3	F4	F5
Control	-	-	-	-	-
Transformants-p	+	+	+	+	+
Transformants-a	+	+	+	+	+

Note: +, grow; -, no grow; 20mg/ml bleomycin; Transformants-p: *P. chrysogenum* transformants by PEG transformation of protoplasts; Transformants-a: *P. chrysogenum* transformants using *A. tumefaciens*; twenty strains were selected from control, transformants-p, transformants-a, respectively.