Supporting Information

Development of a prokaryotic-like polycistronic expression system based on a virus-originated internal ribosome entry site (IRES) in industrial eukaryotic microorganisms

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Experimental Section

Strains, plasmids, media and growth conditions

Strains, plasmids and primers are listed in **Table S1, Table S2 and Table S3**.*Escherichia coli* DH5α was cultivated in LB medium at 37 ℃, 230 rpm and *Pichiapastoris* strain GS115 was grown in YPD medium at 28 ℃, 230 rpm. YEB medium was prepared for the growth of *Agrobacterium tumefaciens*LBA4404 at 28 ℃, 230 rpm and IM medium containing 200 mMacetosyringone (AS) (Sigma, USA) was for transformation. *Acremoniumchrysogenum* was cultivated in YPS medium and fermented in a specific fermentation medium inclued seeds medium ($g \cdot L^{-1}$) (corn steep liquor, 60; sucrose, 35; glucose, 5; DL-Met, 0.5; $(NH_4)_2SO_4$, 8; $CaCO_3$, 0.5; pH 6.5) and fermentation medium ($g \cdot L^{-1}$) (corn steep liquor, 100; starch, 30; dextrin, 60; glucose, 5; DL-Met, 6; K_2HPO_4, 9; $MgSO_4 \cdot 7H_2O$, 3; $(NH_4)_2SO_4$, 13; $CaCO_3$, 10; bean oil, 20 mL; trace elements: $FeSO_4$, 0.16; $MnSO_4$, 0.04; $ZnSO_4$, 0.04; $CuSO_4$, 0.04; pH 6.2) at 28 ℃, 230 rpm.

Construction of the plasmids

pPIC02-vIh:The DNA fragment *vgb*-IRES-*hph* was amplifiedusingplasmid pDH25-vIh as the template and *vgb*-up and *hph*-down as the primers. The PCR fragment was

ligated with T-vector pPIC02-T by T-A ligation. *E.coli* DH5α was selected as the host.Transformants were selected by LB agar medium with 50μg ml^{-1}kanamycin and positive colonies were screened by colony PCR. Detailed scheme for vector construction is depicted in **Figure S1** .

pBI121-vIh: The DNA fragment of *vgb*-IRES-*hph* as well as the promoter(Ptrp) and terminator(Ttrp) was digested by EcoR I/Xba I into three segments and the two shorteroneswere ligated with plasmid pBI121 successively. *E.coli* DH5α was selected as the host. Transformants were selected by LB agar medium with 50μg ml^{-1} kanamycin and positive colonies were screened by colony PCR. A detailed scheme for vector construction is depicted in **Figure S2** .

Transformation of*Pichiapastoris*

Competent*Pichiapastoris* strain GS115 was transformed with linearized pPIC02-vIh by the electroporation according to the methods reported[1].Electro-competent cells (100μL) were mixed with 20μg linearized pPIC02-vIh plasmid in a 0.2 cm electroporation cuvette and pulsed for 6 ms with a field strength of 1.25×10^6 V/m using the electroporation apparatus BTX ECM399. The transformants were screened on the MM plate containing Hygromycin B (200μg/ml) from the colonies emerged after being spread on the MD antibiotic free plate for 2 days. The transformed agar plates compared with the control please refer to **Figure S3.**

Analysis of the hereditary stability of recombinant *Pichiapastoris*

The analysis of the hereditary stability of recombinant *Pichiapastoris* was carried out

by 5 pass-generation tests. For each test, the recombinant*Pichiapastoris* strain was first incubated in a flask of 5ml liquid YPD media at 230 rpm and 28℃ for 24h, and then 5μL broth was transferred to another flask of fresh liquid YPD media for the next test. 10μL broth of each test was diluted 10^7-fold, 200μL of which was then spread on solid YPD medium and incubated at 28℃ for 2 days. 60 colonies were selected randomly from the ones emerged on the solid YPD medium and transferred to the solid MM medium with Hygromycin B (200μg/ml) or without antibiotic. Thehereditary stability was described by the ratio of colony numbers on antibiotic MM media to antibiotic-free MM media for the 5 pass-generation tests.

Agrobacterium tumefaciens*-mediated transformation (ATMT) of *Acremoniumchrysogenum

A. tumefaciens strain LBA4404 was chosen to transform *Acremoniumchrysogenum*. The binary plasmid pBI121-vIh, with vgb-IRES-hph gene under the control of *Aspergillusnidulans*trpC promoter, was transformed into the *A. tumefaciens* competent cells using the freeze–thaw method[2]. The transformant was then grown in YEB liquid medium with kanamycin (50μg/ml) and rifampicin (50μg/ml) for 48h at 230 rpm and 28℃. The bacterial cells were collected by centrifuge, resuspended to OD_{600}=0.15 with IM medium containing 200 mMacetosyringone (AS) (Sigma, USA)[3] and re-incubated at 28℃ until the density reached 0.5 (OD_{600})[4]. The *A. chrysogenum* mycelia, chosen as the receptor, were collected after the incubation in YPS liquid medium at 230 rpm and 28℃for 5 days and then grinded with pestle in order to improve the transformation efficiency. 100μL wet mycelia of *A. chrysogenum* and

100μL suspension of *A.tumefaciens* cells were co-incubated at 230 rpm and 28℃ for 48h and then spread on the YPS medium with 200 μMcefotaxime and 6μg/ml hygromycin B for screening positive transformants. The transformed agar plates compared with the control please refer to **Figure S3**.

Low oxygen stress experiment

The transformant and wild type *A. chrysogenum* were both inoculated in YPS liquid medium at 230 rpm and 28℃ for 5 days. The supernatant of broth containing spores was collected and diluted to OD600=0.2536. The diluted supernatant was first inoculated to seeds medium (g/L) (corn steep liquor, 60; sucrose, 35; glucose, 5; DL-Met, 0.5; $(NH_4)_2SO_4$, 8; $CaCO_3$, 0.5; pH 6.5) and incubated at 230 rpm and 28℃ for 3 days. Fresh fermentation medium (g/L) (corn steep liquor, 100; starch, 30; dextrin, 60; glucose, 5; DL-Met, 6; K_2HPO_4, 9; $MgSO_4{\cdot}7HO_2$, 3; $(NH_4)_2SO_4$, 13; $CaCO_3$, 10; bean oil, 20 mL; trace elements: $FeSO_4$, 0.16; $MnSO_4$, 0.04; $ZnSO_4$, 0.04; $CuSO_4$, 0.04; pH6.2) of different volumes in a 300mL flask was inoculated with the seeds broth by 10% volume ratio. Medium volumes were set to 30, 50, 70, 90, 110mL to create different oxygen availabilities. Each fermentation broth was centrifuged after 7 days' incubation. The supernatant was collected for cephalosporin C (CPC) production analysis and the sediment was dried and weighed.

Analysis of the hereditary stability of recombinant *Acremonium chrysogenum*

The analysis of the hereditary stability of recombinant *A. chrysogenum* was carried out by serial inoculation and incubation for 5 times. The *A. chrysogenum* transformant was first incubated in liquid YPS media at 28℃ and 230rpm for 5 days and then 10μL

broth supernatant (spore suspension) was diluted 10^7-fold, 200μL of which was spread on the solid YPS medium without antibiotics to obtain single colonies and 5μL undiluted broth was transferred to fresh liquid YPS media for the next test. 50 colonies were selected randomly from the ones emerged on the YPS medium without antibiotics and transferred to the YPS medium withHygromycin B (6μg/ml). Thehereditary stability was described by the ratio of colony numbers on antibiotic media to antibiotic free media for the 5 pass-generation tests.

Cylinder plate method

Cup plate method using YPS agar medium was employed, based on the sensibility of the *Bacillus cereus* strain 1.1687 to CPC. 25 mL YPS agar medium was poured into a 100 mm × 20 mm petri dish. After the medium solidified, 200μL broth of *Bacillus cereus* strain 1.1687 was spread. In each plate, 5 oxford cups (6mm × 7.8mm × 10mm) were placed on the surface of medium and each oxford cup was filled with 200μL broth of *A. chrysogenum*. The plates were incubated at 28℃for 24h. The diameters of the growth inhibition zone were carefully measured. All experiments were performed in a clean bench and repeated for three times. As a rapid screening method rather than quantified analysis, the results of the cylinder plate method would be further confirmed by the high performance capillary electrophoresis (HPCE) analysis.

High performance capillary electrophoresis (HPCE) analysis of CPC production

The capillary electrophoresis (CE) analysis was performed by Beckman P/ACETM MDQ Capillary Electrophoresis system (Beckman Coulter, USA) equipped with an on-column UV-visible detector, using 0.2% D-phydroxyphenylglycine as the internal

standard substance and 10 mM phosphate buffer of pH 6.5 as the running buffer. Standard solutions and the running buffer were all pre-filtered through a syringe with the cellulose acetate filter (0.22μm). Each 100μL sample of the culture supernatant of *A. chrysogenum* was diluted with 400μL internal standard substance and filtered through a syringe with the cellulose acetate filter (0.22μm) prior to CE analysis. CE was performed at separation voltage of 30 kV with the running buffer. Capillary temperature was controlled at 25℃ and detection wavelength was set at 254 nm. Sample injection was performed at 0.5 psi for 5 s. Before the next run, the capillary was successively washed with 0.2M HNO_3, deionized water, 0.2M NaOH and deionized water for 3 min each and then rinsed with the running buffer for 3 min. The concentration of CPC was calculated according the peak area of CPC and the internal standard substance on the electrophoretogram.

Tables and Figures

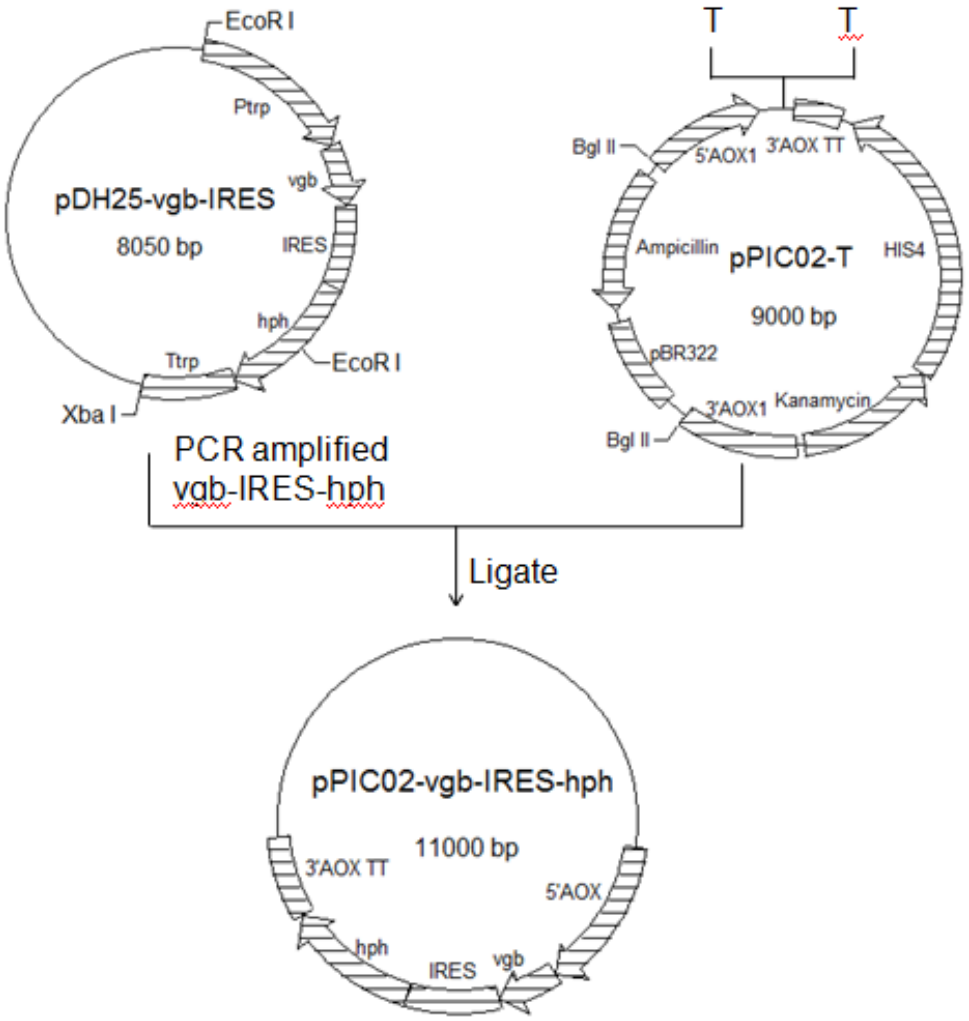

Figure S1. Construction of plasmid pPIC02-vIh

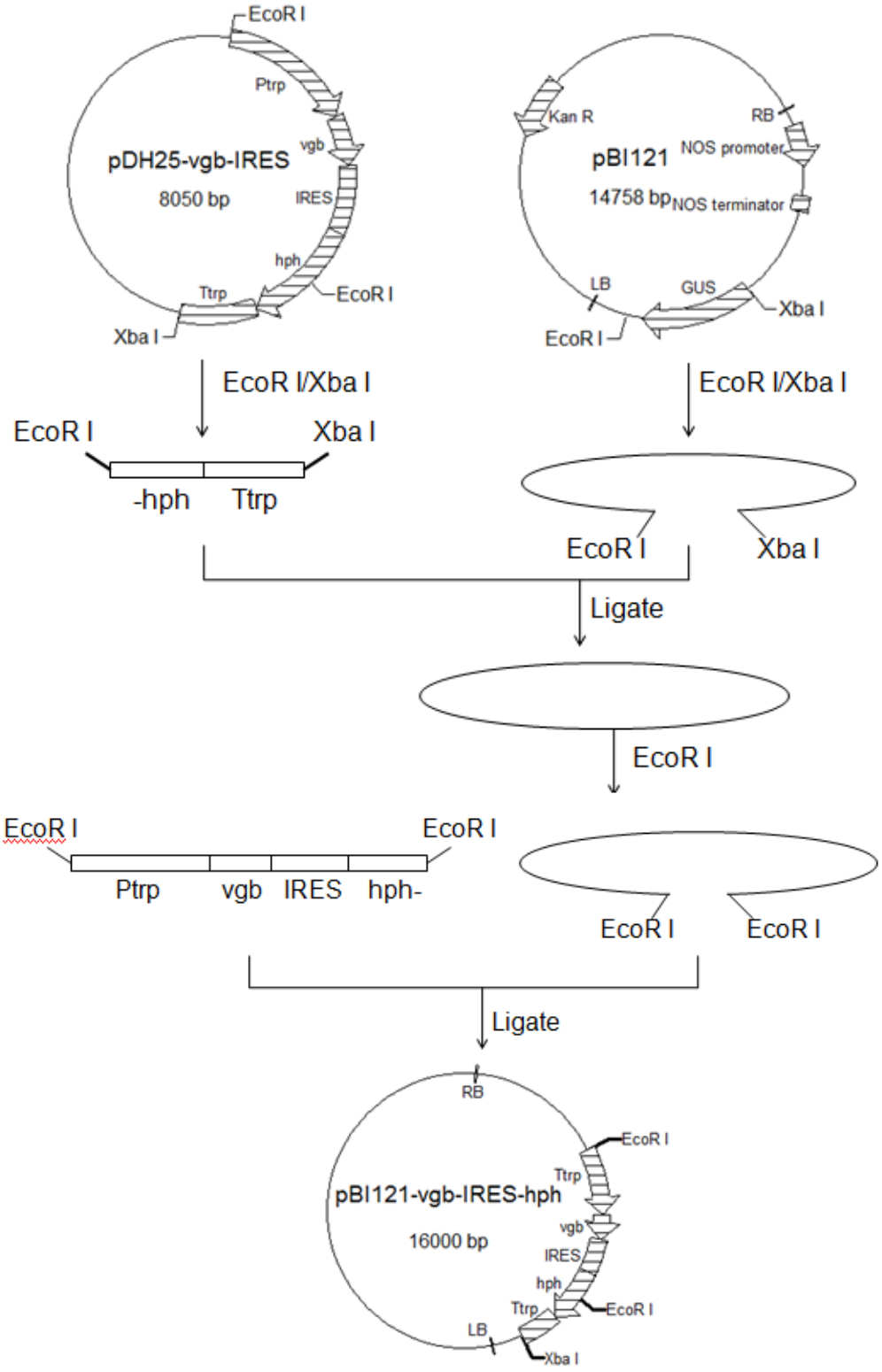

Figure S2. Construction of plasmid pBI121-vIh

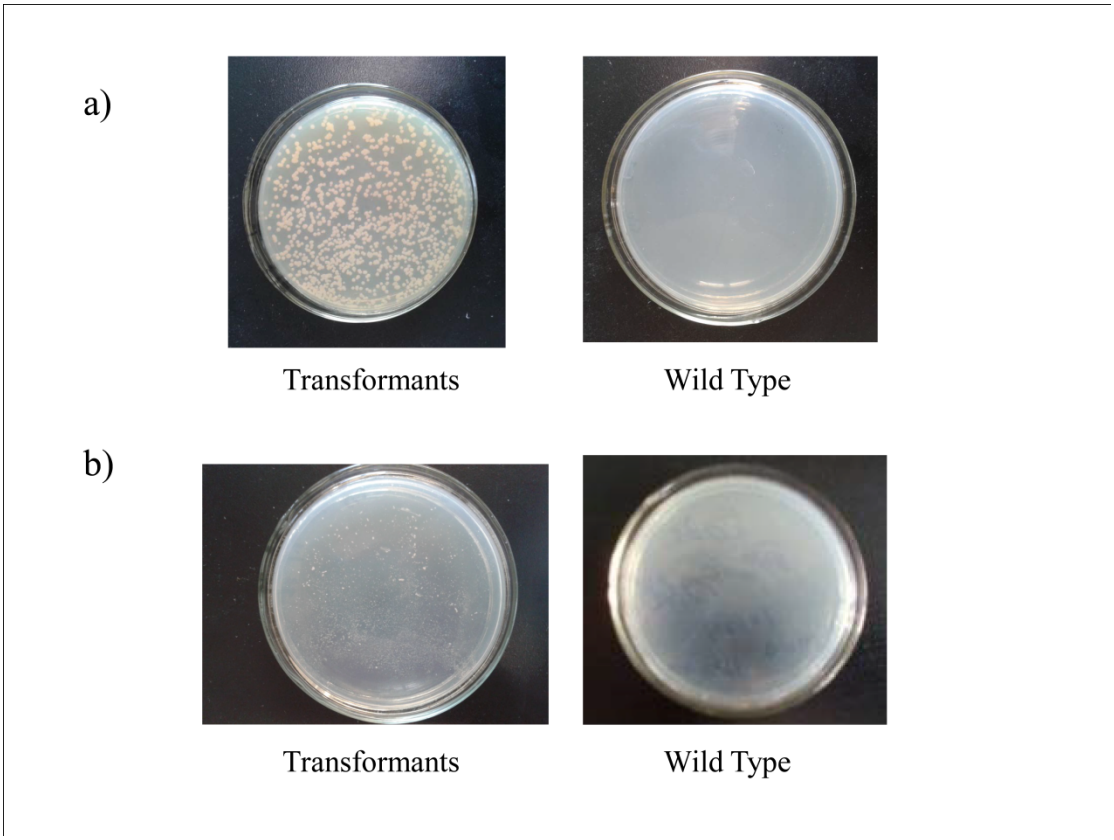

Figure S3. Transformed agar plates compared with the control

a) *Pichia pastoris*

b) *Agrobacterium tumefaciens*-mediated transformation (ATMT) of *Acremonium chrysogenum*

Table S1. Strains used in this study

Name	Description	Source
E. coli DH5α	F -φ80 lacZΔM15Δ（lacZYA-argF）U169 end A1 recA1 hsdR17(rk -,mk -) sup E44 λ- thi-1 gyrA96 relA1 phoA	Biomed
Bacillus cereus 1.1687	Sensitive to CPC	Lab collection
Agrobacterium tumefaciens LBA4404	Used for mediumed transformation of *A.chrysogenum*	Lab collection
Pichiapastoris GS115	Host	Lab collection
Acremoniumchrysogenum	Host	Lab collection

Table S2. Plasmids used in this study

Name	Description	Source
pDH25-vIh	Plasmid pDH25 with the *vgb*-IRES-*hph* gene	This study
pPIC02-T	T-vector from plasmid pPIC3.5K	This study
pBI121	Plasmid coding T-DNA for Agrobacterium-mediated transformation	This study
pPIC02-vIh	Plasmid pPIC02 with the *vgb*-IRES-*hph* gene	This study
pBI121-vIh	Plasmid pBI121 with the *vgb*-IRES-*hph* gene	This study

Table S3. Primers used in this study

Name	**Sequence 5'-3'**
vgb-up	ATGTTAGACCAGCAAACC
hph-down	CGGTCGGCATCTACTCTATTCCTTT
3'AOX	GGCAAATGGCATTCTGACAT
5'AOX	GACTGGTTCCAATTGACAAGC
cefG-up	ATGCTGCCGTCGGCCCAAGTGGCCC
cefG-down	TCACATTAATGACTGATCGAGGAATCCTCTGACGGCATCATTAACCTT GT
vgb-ClaI^{+}	CCATCGATATGTTAGACCAGCAAACCA
hph-SpeI^{-}	GGACTAGTTTATTCAACCGCTTGAGCG

Table S4. Hereditary Stability Test of *Pichiapastori*

Generation of the strains	Colony number			Exogenous gene retention rate
	inoculated	Strains in the MM medium without Hyg B	Strains in the MM medium with Hyg B	
1	60	60	60	100%
2	60	60	60	100%
3	60	60	60	100%
4	60	60	60	100%
5	60	60	60	100%

Table S5. Hereditary Stability Test of *Acremoniumchrysogenum*

passage number	Generation of each passage	Colony number inoculated	Strains in the MM medium with Hyg B	Exogenous gene retention rate
1	5	50	50	100%
2	5	50	50	100%
3	5	50	50	100%
4	5	50	50	100%
5	5	50	50	100%

References

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2. G.Jyothishwaran, D.Kotresha, T.Selvaraj, S.Srideshikan, P. Rajvanshi andC. Jayabaskaran, *Curr. Sci.*2007,**93**, 770-772.

3. C. B.Michielse,P. J. Hooykaas, C. A.van den Hondel andA. F. Ram, *Nat. protoc.* 2008,**3**, 1671-1678.

4.J. P.Combier, D.Melayah, C.Raffier, G. Gay and R.Marmeisse, *FEMS microbial.lett.*2003,**220**, 141-148