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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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A prokaryotic-like polycistronic expression system was developed in *Pichia pastoris* and *Acremonium chrysogenum*. With simpleness and higher effectiveness, this polycistronic expression system is suitable for introducing multiple genes or entire metabolic pathways into industrial eukaryotic microorganisms.

The introduction of multiple target genes or entire metabolic pathways into industrial microorganisms is now a routine procedure of metabolic engineering and synthetic biology, focused on constructing long metabolic pathways with multiple genes simply and effectively[1]. In prokaryotes, linked by ribosome binding sites (RBS), the functionally related genes can be clustered together following a single promoter and multiple protein products can therefore be generated from a single messenger RNA strand called polycistron[2]. In eukaryotic organisms, however, most mRNA is monocistronic, meaning that each gene requires an individual promoter for transcription[3]. In comparison with the polycistronic mechanism in prokaryotes, the monocistronic mechanism in eukaryotes means more DNA cloning/synthesis and multiple rounds of transformation in the genetic engineering process[4], and it also increases the difficulty to identify the expressing level of different genes with independent expression elements, which are important for the metabolic engineering and synthetic biology of industrial eukaryotic microorganisms[5].

Although the vast majority of eukaryotic mRNAs initiate the translation dependent on the 7-methyl-guanosine (m7G) cap structure at the 5' terminal of mRNA[6-8], there is another cap-independent manner for the translation initiation, making the construction of polycistron possible in eukaryotes. Internal ribosome entry sites (IRES), existing in the RNA genome of *Picornaviruses* such as *Poliovirus (PV)*[9],*Encephalomyocarditis virus (EMCV)*[10] and mRNA molecules of yeasts[11], mammals[12], insects, etc, can initiate the translation of mRNA without the assistance of 5' cap structure under the conditions where the cap-dependent translation is absent or suppressed, for instance, during viral infection, cell cycle progression, stress or apoptosis[13]. PV-IRES was the first IRES element utilized to construct polycistron in eukaryotes[9]. However, EMCV-IRES is currently the most widely used for its high efficiency to translate proteins[14] and high adaptability to accommodate different hosts[15, 16]. EMCV-IRES has been applied in cellular transformation[17], production of transgenic animals/plants[18, 19], recombinant protein production[13,20], gene therapy[21, 22], etc. Vectors for co-expression of 3-5 genes can also been constructed with EMCV-IRES[23,24] for the purpose of metabolic engineering. Although EMCV-IRES has already been applied in plant cells, mammalian cells and insects, the application in industrial eukaryotic microorganisms has not been reported. In this paper,a prokaryotic-like polycistronic expression system based on EMCV-IRES was developed in two industrial eukaryotic microorganisms: *Pichia pastoris* and *Acremonium chrysogenum*, in order to investigate the function and application potential of EMCV-IRES in industrial eukaryotic microorganisms.

Figure 1 shows the structure and mechanism of the polycistron called *vgb*-IRES-*hph* based on EMCV-IRES in this study. The sequence of EMCV-IRES was obtained from the NCBI site(GenBank X74312.1) and then synthesized by assembly PCR[25]. *Vitreoscilla* globin (*vgb*) gene encoding *Vitreoscilla* hemoglobin (VHb), which can improve the hypoxic tolerance of host cells, was chosen as the first gene. The bacterial hygromycin B phosphotransferase (*hph*) gene, which can enable the hosts having the resistance to hygromycin B, was chosen as the second gene functioning as a selectable marker. The fragment containing *vgb* gene, EMCV-IRES element and *hph* gene could be transcribed by a single promoter into one mRNA strand with a cap structure at the 5' end. The *vgb* gene could be translated by the 5' cap structure and the*hph* gene by the EMCV-IRES element. Different promoters were chosen for transcription in different hosts. In this study the methanol-inducible promoter P_{AOX1} was chosen for *Pichia pastoris* and the *Aspergillusnidulans* trpC gene promoter *P*trp for *Acremonium chrysogenum*.

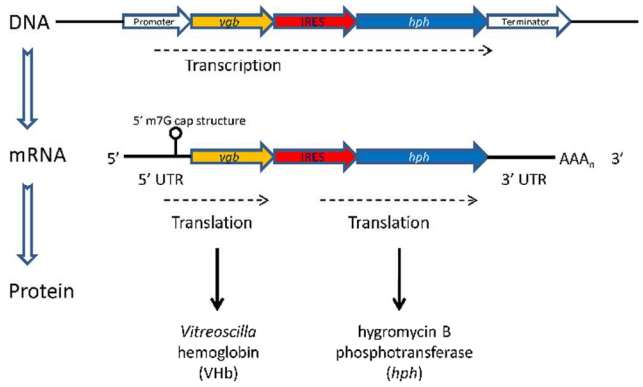

Figure 1. The structure and mechanism of the polycistron *vgb*-IRES-*hph* in this study. The fragment containing *vgb* gene, EMCV-IRES element and *hph* gene is able to be transcribed by a single promoter into a mRNA strand with a cap structure formed at the 5' end. The *vgb* gene can be translated by the 5' cap structure while the *hph* gene by the EMCV-IRES element.

In order to investigate whether the EMCV-IRES could function in *Pichia pastoris*, a shuttle vector for *E coli* and *Pichiapastoris*pPIC02-vIh was constructed by the ligation of *p*PIC02-T with the DNA segment of *vgb*-IRES-*hph* (Figure S1, Supporting Information). The competent yeast cells were electroporated with linearized plasmids of *p*PIC02-vIh. The positive transformants were then transferred to solid MM media containing Hygromycin B (200 μg/ml), supplied with methanol as the carbon resource and inducer at an interval of 24h. All the positive transformants were able to grow on the MM media with Hygromycin B, indicating that EMCV-IRES could initiate the expression of *hph* gene in *Pichia pastoris*. In comparison with the wild type, the 2000bp DNA fragment of *vgb*-IRES-*hph* could be amplified by PCR from the genomic DNA of the transformants, which confirmed the successful integration of heterologous genes into the *Pichia pastoris* genome (Figure 2A). The SDS-PAGE analysis of the cell hydrolysate of the transformant showed a 15kDa band and a 38kDa band, consistent with the molecular weight of VHb and *hph* respectively (Figure 2B). All these results demonstrated that in *Pichia pastoris* EMCV-IRES could initiate the expression of gene in the polycistron effectively in a cap-independent manner. After serial cultivation for 5 times, the transfomants still process Hygromycin B resistance, indicating the stability of heteroulogus genes in *Pichia pastoris* genome (Table S4, Supporting Information).

Following *Pichia pastoris*, the function of EMCV-IRES was investigated in another industrial eukaryotic microorganism, *Acremonium chrysogenum*, as the producer of cephalosporin C (CPC), which can be modified chemically to synthesize several important *β*-lactam antibiotic derivatives, such as 7-aminocephalosporanic acid (7ACA), cefoxitin (CFX), etc. Details of the construction of recombinant plasmid pBI121-vIh for the transformation of *Acremonium chrysogenum* can be found in the Supporting Information Figure S2. *Agrobacterium*-mediated transformation was selected as the transforming method to integrate the DNA fragment of *vgb*-IRES-*hph* into the host genome. The integration of heterologous genes was confirmed by PCR using the genomic DNA of transformants as the template (Figure 2C). Similar to *Pichia pastoris*, the *hph* gene also showed the stability in *Acremonium chrysogenum* after serial cultivation for 5 times (Table S5, Supporting Information). Finally, 19 positive transformants were selected for the further experiments.

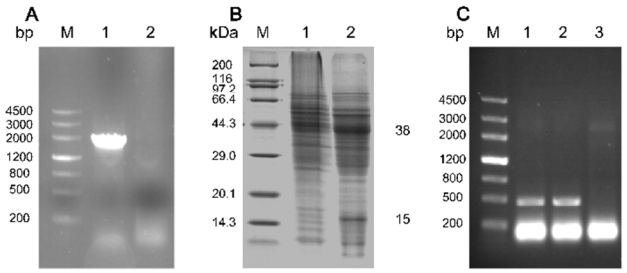

Figure 2. Genomic PCR and SDS-PAGE analysis for the transformants.(A) Amplification of *vgb*-IRES-*hph* fragment from the genomic DNA of *Pichia pastoris*. Lane 1: the transformant, lane 2: wild type as control, M: DNA marker. (B) SDS-PAGE analysis of *Pichia pastoris*. Lane 1: wild type as control, lane 2: the transformant, M: protein molecular weight marker. (C) Amplification of gene *vgb* from the genomic DNA of *Acremonium chrysogenum*. Lane 1~2: the transformants, lane 3: wild type as control, M: DNA marker.

The previous study showed that the protein VHb could improve the production of CPC in *Acremonium chrysogenum*[26], which could be used to test the function of VHb expressed by the polycistronic expression system in this study. Due to the random insertion of T-DNA mediated by *Agrobacterium*, the CPC production of different transformants might vary obviously (Figure 3). Cylinder plate method was applied to measure the CPC yield of these 19 transformants. Figure 3 showed that the CPC yield of different transformants varied significantly. After further confirmation by the high performance capillary electrophoresis (HPCE) analysis, transformant No. 15 which possessed the highest yield was chosen in the ensuing experiments. The low oxygen stress experiment was applied to test the function of VHb protein in the transformant of *Acremonium chrysogenum*. During the fermenting process of filamentous fungi, the growing dispersed hyphae will increase the medium viscosity, which imposed a detrimental effect and limited oxygen transfer. Different liquid volumes of media in a 300mL flask were used to keep different oxygen availability. Both the cylinder plate method (Figure 4A) and the capillary electrophoresis analysis (Figure 4B) indicated that the transformant possessed higher CPC yield than the wild-type under different oxygen available conditions. When the liquid volume was increased from 30mL to 50mL, the CPC yield of the transformant decreased by 52.80% while that of the wild type decreased by 77.63% (Figure 4A). Similar to the change of CPC yield, the dry weight of both the transformant and wild type strain (Figure 4C) decreased with the increase of liquid volume but nevertheless the decrease of transformant was relieved in comparison to that of the wild type. All the experiments demonstrated that the expression of VHb could enhance the tolerance of *Acremonium chrysogenum* to oxygen limited conditions, resulting in higher growth rate and CPC yield than the wild type.

During the transformation of fungal cells, the existence of random insertion of heterologous plasmids caused the false positive colonies [27]. Moreover, transformation of two individual transcription units into host in one plasmid cannot guarantee the translation of both unites together[28]. In this study, the designing strategy of bicistron enable the target gene *vgb* and the selectable marker gene *hph* integrate into the host genome and express simultaneously, and results showed success of the strategy in two eukaryotic hosts(*Pichia pastoris and Acremonium chrysogenum*).Compared with the transformation of two monocistrons (target gene and selectable marker gene), the construction and transformation of the bicistron based on IRES has the following advantage: the target gene and the marker gene share the same promoter, which enables them to be transcribed together so that excludes the case that the marker gene is integrated and expressed but the target gene not. As a result, the false positive rate will be decreased significantly, making the genetic modification of eukaryotic microorganisms more efficient.

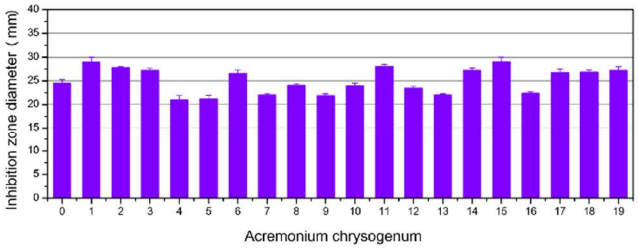

Figure 3. The variation of Cephalosporin C (CPC) yield of different *Acremonium chrysogenum* transformants. No. 0 represents the wild type while No. 1 to 19 represent the transformants.

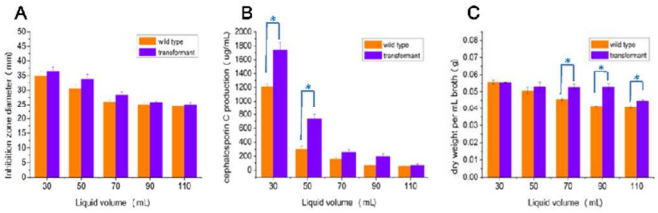

Figure 4. Cephalosporin C (CPC) production and dry weight per mL broth of *Acremonium chrysogenum*.(A) Diameter of the inhibition zone measured by oxford cup method. (B) CPC production analyzed by capillary electrophoresis. (C) Dry weight per mL broth of *Acremonium chrysogenum*. Asterisks (*) indicates the difference at the level of $p<0.05$ and the analytic results in (B) and (C) might be due to the changed priority to use the additional oxygen between the cell growth and the production of secondary metabolite in different oxygen supply conditions.

Due to the simpler and higher efficient operation, construction and transformation of polycistron based on IRES can also be tested in other industrial fungi, which would indicate potential application in metabolic engineering and synthetic biology in the future.

Conclusions

In summary, a prokaryotic-like polycistronic expression system based on EMCV-IRES was first developed in *Pichia pastoris* and *Acremonium chrysogenum, which* are the representatives of important industrial eukaryotic microorganisms. With the simpleness and effectiveness, the polycistronic manner dependent on EMCV-IRES possessed the potential application to construct long metabolic pathways in industrial eukaryotic microorganisms.

Notes and references

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Electronic Supplementary Information (ESI) available: Experimental Section and Hereditary Stability Test of *Acremonium chrysogenum* and *Pichia pastori.*

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