Polycistronic strategy for cyanobacterial expression vector construction: Co-transcription of a human gene and a selective marker gene

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Abstract A polycistronic expression vector, pKGA-NTF1, was constructed for the cyanobacterium. Within this vector, the spectinomycin/streptomycin resistance gene (*aadA*) facilitated the selection of transformants when co-transcribed with favorite genes. A natural *glnA* gene was selected as the platform to introduce the plasmid into a neutral site of the *Synechococcus* sp. PCC 7002 chromosome. Function of the vector was demonstrated by the insertion of a modified human Trefoil factor 3 gene (*NTF1*) to upstream of the *aadA* gene and by the analyses of the transformed strains. Antibiotics resistance assays showed that the dicistronic expression cassette conferred high spectinomycin resistance to both the *E. coli* cells and the *Synechococcus* cells. PCR analysis and Western-blot analysis were carried out to confirm the integration and expression of the *NTF1* gene, respectively. Through simple molecular manipulations, the artificial polycistronic structure described here can be conveniently used to express other favorable genes or operons in cyanobacteria, and to study the cyanobacterial gene expression as well.

Keywords: cyanobacterial transformation, human Trefoil factor 3, polycistronic expression, *Synechococcus* sp. PCC 7002, vector construction.

Cyanobacteria have been becoming more and more attractive as low-cost hosts for the expression of beneficial genes due to their unique characteristics including the high photosynthetic efficiency, low activity of proteases, simple growth requirements and primitive cell construction. Most cyanobacteria are nonpathogenic and nontoxic to human being, but very nutrient. At the same time, the availability of powerful genetic tools[1] and complete genomic sequences enhances the potentialities of the cyanobacterial biotechnology for commercial production or environmental application. Convenient and efficient expression vectors are called for these requirements.

Polycistronic expression is quite useful for the improvement of cyanobacterial expression vector, in addition to the routine considerations. On the contrary to plant and animal monocistronic translation of nuclear genes, most genes in cyanobacteria are co-transcribed[1], which permits the expression of multiple genes for entire pathways or pharmaceutical proteins via a single transformation event. Fewer but stronger transcription regulation elements promise more efficient expression of the favorable gene, while bring the host cells less burden. Recently, several novel pathways were successfully created in cyanobacteria for the production of beneficial organics, for example the eicosapentaenoic acid[2] and ethanol[3]. However, as the shuttle vector they employed, the foreign genes were easy to be lost by the host cells when cultured under stressless circumstances. For stable engineered cyanobacteria, the integrative vector should be more suitable. The gene expression cassette will be integrated into the host chromosomes through DNA recombinant events, where they will be replicated and inherited along with the native genes. In traditional cyanobacterial expression vectors, marker genes are always separated from the target genes. When the total plasmid is integrated into the host chromosome through a single cross-over exchange event, parts of it might be lost[1], which will then result in the false positive or negative phenotypes[4]. De Cosa et al. have constructed a very successful chloroplast expression vector for the expression of *Bacillus thuringiensis* (Bt) cyr2A2a operon in tabacco[5]. This chloroplast vector contained a co-transcription structure of the selectable marker gene and the cyr2A2a operon. As cyanobacteria are evolutionarily linked to the chloroplast, we applied this strategy to cyanobacterial vector construction with

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some modifications. The compact dicistronic expression of the target gene and the antibiotic resistant gene described here can prevent the gene-loss that happens in the single cross-over exchange event.

The well-characterized and highly transformable *Synechococcus* sp. PCC 7002 (*Agmenellum quadruplicatum* PR-6) was selected as a model cyanobacterial system, together with the modified human Trefoil factor 3 as a heterogenous gene to develop a new expression strategy for the production of edible biomedicine or nutrient food. As a species of unicellular marine cyanobacteria, *Synechococcus* 7002 is quite easy to cultivate and is naturally transformable[6]. Trefoil factor 3 (TFF3), also named intestinal trefoil factor (ITF), is supposed to protect the gastrointestinal epithelial layers from damages and participate in the subsequent epithelial reconstruction[7]. Mainly expressed in the intestine of human, rats and adult mice, TFF3 is extremely stable toward acid, heat degradation, and proteolytic digestion due to its compact three-leafed structure, which exists universally in the Trefoil factor family (TFF) peptides[8]. In this study, we developed an N-terminal modified mutant of TFF3 (named NTF1) which is more stable and suitable for genetic engineering than the natural TFF3. A model system to demonstrate the operon expression of favorable gene and selectable marker gene via cyanobacteria was then established.

1 Materials and methods

1.1 Plasmids and genes

Plasmid patpX, provided by Chinese Academy of Agricultural Sciences, conferred the bacterial *aadA* gene cassette with the 5′ promoter and leader sequence (5′ PL) of *atpA* gene and the 3′-untranslated region (3′-UTR) of *rbcL* gene from *Chlamydomonas reinhardtii* chloroplasts[9]. The gene *glnA* from *Synechococcus* sp. strain PCC 7002, as the integration platform, was cloned into plasmid KS to derive the plasmid pKS-GlnA[10]. *NTF1* gene, an N-terminal modified human *TFF3* gene, was cloned in the plasmid pPIC 9K-NTF1 in our laboratory (unpublished data).

1.2 Construction of the expression vector

The *NTF1* gene was amplified by a PCR reaction from the plasmid pPIC 9K-NTF1, using the following two primers: primer 1P (5′-CTACACCATGG-AGGAATACCCGGGCGAA-3′) anneals with the 5′-terminus of the gene, with *Nco*I site underlined and the start codon overstriking; primer 1M (5′-ACCT-GCCATGGCTCGAGCTC**TTACT**AGAAGGTGCAT-TCTGCTTCC-3′) anneals with the 3′ portion of the gene, with *Nco*I and *Sac*I sites underlined and the two tandem stop codons overstriking. After an initial 5 min denaturation at 95 ℃, samples were run for 30 PCR cycles as follows: 95 ℃ for 1 min, 60 ℃ for 1 min and 72 ℃ for 30 sec. The amplified product was digested with the endonuclease *Nco*I, and then inserted into the plasmid patpX to generate the dicistronic structure under the control of the *atpA* promoter and the *rbcL* terminator from *Chlamydomonas reinhardtii* chloroplasts. DNA sequence analysis confirmed the correct orientation of *NTF1* gene, the polylinker sequence, as well as the translational fusions of the ATGs within the two resulting *Nco*I sites with *NTF1* gene and *aadA* gene, respectively. This generated plasmid, named patpX-NTF1, was digested by *Eco*RV and *Sal*I to generate the 2.2 kb atpX-NTF1 cassette fragment to be cloned into the pKS-GlnA plasmid, which had been prepared by the digestion with *Bam*HI, blunt ended with Klenow fragment, and redigested with *Sal*I. The cyanobacterial expression vector, pKGA-NTF1, was finally obtained (Fig. 1(a)).

1.3 Strains, culture conditions and transformation

The marine cyanobacterium *Synechococcus* sp. PCC 7002, from the Institute of Botany, Chinese Academy of Sciences, was cultivated in liquid Medium A at 29 ℃, illuminated by cool white fluorescent lamps (100 $\mu E \cdot m^{-2} \cdot s^{-1}$), oscillated at 135 r/min and subcultivated every 15 days[11]. Cells were also maintained on solid Medium A with 1.2% Bacto agar. The transformation of *Synechococcus* cells was carried out by natural transformation as described previously[6] with a little modification. Following incubation under moderate light for 48 h, the nylon filters covered with *Synechococcus* cells were transferred to selective medium containing either 20 μg ampicillin/mL, or 5 μg spectinomycin/mL, or both of the antibiotics at each concentration. After 2 to 3 weeks incubation under the above growth conditions, resistant transformants appeared and were subsequently restreaked several times onto the fresh selective medium containing 30 μg ampicillin/mL, 10 μg spectinomycin/mL and 10 μg streptomycin/mL, respectively. These antibiotic concentrations were also applied to liquid culture of the transformants.

1.4 PCR confirmation

For PCR analysis, total DNAs were extracted from the putative transgenic samples and wild-type *Synechococcus* as described below. The cells were cultured as described above and 100—500 μL of each mid-log liquid culture was collected. The precipitation was then sufficiently mixed with 20 μL sterile H_2O on a vortex mixer. The suspension was boiled for 5 min, then centrifuged at 4 ℃ (5 min at 12000 r/min), and 10 μL of the supernatants was used as templates in PCR reactions. To assay the integration event, three primers targeting to different positions of the foreign gene expression cassette were employed. Following the same protocols mentioned above, the ~200 bp of *NTF1* gene fragment was amplified with primers 1P and 1M. Meanwhile, the combination of primer 1P and primer 2M (located within the 3′-UTR of *rbcL*, 5′-AAGTTCTGGAGACCATTTAC-3′) produced a 1.1 kb fragment containing both the *NTF1* gene and the *aadA* gene under the following protocol: 1 min at 94 ℃, 1 min at 55 ℃, 2 min at 72 ℃; 30 cycles. All PCR products were separated on 0.9% agarose gels.

1.5 Western blot analysis

Cyanobacterial cultures for assays were harvested at late-log phase. Crude protein was extracted from *Synechococcus* cells as described by Luo et al.[11] 10 μg standard TFF3 protein and 30 μg total proteins from both wild and transgenic cyanobacterial cells were loaded for protein analysis. The protein extract was separated on a 15% SDS-PAGE gel, and then transferred onto nitrocellulose membranes by electroblotting[12]. The blot was probed with the TFF3 antiserum (1∶200 dilution), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Zhong Shan Biotechnology, 1∶5000 dilution).

1.6 Growth of the transgenic *Synechococcus*

For growth rate determination, the growth of both the wild-type and the transgenic *Synechococcus* cells was determined by measuring optical density at 750 nm. The transgenic *Synechococcus* sp. PCC 7002 was cultivated in liquid Medium A supplemented with 30 μg ampicillin/mL, 10 μg spectinomycin/mL and 10 μg streptomycin/mL, respectively. The wild type *Synechococcus* cells were cultured in liquid Medium A with or without these antibiotics.

2 Results

2.1 Construction of the expression vector

The structure of the cyanobacterial expression vector pKGA-NTF1 (6.46 kb) constructed by polycistronic strategy is shown in Fig. 1(a). The *NTF1* gene, encoding a modified human intestinal mucin-associated peptide, was inserted into the unique *Nco*I site within the *aadA* cassette of plasmid patpX to form a dicistron structure. A linker sequence joined the two genes, which contained two tandem stop codons to efficiently terminate the translation of the upstream *NTF1* gene and an additional *Sac*I site to facilitate the future manipulation of gene substitution. The vector contained a 5′ promoter region from chloroplast *atpA* gene cluster driving the *aadA* (aminoglycoside 3′-adenylyltransferase) gene for spectinomycin selection[9] and the *NTF1* gene. The *atpA* gene encoding the α-subunit of the coupling-factor-1 ATP synthase has a high homology (71.84%) between the cyanobacteria genomes and the higher plant chloroplast genomes[13], and this promoter transcribes a natural chloroplast gene cluster in *Chlamydomonas reinhardtii*[14]. These dicistronic transcripts were stabilized by a chloroplast *rbcL* 3′ end. Through a single-crossover event, the recombination platform—a *glnA* gene fragment from the host cell, allowed the integration of the atpX-NTF1 expression cassette into a neutral site of the *Synechococcus* sp. PCC 7002 genome. The *glnA* gene encodes a key enzyme, glutamine synthetase (GS), involved in the assimilation of newly fixed nitrogen, but *Synechococcus* cells have another enzyme—carbamoyl phosphate synthetase to maintain this assimilation activity when *glnA* is inactivated[15]. Our previous work also confirmed that the insertion of the foreign DNA immediately upstream of *glnA* gene had no significant influence on the cyanobacteria growth[10].

2.2 Transformation of *Synechococcus* sp. and screening

The pKGA-NTF1 expression vector and the empty plasmid pKS-GlnA were transferred to *Synechococcus* cells and the correct transformants were selected with ampicillin or spectinomycin or both the antibiotics. Correspondently, different yields of transformants implied the diversity of the markers themselves as well as the different expression strategies that they represented. The expression vector pKGA-NTF1 contains two selectable marker genes,

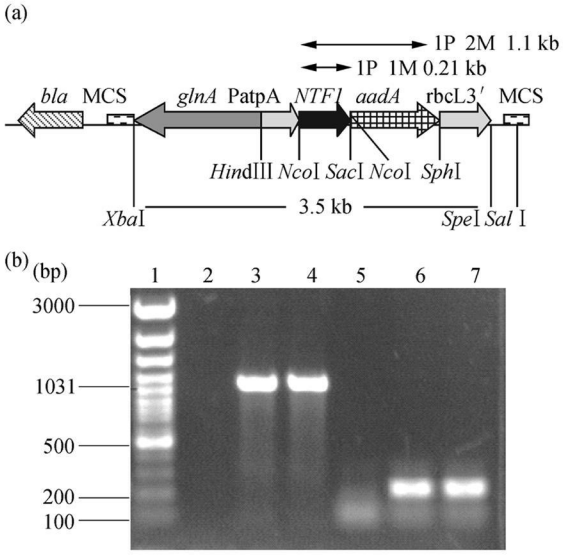

Fig. 1. Expression vector pKGA-NTF1 and PCR assays of the *Synechococcus* transformants. (a) Plasmid pKGA-NTF1 (6.46 kb) with PCR primer targeting sites and expected fragment sizes. (b) PCR products of the wild type and putative transformed *Synechococcus* using two primer sets: 1P2M (lanes 2-4) and 1P1M (lanes 5-7). Lane 1, 1 kb DNA ladder (MBI); lane≤2, 5, wild-type genomic DNAs were taken as templates and no products were shown; lanes 3, 6, genomic DNAs of putative transformants were used as PCR templates and corresponding products were detected; lane≤4, 7, pKGA-NTF1 plasmid DNA was taken as the positive control.

one is the *E. coli bla* gene inherited from pKS-GlnA, and the other is *aadA* gene acquired by the insertion of the atpX-NTF1 expression cassette. According to pET System Manual (10th ed. 2003), the *bla* gene, encoding β-lactamase, is considered to be an inefficient selectable marker. In this experiment, the single ampicillin selection gave birth to the overgrowth of the wild-type cells during a long-term culture (Fig. 2(a)). Furthermore, as the *bla* gene is not tightly linked to the target gene expression cassette in pKGA-NTF1 (Fig. 1(a)), DNA loss might have taken place during the integration into the cyanobacteria chromosomes[1]. There were some reports about the invalidation of ampicillin resistance phenotype due to the random losses of the plasmid DNA accompanying the transformation[4]. From these points on, high screening efficiency calls for stable selective markers in a tight-link fashion for integrative vector construction. In order to meet this requirement, we constructed the dicistronic cyanobacterial expression vector pKGA-NTF1 as described above. Compared to the individual expression of *bla* gene, the co-transcribed *aadA* gene was quite more efficient (Fig. 2), and the separation events accompanying the single-cross recombination have been successively overcome. About 32 to 155 transformants were obtained per μg DNA. The co-transcription of selective marker gene and interest gene was first reported here in the cyanobacterial transgene study.

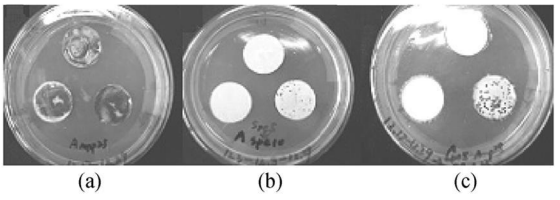

Fig. 2. Selection of the transgenic *Synechococcus* sp. strain PCC 7002 on the Medium A plate containing different kinds of antibiotics: (a) 25μg ampicillin/mL, (b) 5—10μg spectinomycin/mL and (c) 5μg spectinomycin/mL together with 25μg ampicillin/mL. In every plate, the top nylon film is spread with wild-type *Synechococcus* cells, the left below with empty vector pKS-GlnA transformed cells, and the right below with the expression vector pKGA-NTF1 transformed cells.

2.3 PCR and Western blot analysis

PCR analysis confirmed the successful integration of the foreign genes. Two sets of PCR primers were employed to test the integration of the pKGA-NTF1: the primer pair 1P/1M generated the ~0.2 kb *NTF1* gene fragment and the 1P/2M pair could amplify a ~1.1 kb DNA fragment containing both the *NTF1* and the *aadA* genes. When total DNA from the supposed transgenic *Synechococcus* cells was taken as templates, both the PCR products in each size could be obtained, just like the positive control plasmid pKGA-NTF1 did. Untransformed cells, as expected, showed neither PCR products (Fig. 1(b)).

In order to verify the expression of the *NTF1* gene, whole-cell extracts were prepared from the pKGA-NTF1 transformed cyanobacteria cultures, and those from wild-type *Synechococcus* were taken as control. Western blotting with anti-TFF3 antiserum was carried out for the immunological analysis (Fig. 3). Specific cross-reaction was only observed in the transformed cyanobacterial cell extracts (lane 3), whereas no significant signal was detected in the wild-type total protein sample (lane 2). The precipitate of culture medium of the transformants did not cross-react with the NTF1 antiserum either (lane 4). Compared to the strong signal given by the yeast-produced NTF1 dimers (lane 1), the extracts from transformed cyanobacteria cells gave a much fainter immunostaining band representing a lower molecular weight. One reasonable explanation could be that the

NTF1 gene was translationally fused to the 5′ coding region of the chloroplast *atpA* gene, which resulted in an appendant 28 extra amino acids to the N terminal of the protein product. Therefore the molecular mass of the fusion protein was 11.8 kD, bigger than that of the NTF1 monomer (6.5 kD), but slightly smaller than that of the dimer (13.6 kD). At the same time, the relatively large appendent might have influenced the antigenic integrity of the 59-amino-acid NTF1 peptide. Based on these results, conclusions can be made that the NTF1 peptide has been expressed in these transgenic *Synechococcus* cells as a fusion protein, which presented in monomer form and reserved partial antigenicity.

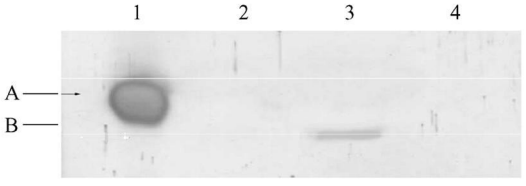

Fig. 3. Western blots of transgenic *Synechococcus* using anti-hTFF3 poly-clonal antibody. Lane 1, standard TFF3 dimmer; lane 2, the extracts from the wild type as a control; lane 3, total proteins from the pKGA-NTF1 transformed *Synechococcus*; lane 4, the precipitate of the transformants culture medium. Arrow A indicates the TFF3 dimer and arrow B indicates the fusion form NTF1 produced by the transformed cyanobacteria.

2.4 Growth and stability analysis

In an attempt to determine whether the expression of *NTF1* and *aadA* had any side-effects on the physiology of the host cells, growth curves of the pKGA-NTF1 transformants and the wild-type *Synechococcus* cells were measured. As expected, in liquid Medium A containing 30 μg ampicillin/mL, 10 μg spectinomycin/mL and 10 μg streptomycin/mL, respectively, the wild-type *Synechococcus* cells were not able to grow, while the transformants grew rapidly, which was only somewhat lower than the wild-type cells in Medium A without any antibiotics (Fig. 4), indicating that the insertion and expression of heterogenous genes had little influence on the physiological functions of the host cells.

The transformants obtained extremely high spectinomycin resistance and the foreign genes were inherited stably. To estimate the antibiotic resistance ability, the transformed *Synechococcus* cells and the wild-type cells were cultivated on solid or in liquid Medium A containing increasing concentrations of spectinomycin. The transformants were able to grow on solid medium at concentrations of spectinomycin as high as 200 μg/mL and in liquid medium up to 300 μg/mL (data not shown), respectively, while the wild-type *Synechococcus* were unable to grow at a level of spectinomycin as low as 5 μg/mL. Spectinomycin-resistant colonies could still be obtained on selective plates after 5-week free antibiotic culture. These results correspondingly confirmed that the foreign gene expression cassette had been stably integrated into the *Synechococcus* genome and inherited along with the host genes.

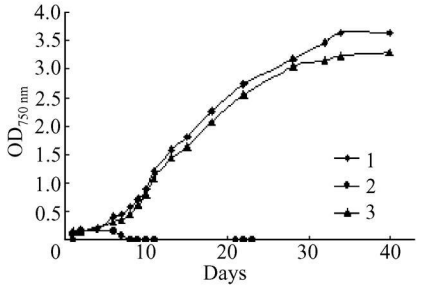

Fig. 4. The growth curve of the transgenic *Synechococcus* sp. strain PCC 7002 in 100 mL liquid Medium A supplemented with 30 μg/mL ampicillin, 10 μg/mL spectinomycin and 10 μg/mL streptomycin respectively. 1, Wild type without antibiotics; 2, wild type with antibiotics; 3, pKGA-NTF1 transformed *Synechococcus* with antibiotics.

3 Discussion

Polycistronic expression has many advantages over the traditional cyanobacterial expression system. First of all, multiple genes, single- or multiple-origin, from plants or animals, could be transferred via a single transformation event, which significantly improves the efficiency of bioengineering and affords the transformants much more beneficial properties. Secondly, stronger but fewer transcription regulation elements not only ensure the efficient expression of the favorable gene, but also bring less foreign DNA into the host genome. What is more, since the favorite gene (*NTF1*) was placed upstream of the selectable marker gene (*aadA*) in pKGA-NTF1, the spectinomycin-resistant phenotype here could also be considered as a convincing proof for the correct expression of the proceeding gene, in addition to its selection function. The foreign DNA size can be further reduced when double integration platform is used to integrate the polycistronic expression cassette into the *Synechococcus* chromosome, and which is a more appropriate strategy for cyanobacterial transformation. Finally, the expression activity of the cyanobacterial vec-

tors in *E. coli* cells is quite helpful for the manipulation of the vector construction. We observed that *E. coli* DH5α cells carrying pKGA-NTF1 were able to tolerate high spectinomycin concentrations (data not shown).

This cyanobactrerial expression vector can be conveniently used for different experimental purposes. Some restriction enzyme recognition sites were designed to ensure the easy substitutions of single or multiple elements by a single step cloning process performed in *E. coli*. First of all, as an expression vector, the correct reading frame allows simple beneficial gene insertion. Different promoter or terminator sequences could be tested to achieve the highest expression of the target gene in *Synechococcus*. If one wants to study the activity of a special promoter, a reporter gene like *gfp* (green fluorescence protein) could be inserted between the promoter and the marker gene. In addition, the antibiotic resistance gene could also be changed when necessary. Mutations can be easily obtained with this vector when the target cyanobacterial gene is taken as the integration platform.

We describe here an integrative expression system that has successfully expressed a therapeutic protein coding gene (*NTF1*) and a selectable marker gene (*aadA*) in a dicistron fashion in *Synechococcus* sp. PCC 7002. Because the conformation and biological activity of the NTF1 peptide is influenced by an unwanted fusion, promoters without coding sequences should be tested in the future. Since the co-transcribed spectinomycin-resistance (*aadA*) gene is highly efficient for the recovery of cyanobacteria transformation, it is more appropriate to integrate the portable expression cassette into the host chromosome via a double-recombination than to integrate the whole plasmid mediated by single-crossover event employed here. Attentions should also be paid to the effects of culture conditions to improve the expression level of NTF1 in *Synechococcus* cells. With some further optimizations, we hope to develop an excellent expression system for the production of biologically active therapeutic proteins based on the simple photoautotrophic organism.

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