ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2005, **37**(10): 709–712 **CN** 31-1940/Q

Short Communication

Recombination and Heterologous Expression of Allophycocyanin Gene in the Chloroplast of *Chlamydomonas reinhardtii*

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Abstract Heterogeneous expression of multiple genes in the nucleus of transgenic plants requires the introduction of an individual gene and the subsequent backcross to reconstitute multi-subunit proteins or metabolic pathways. In order to accomplish the expression of multiple genes in a single transformation event, we inserted both large and small subunits of allophycocyanin gene (*apcA* and *apcB*) into *Chlamydomonas reinhardtii* chloroplast expression vector, resulting in papc-S. The constructed vector was then introduced into the chloroplast of *C. reinhardtii* by micro-particle bombardment. Polymerase chain reaction and Southern blot analysis revealed that the two genes had integrated into the chloroplast genome. Western blot and enzyme-linked immunosorbent assay showed that the two genes from the prokaryotic cyanobacteria could be correctly expressed in the chloroplasts of *C. reinhardtii*. The expressed foreign protein in transformants accounted for about 2%–3% of total soluble proteins. These findings pave the way to the reconstitution of multi-subunit proteins or metabolic pathways in transgenic *C. reinhardtii* chloroplasts in a single transformation event.

Key words *Chlamydomonas reinhardtii*; chloroplast transformation; allophycocyanin gene

Expression of multiple genes in the nucleus of transgenic plants is complicated and time-consuming due to the monocistronic translation of nuclear mRNAs. For example, in order to express the polyhydroxybutyrate polymer or Guy's 13 antibody, a single gene was first introduced into the nuclear genome of an individual transgenic plant, then the plant was backcrossed to reconstitute the entire pathway or the complete multi-subunit protein [1,2]. In contrast, most chloroplast genes of plants are co-transcribed. This provides the possibility of expressing foreign polycistrons using the *Chlamydomonas reinhardtii* chloroplast and reconstituting entire metabolic pathways or multi-subunit proteins in a single transformation event. Similar work was successful in tobacco chloroplasts [3].

Allophycocyanin is one of the photosynthetic antenna proteins in cyanobacteria and red algae [4]. The basic unit of allophycocyanin is a heterodimer composed of an alpha subunit and a beta subunit with molecular mass between 15 kDa and 23 kDa [5]. Our previous studies suggested that the recombinant allophycocyanin (rAPC) could remarkably inhibit the S-180 carcinoma in mice with an inhibitory rate ranging from 45% to 64%, without any obvious effect on the thymus index or leukocyte count [6,7]. This indicates that the expression of APC in the *C. reinhardtii* chloroplast may facilitate the production of a new valuable plant-derived protein.

In this study, the allophycocyanin gene *apc* (containing the fragments encoding alpha subunit, *apcA*, and beta subunit, *apcB*) was used as a model gene to demonstrate

Received: January 18, 2005 Accepted: June 24, 2005
This work was supported by a grant from the Key Innovative Project (KZCX-3-SW-215) of the Chinese Academy of Sciences
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DOI: 10.1111/j.1745-7270.2005.00092.x

the possibility of multiple genes co-expression in the *C. reinhardtii* chloroplast.

Experimental Procedures

The wild-type *C. reinhardtii* strain 137cc was kindly provided by the Biotechnology Research Institute, Chinese Academy of Agricultural Sciences (Beijing, China). This alga was cultured in Tris-acetate-phosphate (TAP) medium [8] with a cycle of 16 h light:8 h dark (30 μmol·m^{-2}·s^{-1}) at 25 ºC. Then it was cultured on solid medium by adding 2% agar.

pUC18 and pBluescriptII SK (+) were kept in our laboratory. *apcA* and *apcB* were cloned from the cyanobacterium *Spirulina maxima* and then subcloned into pUC18 to obtain plasmid pUC18-*apc*. Plasmid p64D containing the *chlL* homologous fragment of the *C. reinhardtii* chloroplast and aminoglycoside adenine transferase gene (*aadA*) cassette (including the *atpA* promoter and *aadA*-*rbcL* terminator) was obtained from the Biotechnology Research Institute. All restriction enzymes, *Taq* DNA polymerase for polymerase chain reaction (PCR), T4 DNA ligase and Klenow fragment were purchased from TaKaRa (Dalian, China).

The *aadA* cassette was cleaved from p64D with *Eco*RV and *Sac*I, and ligated to pBluescriptII SK (+) digested with the same enzymes to create the plasmid pSK-*aadA*. The plasmid pSK-*apc* was constructed by inserting *apc* cleaved from pUC18-*apc* with *Sma*I and *Sph*I into pSK-*aadA* to replace *aadA*. Consequently, *apc* was driven by the *atpA* promoter and terminated by the *rbcL* terminator of the *C. reinhardtii* chloroplast. To obtain the plasmid pSK-*apc*-*aadA*, the *aadA* cassette, which was cleaved from p64D as described above, was inserted into pSK-*apc*, following the *apc* cassette. Both *apc* and *aadA* cassettes were cut from pSK-*apc*-*aadA* and used to replace the *aadA* cassette in the plasmid p64D to make a *C. reinhardtii* chloroplast homologous integration vector papc-S. DNA sequencing was used to check the open reading frame (ORF) of *apc* to ensure its correct expression.

Gold particles coated with plasmid papc-S (containing *apcA* and *apcB*) were bombarded into *C. reinhardtii* using the biolistic bombardment equipment PDS1000/He (Bio-Rad, Hercules, USA) as described by Kindle *et al.* [9]. After transformation, cells were incubated at 21 ºC in dim light for 24 h, then washed with TAP liquid medium before transferring them onto fresh TAP plates containing 100 μg/ml spectinomycin (Sigma, St. Louis, USA). After a two-week culturing period at 25 ºC, colonies were picked out and inoculated in 50 ml liquid TAP selective medium containing 100 μg/ml spectinomycin for 7 d on a gyratory shaker at 160 rpm. In order to improve homogeneity, the solid-liquid selection procedure was repeated twice.

Total DNA of *C. reinhardtii* was isolated as described by Goldschmidt-Clermont [10]. In order to verify the integration of *apc* into chloroplast genomes of *C. reinhardtii* transformants, two PCR primers were designed according to the 5′ downstream sequence of *chlL* and the 3′ upstream sequence of *apc*: primer *chlL*-F, 5′-GTTTT-TATTCCTGGAGTTTG-3′; and primer *apc*-R, 5′-TATGCATGCTTGGAAGCTTAG-3′. The protocol for PCR was: 30 cycles of 95 ºC for 1 min, 50 ºC for 1 min and 72 ºC for 2 min. The PCR products were visualized on 1% agarose gel.

For Southern blot analysis, *C. reinhardtii* total DNA was digested with *Eco*RV and *Sac*I, then loaded onto 1% agarose gel. The gel was transferred to a nylon filter using the Mini protean II cell blotter system (Bio-Rad) Southern blot was carried out with intact *apc*, which was cleaved from pUC18-*apc*, as the probe using the DIG DNA labeling and detection kit (Roche, Basel, Switzerland).

The standard APC antigen was prepared as described by Zhang and Chen [11]. Rabbit anti-APC polyclonal antibodies were obtained according to the method of Krakauer *et al.* [12]. Crude protein was extracted from *C. reinhardtii* as described by Goldschmidt-Clermont [10]. The concentration of total soluble proteins (TSP) from the *C. reinhardtii* transformants was quantified according to Bradford [13]. The content of recombinant APC in transgenic *C. reinhardtii* was determined using quantitative enzyme-linked immunosorbent assays (ELISA) as described by Sun *et al.* [14].

After centrifugation at 6600 *g* for 2 min, the supernatant of crude protein was subjected to 20% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto nitrocellulose membrane by electro-blotting (Bio-Rad). Rabbit anti-APC (1:1000) was used as the primary antibody and alkaline phosphatase-conjugated goat anti-rabbit IgG (1:500) was used as the secondary antibody. The antigen-antibody complexes were visualized by 3,3′-diaminobenzidine (DAB; Amresco, Solon, USA).

Results and Discussion

DNA sequencing showed that the ORF of *apc* in papc-S was in the right orientation. Fifteen colonies were obtained

after the first round of spectinomycin selection of the transformants. When the transformants and wild-type *C. reinhardtii* were incubated in dim light, all the transformants became yellow; in contrast, the wild-type strain showed distinct green phenotype. According to the report of Suzuki and Bauer [15], the transformants will become yellow when they are incubated in dim light if *chlL* is replaced by a foreign gene. The same phenomenon was observed in our experiments, which suggested that the target gene cassettes had been integrated into the directed site of the *C. reinhardtii* chloroplast genome through homologous recombination.

After three rounds of spectinomycin selection, two colonies were picked out randomly. The result of PCR amplification using the pair of primers (*chlL*-F and *apc*-R) showed that an expected 1.7 kb band covering the *atpA* promoter and *apc* fragment was amplified in these two transformants, but no band was obtained in wild-type *C. reinhardtii* [**Fig. 1(A,B)**]. After digestion and Southern blot analysis, a band of approximately 3.8 kb representing the *apc* and *aadA* cassettes was visualized in the total DNA of both transformants (**Fig. 2**). The result of PCR and Southern analysis suggested that fragments of the *apc* and *aadA* cassettes had been integrated into the chloroplast genome of *C. reinhardtii*.

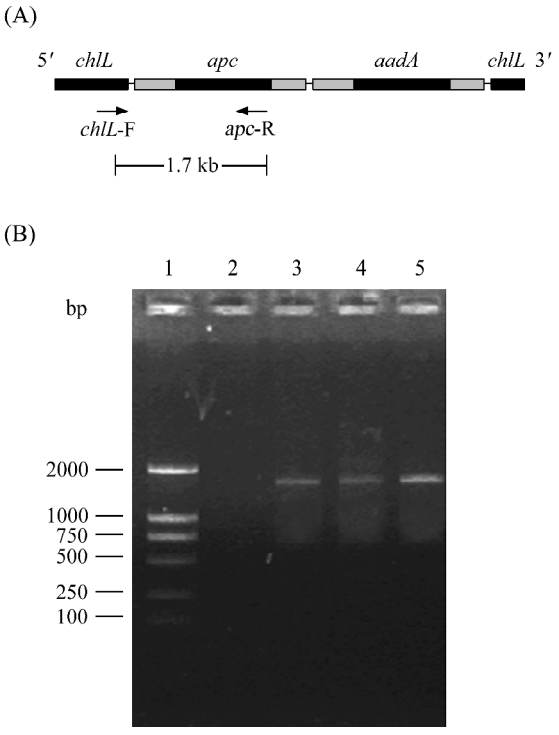

Fig. 1 Schematic diagram of *Chlamydomonas reinhardtii* chloroplast transformation and the PCR assay of *C. reinhardtii* transformants

(A) Schematic diagram of *C. reinhardtii* chloroplast transformation, transformants containing the foreign gene and the disrupted *chlL* gene. (B) PCR amplification of *atpA* promoter-*apc* in *C. reinhardtii* transformants. 1, marker; 2, wild-type cells; 3 and 4, two transformants; 5, positive control (the PCR products of the plasmid papc-S).

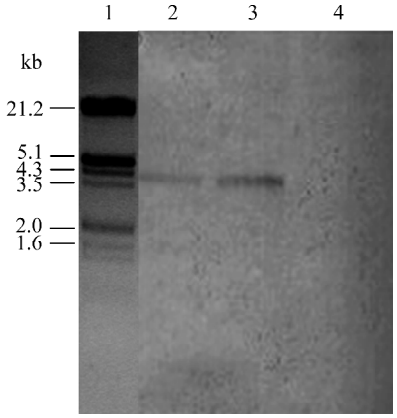

Fig. 2 Southern blot analysis of genomic DNA from *Chlamydomonas reinhardtii* transformants

Genomic DNA was digested with restriction endonucleases *Eco*RV and *Sac*I, then hybridized with intact *apc* as the probe. 1, marker; 2 and 3, two transformants; 4, wild-type cells.

In Western blot analysis, two bands of approximately 21 kDa and 17 kDa were detected (**Fig. 3**), corresponding to the alpha and beta subunits of rAPC in molecular weight, respectively. This indicates that the two foreign genes

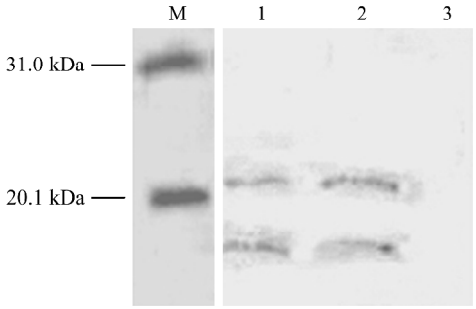

Fig. 3 Western blot analysis using total proteins from wild-type cells and transformants

M, marker; 1 and 2, two transformants; 3, wild-type cells.

(*apcA* and *apcB*) were correctly expressed in the chloroplast of *C. reinhardtii*. Determination of expressed APC in the two transformants using quantitative ELISA techniques revealed that they constitute 23.6±0.1 μg and 26.0±0.2 μg per milligram of TSP, respectively (**Fig. 4**).

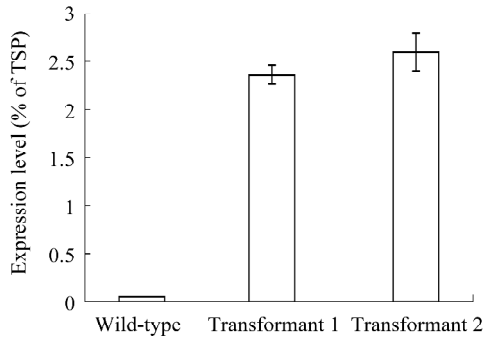

Fig. 4 Expression levels of APC in transformants of *Chlamydomonas reinhardtii* using quantitative ELISA

Three parallel quantifications were made for the wild-type and transformant samples. TSP, total soluble proteins.

In this research work, we successfully expressed prokaryotic *apcA* and *apcB* genes using a single *atpA* promoter in the *C. reinhardtii* chloroplast, indicating that prokaryotic cyanobacteria polycistrons can be correctly translated in eukaryotic chloroplasts. This result provides a foundation for the expression of foreign pathways or pharmaceutical proteins involving multiple genes in the *C. reinhardtii* chloroplast. The expression level of APC in the *C. reinhardtii* transformants accounted for 2%–3% (*W*/*W*) of TSP, showing the feasibility of using transgenic *C. reinhardtii* chloroplasts as a kind of bioreactor to produce functional proteins. Transgenic plants, as the recombinant functional protein source, have several advantages. For example, they are relatively inexpensive and safe [16]. The recombinant protein expressed in the algal chloroplast will be further tested for its biological activity and its potential application in pharmacology.

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Edited by
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