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

Zhong-Liang SU\(^1,3\), Kai-Xian QIAN\(^1\)*, Cong-Ping TAN\(^2,4\), Chun-Xiao MENG\(^2,4\), and Song QIN\(^2\)*

\(^1\) Department of Biotechnology, College of Life Sciences, Zhejiang University, Hangzhou 310027, China;
\(^2\) Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China;
\(^3\) Qingdao University of Science and Technology, Qingdao 266061, China;
\(^4\) Graduate University of Chinese Academy of Sciences, Beijing 100039, China

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...
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 pBluescript II 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 *EcoRV* and *SacI*, and ligated to pBluescript II SK (+) (as described above) to create the plasmid pSK-*apc*-aadA. The plasmid pSK-*apc* was constructed by inserting *apc* cleaved from pUC18-*apc* with *SmaI* and *SphI* 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 *EcoRV* and *SacI*, 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*.

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

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**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 and 3, two transformants; 4, wild-type cells.

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

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

**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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