

# Electrophoretic karyotyping and chromosomal gene mapping of *Chlorella*

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## ABSTRACT

**Molecular karyotypes for six strains of four *Chlorella* species were obtained by using an alternating-field gel electrophoresis system which employs contour-clamped homogenous electric fields (CHEF). The number and migration pattern of the chromosomal DNA molecules varied greatly from strain to strain: for example, nine separated chromosomes of *C. ellipsoidea* C87 ranged from 2.5 to 6.5 megabase pairs (mbp) in size, whereas 16 chromosomes of *C. vulgaris* C169 were from 980 kilobase pairs (kbp) to 4.0 mbp. Depending on the chromosome migration patterns, the six strains were classified into two major chromosome-length polymorphism groups. Using hybridization techniques, the genes for  $\alpha$ -tubulin, chlorophyll-a, b-binding proteins, ribosomal RNAs, and the small subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) were mapped on the separated chromosomes of *C. vulgaris* C169. Since *Chlorella* chromosomes are small enough to separate and isolate individually by CHEF gel electrophoresis under ordinary conditions, they should serve as excellent materials to study the fundamental molecular structure of plant-type chromosomes.**

## INTRODUCTION

The unicellular green algal genus *Chlorella* includes a variety of species (1), some of which have served as model organisms in plant physiological and biochemical studies for several decades (2). So far, a number of *Chlorella* mutants have been isolated which are related to various photosynthetic aspects, including the synthesis of chlorophylls and of plastids, the degradation of chlorophylls, the role of carotenoids and of chlorophylls, and the size of the photosynthetic units (3). However, molecular biological and genetic information about those mutants is lacking because of no appropriate systems for genetic analysis in *Chlorella*. In order to identify and isolate genes involved in the mutations, it would be helpful to establish in *Chlorella* a molecular karyotype combined with physical and genetic mapping.

The recent advance in alternating or pulsed-field gel electrophoresis technology has made it possible to resolve large chromosomal DNA molecules of various organisms. The electrophoretic karyotyping by this technique has been obtained for yeasts (4–9), fungi (10, 11), and protozoa (12–14). There may be, however, two major limits in application of this method to *Chlorella*: (i) the preparation of unbroken chromosome molecules and (ii) the upper limit of resolution to the size of molecules (7). We have already developed a method to prepare protoplasts from several strains of *Chlorella* (15). The protoplasts would be a good source of intact chromosomes. According to Walbot and Goldberg (16), the genome size of *C. ellipsoidea* is almost twice that of *Saccharomyces cerevisiae* haploid cells and seems to be within the range of resolution by CHEF gel electrophoresis under ordinary conditions (7). In this study, we have separated the chromosomal DNA molecules of six *Chlorella* strains by CHEF gel electrophoresis and revealed two major chromosome length polymorphism (CLP) groups. Furthermore, several genes have been mapped by hybridization on the separated chromosomes of *C. vulgaris* C169.

## MATERIALS AND METHODS

### *Chlorella* strains

*C. ellipsoidea* (C87), *C. saccharophila* (C211), and *C. vulgaris* (C135, C150 and C169) were obtained from the algal culture collection of the Institute of Applied Microbiology (IAM), the University of Tokyo (15). *Chlorella* sp. NC64A (17) was kindly given by Dr. J. Van Etten, Lincoln, Nebraska, USA. *Chlorella* cells were cultured in a modified Bristol medium supplemented with 0.1% proteose peptone (15), except for strain NC64A that was grown in a modified Bold's basal medium as described (17).

### Preparation of intact chromosomal DNA

Cells were grown to late logarithmic phase ( $5 \times 10^7$  cells/ml) in a liquid medium (100 ml), collected by centrifugation at  $1,000 \times g$  for 5 min, washed and resuspended in 4 ml protoplast forming medium containing 0.1 M sodium citrate, 1 M sorbitol, 60 mM EDTA, 10 mM 2-mercaptoethanol, 4% cellulase Onozuka R-10 (Yakult) and 2% Macerozyme R-10 (Yakult). After mixing quickly

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with 5 ml of 1% low-melting-point agarose (BRL) in 0.125 M EDTA and 10 mM Tris-HCl (pH 7.5) solution at 50°C, the cell suspension was poured into plug moulds and incubated overnight at 37°C. The plugs were then transferred into 10 mM Tris-HCl (pH 8.0) containing 0.5 M EDTA, 1% sodium lauroylsarcosinate (Nippon Gene) and proteinase K (1 mg/ml, Merck) and incubated at 37°C for 24 h. After washing three times in 0.5 M EDTA (pH 8.0) at 37°C for 4 h, the plugs were stored in 0.5 M EDTA (pH 8.0) at 4°C before CHEF gel electrophoresis.

### CHEF gel electrophoresis

The electrophoresis chamber contains 24 electrodes in a closed-contour, with the dimensions of the chamber and the positions of the electrodes as described by Chu, *et al.*(5). Constant temperature was maintained by circulating cool water beneath the chamber connected to an Eyela cooling system (Tokyo Rikakikai). Unless otherwise specified all gels were 1% agarose in 0.5×TBE (45 mM Tris, 45mM boric acid, 1 mM EDTA, pH 8.3), run at 3.3 V/cm (13°C) in 0.5×TBE, with switching every 7.5 min between two electric fields whose orientation differs by 120°. The gels were then stained with ethidium bromide (0.5 µg/ml) for 20 min and destained in distilled water for 20 min.

### DNA transfer and hybridization

The gel was irradiated for 1 min with UV light (254 nm) and treated with 0.25 N HCl for 20 min to nick the DNA. The gel was then incubated in 0.5 N NaOH-1.5 M NaCl for 30 min to denature the DNA and neutralized for 30 min in 0.5 M Tris-HCl (pH 7.5)-1.5 M NaCl. The DNA was transferred to a nylon filter (Pall BioSupply) by the standard method (18).

For the specific detection of filter bound sequences, nonradioactive digoxigenin-dUTP labeled DNA probes were used. Labeling and immunodetection were carried out with the Boehringer kit (Boehringer Mannheim) according to the manufacturers manual. Hybridization was performed in a mixture containing 50% formamide, 5×SSC, 5% blocking reagents, 0.1% sodium lauroylsarcosinate and 0.02% SDS for 20 h at 42°C with a homologous probe or at 28°C with a heterologous probe.

### Hybridization probes

DNA clones used as probes to map specific genes onto the *Chlorella* chromosomes were as follows:  $\alpha$ 10-2, the  $\alpha$ -tubulin gene of *Chlamydomonas reinhardtii* (19); pAB30, the gene for light-harvesting chlorophyll a/b-binding proteins of photosystem II from *Lemna gibba* (20); CS2.1, the gene for the small subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase of *C. reinhardtii* (21); pRRN4, the cytoplasmic ribosomal RNA genes of *Chlorella ellipsoidea* (T. Aimi and T. Yamada, in preparation); pCCS14, the *psbA* gene of *C. ellipsoidea* chloroplast DNA (22); pMCOII, the gene for mitochondrial cytochrome c oxidase subunit II from pea (K. Nakamura, personal communication). For identification of the individual chromosomal DNAs of *C. vulgaris* C169 separated by CHEF gel electrophoresis under different conditions, chromosome-specific DNA clones for hybridization probes were prepared as follows: a gene library of *C. vulgaris* C169 nuclear DNA was constructed by ligating *Sau*3A-partial digests (4–6 kbp) of C169 DNA and *Bam*HI-digested pUC18 DNA and transformed into *E. coli* NM522 (18). Chromosome-specific clones were screened by colony hybridization with probes of the isolated individual chromosomes.

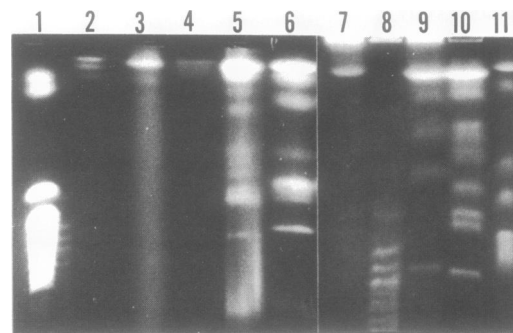
## RESULTS

### Separation of chromosomal DNAs of *Chlorella* strains by CHEF gel electrophoresis

Cells of *Chlorella* strains treated with cell wall-degrading enzymes and proteinase K in agarose gel produced intact chromosomal DNAs that could be reproducibly separated by CHEF gel electrophoresis. Examples of the resolution are demonstrated in Fig. 1, where electrophoresis was carried out with a switching interval of 7.5 min at 3.3 V/cm for 72 h for lanes 1–6 or for 96 h for lanes 7–12. Under these conditions, several chromosomal bands could be resolved for the strains of C211 (lane 5), C169 (lane 6 and 10) and NC64A (lane 9), whereas the chromosomes of C87 (lane 3), C135 (lane 4) and C150 (lane 7) did not separate well. The upper size limit of resolution under these conditions was 2–3 mbp judged from the separation pattern of the *Schizosaccharomyces pombe* chromosome markers (lane 2). This indicates that there are two major distinct karyotypes or chromosome-length polymorphism (CLP) groups among these 6 strains of *Chlorella*: one with larger chromosomes more than 2 mbp, designated as the C87 group after a representative strain *C. ellipsoidea* C87 and the other with chromosomes subdivided into smaller pieces, designated as the C169 group after *C. vulgaris* C169.

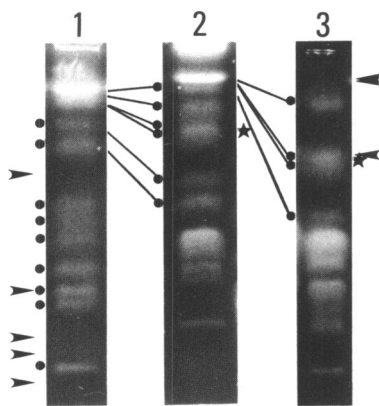
### Karyotyping of *C. vulgaris* C169

As a representative of the CLP group with smaller chromosomes, the chromosomes of *C. vulgaris* C169 were further separated. In addition to the several chromosomes separated in Fig. 1, there was a thick band at the topmost part of the gel, indicating the existence of chromosomal DNA molecules larger than 2–3 mbp. The molecular weight range in which CHEF gel electrophoresis gives maximal resolution can be shifted upward by increasing the switching intervals. To separate and identify unambiguously each chromosomal DNA molecules, we adopted three different windows of resolution by CHEF gel electrophoresis: (i) a window for smaller molecular weights (800 kbp–2.5 mbp) obtained under the electrophoresis condition of a combination of a 5 min switching interval at the field strength of 3.3 V/cm for 24 h, a 7 min interval at 3 V/cm for 24 h and a 8 min interval at 2.6 V/cm for 24 h, (ii) a window for middle molecular weights (2–3



**Figure 1.** Separation of chromosomal DNAs of *Chlorella* strains by CHEF gel electrophoresis. Lanes: 1, *S. cerevisiae* (size markers); 2, *S. pombe* (size markers); 3, *C. ellipsoidea* C87; 4, *C. vulgaris* C135; 5, *C. saccharophila* C211; 6, *C. vulgaris* C169; 7, *C. vulgaris* C150; 8, *S. cerevisiae* (size markers); 9, *C. sp* NC64A; 10, *C. vulgaris* C169; 11, *Candida albicans* (size markers). CHEF gel electrophoresis was carried out with a switching interval of 7.5 min at 3.3 V/cm for 72 h (lanes 1–6) or for 96 h (lanes 7–11).

mbp) by electrophoresis with a 12 min switching interval at 2 V/cm for 4 days, and (iii) a window for larger molecular weights (more than 2.5 mbp) by electrophoresis with a switching interval of 25 min at 1.6 V/cm for 3 days and then with a 20 min interval at 2 V/cm for 2 days. Typical resolution patterns of the chromosomes of the C169 strain under these three conditions are compared in Fig. 2. The correspondence between the individual chromosomal DNA bands separated under different conditions were determined by hybridization with chromosome specific probes (data not shown). Most of the chromosomal DNAs were well separated from one another under at least one of the three conditions. However, there were a few diffused or expanded thick bands in the higher molecular weight range (Fig. 2, lanes 2 and 3, asterisks). Further changing the switching interval did not resolve these bands even though they migrated in the region of highest resolution. Chromosome-specific DNA clones always showed the similar broad hybridization patterns (data not shown). Because of the band strength, we concluded that they were comigrating doublets with similar molecular sizes. An abnormal broad banding of yeast chromosome XII was reported and thought to be due to the existence of highly repeated rDNAs on it (4, 5). In fact, as shown below, one of the C169 broad bands hybridized to the rDNA probe (chromosome XII in Fig. 3). Likewise, the broad banding behaviors of the chromosomal DNAs of X, XI, XIV and XV (Fig. 3) may due to some highly repetitive sequences located on them. Probes for chloroplast DNA (*psbA*) and mitochondrial DNA (*coII*) did not hybridized with any separated bands but hybridized to the well of the gel (data not shown). This result indicates that chloroplast DNA and mitochondrial DNA did not enter the gel. Nine chromosomal DNA bands were separated in lane 1 of Fig. 2. Additional 4 bands in the higher molecular weights range were further separated in lane 2 of Fig. 2. From the topmost band of lane 2, 3 bands were finally separated in lane 3 of Fig. 2. We conclude from these results that the genome of *C. vulgaris* C169 consists



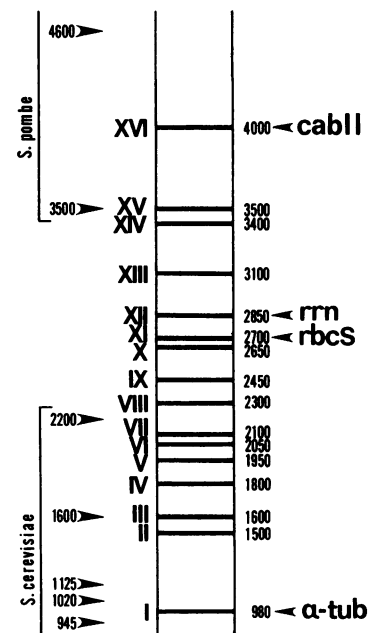
**Figure 2.** Karyotyping of *C. vulgaris* C169. The chromosomal DNA molecules of strain C169 were separated by CHEF gel electrophoresis under three different conditions: a combination of a 5 min switching interval at 3.3 V/cm for 24 h, a 7 min interval at 3 V/cm for 24 h and a 8 min interval at 2.6 V/cm for 24 h (lane 1); a 12 min switching interval at 2 V/cm for 4 days (lane 2); a switching interval of 25 min at 1.6 V/cm for 3 days and then with a 20 min interval at 2 V/cm for 2 days (lane 3). Dots indicate individual chromosomal DNA bands. The corresponding chromosomal DNA bands at the higher molecular weight range in different lanes are connected to each other by lines. Asterisks indicate abnormal broad bands. Arrowheads indicate the positions of size markers of *S. cerevisiae* chromosomes XII, IV, XV, VII and XVI from top to bottom (lane 1) and of *S. pombe* chromosomes (lane 3).

of 16 chromosomal DNAs. The relative mobilities and sizes of *C. vulgaris* C169 chromosomes estimated based on the mobilities of *S. pombe* and *S. cerevisiae* chromosomes as size markers are schematically summarized in Fig. 3.

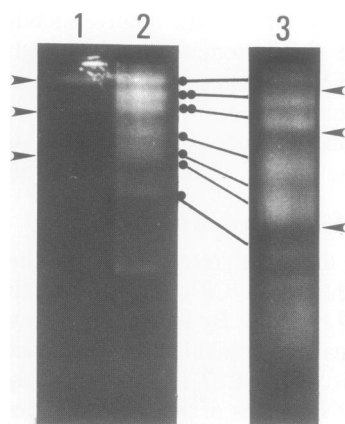
Summing up the sizes of individual chromosomal DNAs gave the total genome size of 38.8 mbp for *C. vulgaris* C169.

### Karyotyping of *C. ellipsoidea* C87

It was relatively difficult to resolve the large chromosomal DNA molecules of the strain C87, a representative of the large chromosome CLP group. By increasing the switching interval, the window of maximal resolution was positioned to resolve large DNA molecules of the C87 chromosomal size range. Seven chromosomal DNA bands of the strain C87 could be separated by using a 60 min switching interval at a field strength of 2 V/cm for 4 days and then a 90 min interval at 1.3 V/cm for 4 days (Fig. 4, lane 2). The resolution was not improved by extending the running period to 14 days at a 90 min switching interval at 1.3 V/cm because of gradual degradation of the larger chromosomal DNAs (Fig. 4, lane 3). Under these conditions, the second and the third largest bands were comigrating doublets based on a densitometric scanning of the gel. Apparently, the largest C87 chromosomal DNA exhibited a lower mobility than that of *S. pombe* chromosome I DNA of 5.7 mbp. Since no molecular weight markers exist in this size range, the size of this chromosome was tentatively estimated to be 6.5 mbp by extending the mobility-size correlation of *S. pombe* chromosomal markers. The sizes of the other eight chromosomal DNAs were 5.2 (doublet), 4.7 (doublet), 4.0, 3.0, 2.9 and 2.5 mbp based on their mobility relative to those of *S. pombe* chromosomes (5.7, 4.6 and 3.5 mbp) and of the largest chromosome of *S. cerevisiae* (2.2 mbp). These combined estimates indicate that the total



**Figure 3.** Schematic representation of the *C. vulgaris* C169 karyotype. The chromosomal DNAs indicated by bars are numbered from I to XVI in order of size. The relative positions of size markers, *S. cerevisiae* chromosomes (4, 5) and *S. pombe* chromosomes (6) are shown by arrowheads on the left side of the lane. Arrowheads on the right side indicate the chromosomes where the specific genes were mapped. Size is shown in kilobase pairs.



**Figure 4.** Karyotyping of *C. ellipsoidea* C87. The chromosomal DNA molecules of the strain C169 (indicated by dots) were separated by CHEF gel electrophoresis with a switching interval of 60 min at 2 V/cm for 4 days and successively with a 90 min interval at 1.3 V/cm for 4 days (lane 2) or a 90 min interval at 1.3 V/cm for 14 days (lane 3). The corresponding bands between lanes 2 and 3 are connected by lines to each other. The bottom bands in lanes 2 and 3 are those derived from degradation products of the chromosomal DNAs because they appeared occasionally and correspond to DNA molecules less than 50 kbp in size. Lane 1 contained *S. pombe* chromosome size markers (arrowheads). For lane 3, the positions of size markers (*S. pombe* chromosomes) are indicated by arrowheads.

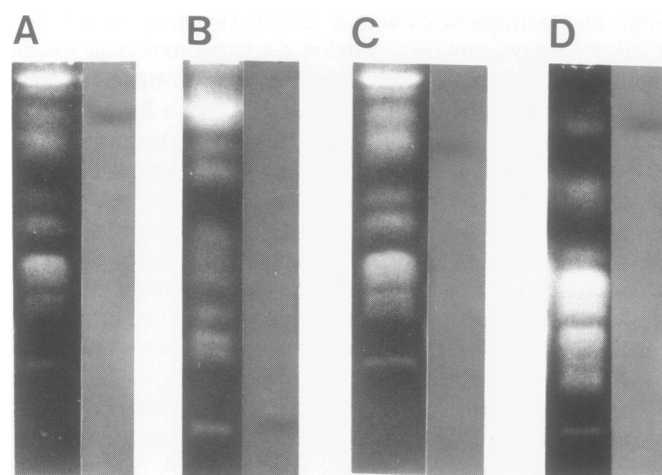
genome of *C. ellipsoidea* C87 is approximately 38.7 mbp. The strains of *C. vulgaris* C135 and C150 gave similar chromosome separation patterns to that of C87 with minor differences in the chromosome mobilities under the same electrophoresis condition (data not shown).

#### Mapping of genes onto the *Chlorella* chromosomes

Resolving the chromosomal DNA molecules makes it possible to map specific genes to the individual chromosomal DNAs of *Chlorella* by hybridization. To demonstrate this, 4 genes were mapped to *C. vulgaris* C169 chromosomal DNAs. Figure 5 shows the hybridization patterns of the C169 chromosomal DNAs separated by CHEF gel electrophoresis with different DNA probes. The pRRN4 containing a complete set of the *Chlorella* rDNA repeating unit of approximately 8.5 kbp hybridized solely to chromosome XII under stringent conditions (Fig. 5A), indicating that the genes for rRNAs (*rrn*) are coded by only this chromosome. The probe  $\alpha$ 10-2 for the  $\alpha$ -tubulin gene ( $\alpha$ -tub) hybridized specifically to chromosome I (Fig. 5B). This indicates that even if there are more than two genes for  $\alpha$ -tubulin, like in *Chlamydomonas reinhardtii* (19), they are located on chromosome I in *Chlorella vulgaris* C169. The gene for the small subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase, *rbcS* could be mapped solely to chromosome XI (Fig. 5C). The probe pAB30, containing the *Lemma cabII* sequence hybridized specifically to chromosome XVI (Fig. 5E). This suggests that at least one *cabII* gene is encoded on chromosome XVI. A summary of the hybridization results is shown in Fig. 3.

#### DISCUSSION

The unicellular green algal genus *Chlorella* includes extraordinarily heterogeneous species (1) and its taxonomy has remained in a state of chaos (23). The *Chlorella* strains used in this work for karyotyping (except for NC64A) are those which



**Figure 5.** Hybridization of separated chromosomal DNAs of *C. vulgaris* C169 with probes of the following genes: (A) *rrn* for cytoplasmic rRNAs, (B)  $\alpha$ -tub for  $\alpha$ -tubulin, (C) *rbcS* for small subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase and (D) *cabII* for chlorophyll a, b-binding protein. For each hybridization, a separation pattern where the hybridizing band migrated in the range of highest resolution was used; namely the patterns of lane 2, lane 1, lane 2 and lane 3 of Fig. 2 were hybridized with *rrn* (A),  $\alpha$ -tub (B), *rbcS* (C) and *cabII* (D), respectively. A summary of the hybridization results is shown in Fig. 3.

were previously shown to be sensitive to cell wall-degrading enzymes (15) and belong to three taxonomically closely-related species *C. ellipsoidea*, *C. saccharophila* and *C. vulgaris* (23). Unexpectedly, two major chromosome-length polymorphism groups, namely the C87 group and the C169 group were revealed among these strains. The nine chromosomes of C87, a representative strain of the former, ranged from 2.5 to 6.5 mbp, whereas in C169, a representative of the latter, the 16 chromosomes were detected ranging from 980 kbp to 4.0 mbp. Preliminary hybridization experiments with the C169 chromosome-specific probes to correlate chromosomes between the two strains suggested very complicated relationships of the chromosomes; for example, three different chromosome I-specific probes of C169 hybridized with different chromosomes of C87 (T. Higashiyama and T. Yamada, unpublished result). Dynamic rearrangements among the chromosomes may explain such a mosaic correlation. This is also true within the C169 group since the gene for  $\alpha$ -tubulin hybridized to chromosome I (980 kbp) of C169 (Fig. 5), while it hybridized to a much larger chromosome (>2 mbp) in NC64A (T. Higashiyama and T. Yamada, unpublished result). The nature of the chromosome-length polymorphism and possible chromosome rearrangements can be clarified by comparing detailed physical and genetic maps constructed for each *Chlorella* chromosomal DNAs.

In spite of the different chromosome separation patterns, the sum of the chromosome sizes was almost the same, approximately 38–39 mbp for *C. ellipsoidea* C87 and *C. vulgaris* C169 and also for four other *Chlorella* strains (data not shown). The total DNA contents per cell determined for C87 and C169 were 42 mbp and 45 mbp, respectively (T. Yamada, unpublished result) and are in good accordance with this value. This genome size is approximately twice that of *Saccharomyces cerevisiae* (4, 5), three times that of blue-green algae and half the size of *Arabidopsis thaliana* (24). To our knowledge, the *Chlorella* genome is the smallest of eukaryotic photosynthetic organisms so far characterized. Therefore, the *Chlorella* chromosomes

should serve as a good source for cloning specific genes involved in photosynthesis. Especially, the strain C169 contains 16 chromosomes all of which could be easily separated by CHEF gel electrophoresis, so that its individual chromosomal DNAs will be able to be purified and used to prepare chromosome-specific DNA libraries. Moreover, its small chromosomal DNAs could be an interesting subject for characterizing the molecular organization of plant-type chromosomes.

By hybridization, 4 genes were mapped to the C169 chromosomes. The precise location of the genes could be established in combination with physical mapping. Theoretically, all *Chlorella* genes can be mapped to the *Chlorella* chromosomes. So far, there has been no appropriate system for genetic analysis in *Chlorella* cells, in spite of their frequent use as a model organism in a wide range of photosynthetic studies. Here, we have established a powerful way to accumulate genetic information about *Chlorella* cells.

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