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Expressed Sequence Tags from the *Closterium peracerosum-strigosum-littorale* complex, a Unicellular Charophycean Alga, in the Sexual Reproduction Process

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Abstract

We obtained genetic information on sexual reproduction of the *Closterium peracerosum-strigosum-littorale* complex, a unicellular charophycean alga. Normalized cDNA libraries were constructed from cells in the sexual reproduction process, and a total of 1190 5′-end expressed sequence tags were established. Since 604 of these ESTs were classified into 174 non-redundant sequences, these 1190 ESTs include 760 unique sequences. Similarity search against a public non-redundant protein database indicated that 390 unique sequences had significant similarity to registered sequences. Among these 390 sequences, 3 were identical to and 4 were homologous to previously identified sex-pheromone genes. According to our study, 370 of 760 unique sequences are likely to be novel transcripts. These cDNA clones and EST sequence information may would be helpful for future functional analyses using DNA array technologies.

Key words: *Closterium*; conjugation; EST; sexual reproduction

1. Introduction

Charophycean green algae are closely related to land plants and comprise at least five lineages (orders) of freshwater green algae (Charales, Coleochaetales, Zygnematales, Klebsormidiales, and Chlorokybales). Among these charophycean algae, Charales and Coleochaetales, which are most closely related to land plants, have multicellular bodies and form eggs and sperm unlike other unicellular or filamentous charophyceans. The monophyletic clade consisting of charophyceans and land plants is phylogenetically distant from other major green algal lineages, i.e., the Chlorophyceae and the Ulvophyceae.[1,2] A large-scale EST project of *Chlamydomonas reinhardtii*, which is a fresh water green alga belonging to the Chlorophyceae, is in progress.[3,4] However, no information is available on the charophycean genome as a whole or on cDNA sequences although analyses of their genomes are crucial to investigate the evolutional development mechanism from green algae to land plants.

In this study, we focused on the desmid *Closterium*, because this is a unicellullar charophycean alga and is easily grown and maintained under laboratory conditions, and moreover, there have been many reports on its sexual reproduction process.[5–14] Heterothallic strains of *Closterium* have two morphologically indistinguishable sexes: mating type plus (mt^+) and mating type minus (mt^-). Sexual reproduction is easily induced when cells of these two sexes are cultured together in nitrogen-depleted medium under light.[15] A hypothetical sexual reproduction mechanism of the *Closterium peracerosum-strigosum-littorale* complex (*C. pslc*) has been reported.[16]

As the first step to understand the sexual reproduction mechanism of *C. pslc*, we established and analysed ESTs from cells in the sexual reproduction process. This study is the first EST analysis of charophycean algae and will help our understanding of the evolution of sexual reproduction from green algae to land plants.

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2. Materials and Methods

2.1. Strains and cell culture conditions

The strains of heterothallic *C. pslc* used in this work were NIES-67 (mt^+) and NIES-68 (mt^-) obtained from the National Institute for Environmental Studies (Ibaraki, Japan). Clonal cultures were grown in nitrogen-supplemented medium (C medium[15]) as previously described.[17]

Sexual reproduction of *C. pslc* was induced according to the following steps. Vegetatively growing cells of the two mating types were harvested, washed three times with nitrogen-depleted medium (MI medium[15]) and suspended separately in MI medium under continuous light condition (130 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After 24 hr of incubation, cells of both mating types (5.4×10^5 each) were mixed in fresh 75-ml MI medium in 300-ml Erlenmeyer flasks and incubated under light. At various intervals (0, 2, 4, 8, 12, 24, 48, and 72 hr), cells were harvested, frozen in liquid nitrogen and stored at −80°C.

2.2. Preparation of polyadenylated RNA

Frozen *C. pslc* cells collected from various stages of sexual reproduction were mingled, ground to powder in liquid nitrogen and treated with TRIZOL reagent (Invitrogen Corp., Carlsbad, CA, USA) to extract total RNA, in accordance with the supplier's instructions. Poly(A)$^+$ RNA was further purified using the PolyATtract mRNA isolation system (Promega Corp., Madison, WI, USA) following the supplier's instructions.

2.3. Construction of cDNA libraries

We synthesized cDNAs using a cDNA library synthesis kit (Superscript Plasmid System; Invitrogen), according to the supplier's instructions. Synthesized cDNAs were resolved by 1.5% agarose gel electrophoresis, and fractions ranging from 0.5 to 3 kb were separately recovered from the gel using Suprec-01 columns (TAKARA, Kusatsu, Shiga, Japan). The recovered fragments were cloned into *Sal* I-*Not* I sites of pSPORT1-plasmid vectors and transformed into an *Escherichia coli* DH10B strain (Invitrogen) by electroporation.

Normalization was performed as described by Asamizu et al.[3] A single-stranded library was prepared from 5 μg of plasmid DNA by the combined action of gene II endonuclease of phage F1 and exonuclease III (Gene Trapper cDNA Positive Selection System; Invitrogen), according to the manufacture's instructions. The remaining single-stranded plasmids were purified using hydroxyapatite chromatography (Macro-Prep Ceramic Hydroxyapatite TYPE II 40 μm, Bio-Rad Lab., Hercules, CA, USA) at 60°C as described previously.[18] The sample was extracted four times with 2-butanol and twice with ether. Residual ether was evaporated by vacuum and the sample was ethanol-precipitated and dissolved in 50 μl of TE buffer. The sample was desalted by passage through a microspin S-300 column (Amersham Biosciences Corp., Piscataway, NJ, USA), concentrated by ethanol-precipitation, and dissolved in 5.5 μl of water.

For amplification of the inserts, 0.5 μl of purified single-stranded plasmids were mixed with 10 μl of 10 μM pSPORT-sense primers (5′-CGTAAGCTTGGATCCTCTAGA-3′) and 10 μl of 10 μM pSPORT-anti primers (5′-TGCAGGTACCGGTCCGGAAT-3′), then PCR was performed in a total volume of 100 μl by AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) or Platinum *Pfx* DNA polymerase (Invitrogen) using a GeneAmp 9700 Thermal Cycler. The PCR product was ethanol-precipitated and dissolved in 1.5 μl of water.

The remaining 5 μl of single stranded plasmids were evaporated, dissolved in 5 μl of formamide, and then mixed with the PCR product, 0.5 μl (10 μg) of 5′-blocking oligo nucleotide (5′-CGGACGCGTGGGTCGACCCGGGAATTCCGGACCGGTACCTGCA-3′) and 0.5 μl (10 μg) of 3′-blocking oligo nucleotide (5′-CGTAAGCTTGGATCCTCTAGAGCGGCCGCCC-3′). After heating at 80°C for 3 min, 1 μl of 10 × buffer [1.2 M NaCl, 0.1 M Tris-HCl (pH 8.0), 50 mM EDTA, and 10% SDS] and 1.5 μl of water was added, and hybridization was performed at 30°C for 24 hr. The remaining single-stranded plasmids were purified using hydroxyapatite chromatography and converted to double strands using repair enzyme (Gene Trapper System) in accordance with the supplier's instructions. The obtained double-stranded plasmids were transformed into host *E. coli* by electroporation.

2.4. Nucleotide sequencing

Plasmid DNA for the sequencing reaction was prepared from 2-ml overnight cultures using a QIAprep Spin Miniprep kit (Qiagen; Hilden, Germany). Sequence reactions were performed using a CEQ DTCS Quick Start kit (Beckman Coulter, Inc., Fullerton, CA, USA) followed by an automated sequencer CEQ 2000XL (Beckman Coulter).

The remaining EST sequence information was commercially obtained from Shimadzu Corp. (Kyoto, Japan) using multi-capillary automated DNA sequencers RISA-384 (Shimadzu).

2.5. Sequence data analysis

Vector-derived sequences and ambiguous sequences were manually removed from the collected EST sequences prior to the computer-aided analyses. Then, each sequence was subjected to similarity search against the non-redundant protein database, nr, provided by NCBI using the BLASTx algorithm.[19] Sequence similarities were considered to be significant when the expected value was below 1.0^{-14} at the amino acid sequence level. Redundancy of the ESTs was checked with a

homology searching program (PrivateBioDB; Software Development Co., Shibuya, Tokyo, Japan), with a data set of themselves. Clones that showed over 95% identity for more than 100 bp were grouped together.

2.6. Phylogenetic analysis

To reconstruct phylogenetic trees of *EREBP* and *PGPD14* genes, the amino acid sequences shown in Figs. 2 and 3 were obtained from EMBL/DDBJ/GenBank DNA databases and aligned using the program Clustal W, version 1.6.[20] The maximum likelihood distances were calculated with the ProtML program[21] under the condition of the JTT-model,[22] and a Neighbor-Joining tree was obtained. The trees were further analyzed with local rearrangement search using the program ProtML to obtain the maximum likelihood tree. Bootstrap values calculated with the RELL method[23] are indicated above the nodes reconstructed by both the maximum likelihood and Neighbor-Joining methods.

3. Results and Discussion

3.1. Sexual materials for construction of normalized cDNA libraries

Several sex pheromones induce and promote progress of sexual reproductive steps in *C. pslc*. It is considered that known and unknown sex pheromones regulate the gene expression during early stages (0–8 hr) after mixing, although no morphological changes are observed. Morphological changes can be seen at 12 hr (sexual cell division for forming gametangial cells) and 24 hr (sexual pair formation for forming zygospores followed by release and fusion of gametic protoplasts) after mixing. Cells in various stages of sexual reproduction (0, 2, 4, 8, 12, 24, 48, 72 hr after mixing) were collected, mingled, and used as primary materials to obtain various ESTs related to sexual reproduction. Two primary cDNA libraries respectively containing 1.6×10^7 and 6.5×10^6 independent clones were constructed. Then, normalized cDNA libraries (Nos. 1 and 2) respectively containing 6×10^4 and 5×10^5 independent clones were established from these primary cDNA libraries, thereby reducing redundancy.

3.2. Features of the generated ESTs

Single-pass sequencing from the 5′ end was performed on 642 and 548 clones randomly isolated from library Nos. 1 and 2, respectively. The average GC content of the ESTs was 59.3%, estimated from the sequences of 30 randomly selected ESTs. The 5′-end sequences were compared with a data set of themselves using an analysis program to identify the number of independent clones. Clones showing identity of over 95% over more than 100 bp were grouped together. We revealed that 339 and 265 ESTs from library Nos. 1 and 2, respectively, overlapped with one or more of the 1190 EST clones. Meanwhile, ESTs were divided into 760 non-redundant sequences, yet the real number of independent genes would be less than 760 since they could contain non-overlapping fragments deriving from the same genes.

3.3. Database search

The EST sequences were compared with public non-redundant protein sequence databases with the BLASTx program, and $P < 1.0^{-14}$ was taken as the level of significance. As a result, 390 sequences among the 760 non-redundant sequences showed similarity to previously registered genes in the public databases. Since there are few reports in the literature concerning genes from *C. pslc*, almost all ESTs showed similarities to sequences derived from other organisms (Table 1). The source group showing the highest similarity was land plants including *Arabidopsis thaliana*, while sequences from algae showed similarity to only 17 of the non-redundant sequences.

Genes whose functions could be estimated from the similarity search were classified into 16 categories based on their biological roles. The largest functional group in the libraries was 108 non-redundant sequences involved in translation, i.e. genes encoding ribosomal proteins (Table 2). Since the remaining 370 unique sequences comprised only 465 clones, they possibly include genes specific to *C. pslc* as well as genes expressed at low levels in other organisms. The search results including the names of proteins obtained through our search and putatively encoded by EST clones as well as the accession numbers of ESTs are provided at our Web page (http://foj.c.u-tokyo.ac.jp/closterium/).

3.4. Characterization of respective ESTs

ESTs obtained in this study included previously reported sex pheromone genes, i.e. PR-IP Inducer and two subunits of PR-IP. PR-IP Inducers are released from mt^- cells of *C. pslc* and are responsible for PR-IP gene expression in mt^+ cells.[24–26] It was reported that gene expression of PR-IP Inducer surged immediately after the cells were applied to nitrogen-depleted medium under light even if the cells were not mixed and was specifically observed in mt^- cells.[27] Furthermore, PR-IP Inducer transiently boosts gene expression of PR-IP in mt^+ cells.[26] These results indicate that present libraries include variable conjugation-related sequences.

Two non-redundant sequences, 3-01_E08 (AU294823) and 4-02_D09 (AU295448 etc.), showed high levels of sequence similarity with the PR-IP Inducer gene (Fig. 1). This result coincides with our previous paper,[27] which reported the existence of more than one gene homologous to the PR-IP Inducer gene in the genome. In the meantime, Fukumoto et al. showed that the partial amino acid

Table 1. The result of similarity search against public non-redundant protein databases. The numbers of non-redundant sequences and clones showing similarity to previously reported *C. pslc* genes and known genes from other organisms are indicated.

Similarity	Number of non-redundant sequences	Number of Clones
Genes from *C. pslc*	7	63
Genes from other algae	9	14
Genes from land plants	341	605
Genes from cyanobacteria	7	10
Genes from other organisms	26	33
No similarity	370	465
Total	760	1190

Table 2. Classification of deduced functions of 390 non-redundant sequences.

Functional categories	Number of non-redundant sequences
Amino acid biosynthesis	12
Biosynthesis of cofactors, prothetic groups, and carriers	16
Cell envelope	7
Cellular processes	21
Central intermediary metabolism	6
Energy metabolism	27
Fatty acid, phospholipid and sterol metabolism	5
Photosynthesis and respiration	31
Purines, pyrimidines, nucleosides, and nucleotides	2
Regulatory functions	39
DNA replication, recombination, and repair	4
Transcription	3
Translation	108
Transport and binding proteins	32
Other categories	14
Hypothetical	63
Total	390

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PR-IP_inducer     1 MAC---------LARVLLLAA------------------LAVGLLAAS---VSGARDIVP
3-01_E08          1 MAP--------LAARLLLAA------------------LAVCLLAAP--AHAGRDLGVK
4-02_D09          1 MALPRGLSFASALALIVLLAASLAPAAHARNGALVSDEEISSALLAASGAARSGADGGVP

PR-IP_inducer    31 RAAGKGVGGQ----QRKGGRILAAETFSHHDTLPDYLSHI-PRRKALATTC------GGS
3-01_E08         33 QAGP---------------RILAAETYSHDQELPDYLDHI-PRRKALATTC------GRT
4-02_D09         61 GATAGGVAEIAESIQRQGPRILNVETYEVDEPLPDYLSHVEPHRRELQTSWKLDSKCDGS

PR-IP_inducer    80 TVTVRSQYLGPFDLHSYIFNMTFYNGCAYNVTSPLVVFNCFEFGTLWDGFLENPAPNPTQ
3-01_E08         71 TVVIKSQYLGPYDLHNYIFNMTFYNGCAFNVTSPLRVFQCQDFGNVWQGILKNPAKNPTI
4-02_D09        121 NLILASKFVSVFHRANYLFNFTMVNTCKFSLVSPLVVFNWFEFGKVVV------------

PR-IP_inducer   140 YQTGPIFGQTLPGYCEMWMNRGAAGNVQMFPGETVNILYAWAYDFRPCIEELHFSNSNSY
3-01_E08        131 YQTG------------------------------------------------------
4-02_D09            ------------------------------------------------------

PR-IP_inducer   200 IRGNNTCVPAATI
3-01_E08            -------------
4-02_D09            -------------
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Figure 1. Comparison of the deduced amino acid sequences of PR-IP Inducer and its homologous genes. Identical amino acid residues are indicated by black boxes with white letters, whereas residues with similar chemical characteristics are given in gray boxes with black letters. Gaps have been introduced to maximize similarities among sequences.

sequences of a sexual cell division-inducing pheromone (SCD-IP) from *C. ehrenbergii* had significant similarity with the PR-IP Inducer gene, indicating these two sex pheromones are evolutionally related to each other.[28] Recently, we developed an assay system for sexual cell division of *C. pslc* and detected the SCD-IP released from mt^- cells (SCD-IP-minus), whose biochemical characteristics are quite similar to PR-IP Inducer.[29] The production of recombinant proteins of the two non-redundant sequences homologous to the PR-IP Inducer gene is necessary in order to understand their possible pheromonal activities.

PR-IP consists of two subunits of 19 kD[30] and 42 kD.[31] Eight clones (clone ID: 3-04_H10, accession number: AU295130 etc.) with the same non-redundant sequence showed high levels of sequence similarity to the 19-kD subunit gene, whereas one clone (clone ID: 3-06_H09, accession number: AU295316) showed a high level of sequence similarity to the 42-kD subunit gene. Since SCD-IP from mt^+ cells (SCD-IP-plus) likely has biochemical characteristics similar to PR-IP,[29] the non-redundant sequences of PR-IP-like genes might function as SCD-IP-plus.

Furthermore, we found transcription factor genes from the EST libraries above. We isolated two non-redundant sequences encoding single-AP2-repeat-containing proteins (AP2/EREBP proteins). AP2/EREBP proteins are divided into three classes: an ethylene-response element-binding protein (EREBP) group, a dehydration-response element-binding protein (DREBP) group, and a B3-like-domain-containing DNA-binding protein group.[32,33] As shown in Fig. 2, both *Closterium* AP2/EREBP proteins were closely related to the *DREB* gene group. One of these proteins (clone ID: 4-01_H11, accession number: AU295402, *CpDREB1-like1*) formed a clade with *Arabidopsis DREB1A*, *B*, *C*, *TINY*, *RAP2.1*, and *RAP2.10* genes. The expression of *DREB1A*, *B*, *C* genes are enhanced by low temperature and dehydration. On the other hand, the other AP2/EREBP protein (clone ID: 3-01_E07, accession number: AU294822, *CpDREB2-like1*) formed another clade with *DREB2* genes, whose expression is induced by drought and high salt stress. This result suggests that the common ancestor of land plants and desmids already possessed two ancestral *DREB-like* homologues. Studies on the expression patterns and functions of both genes would help clarify the evolution of environmental stress genes.

In the meantime, we found an EST clone (clone ID: 4-03_C09, accession number: AU295523) with a deduced amino acid sequence similar to the *PGPD14* gene, which is involved in pollen germination in *Petunia* and encodes a transcription factor with zinc-finger motif.[34] Accordingly, the *Closterium* EST clone was termed *CpPGPD14-like1*. Phylogenetic analysis (Fig. 3) indicated that the *CpPGPD14-like1* formed a clade with angiosperm *PGPD14* genes and was a homologue of

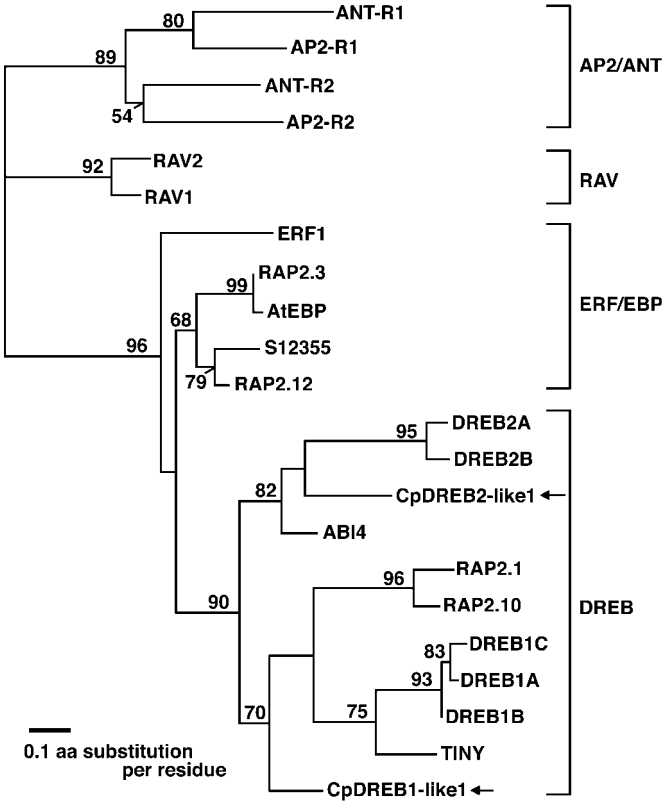

Figure 2. A gene tree of the *AP2/EREBP* family based on the maximum likelihood method. The arrows indicate the genes from *C. pslc*. The clones identified as 4-01_H11 and 3-01_E07 were termed *CpDREB1-like1* and *CpDREB2-like1*, respectively. The others are *Arabidopsis thaliana AP2/EREBP* genes. The maximum likelihood distances were calculated based on aligned 54 amino acid residues corresponding to the well-conserved AP2 domain.

the human *ARNIP* gene encoding androgen-receptor N-terminal-interacting protein. Land plants and metazoans independently obtained oogamies in their lineages. Pollens, which are the micro-gametophyte of seed plants, contain male haploid reproductive cells, or sperm. Sperm correspond to protoplasts released during sexual reproduction of isogametic *Closterium*.[17] We need to reveal whether the *CpPGPD14-like1* gene functions in only one or both of the two mating type cells in order to understand the evolution of male sexual reproductive cells in the land plant lineage.

In a preliminary experiment, the expression patterns of non-redundant sequences obtained in this study was analyzed using cDNA microarray. Gene expression of some unique sequences, e.g. clone ID: 3-03_A10 (accession number: AU294961), 4-03_G04 (AU295565), 4-04_F09 (AU295645), and 4-05_B03 (AU295685), as well as known sex pheromones soared at least five times from 0 to 8 hr after mixing, when sexual interaction between the two mating type cells is enhanced (data not shown). From these results, we confirmed that present

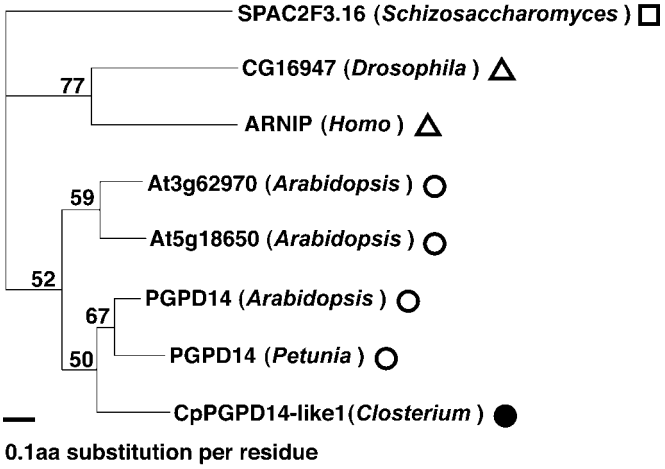

Figure 3. A gene tree of *PGPD14* and its homologues based on the maximum likelihood method. The closed circle indicates the gene from *C. pslc.* Open circles, squares, and triangles represent angiosperms (*Arabidopsis thaliana* and *Petunia hybrida*), yeast (*Schizosaccharomyces pombe*), and metazoans (*Homo sapiens* and *Drosophila melanogaster*), respectively. Aligned 94 amino acid residues were used for calculation of the maximum likelihood distances.

non-redundant sequences, which actually included sexual interaction-related sequences, would be quite useful for analyzing the sexual reproduction process of *C. pslc.*

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