Running title: Microarray analyses in *Closterium*

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Journal research area: Genetics, Genomics, and Molecular Evolution
Gene expression profiling using cDNA microarray analysis of the sexual reproduction stage of the unicellular charophycean alga *Closterium peracerosum-strigosum-littorale* complex

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This work was supported by a grant-in-aid from the 21st Century Center of Excellence program, Research Center for Integrated Science, of the Minister of Education, Culture, Sports, Science and Technology, Japan, to H.Se., and from the Japan Society for the Promotion of Science to M.I. and H.Se.

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The desmid *Closterium peracerosum-strigosum-littorale* complex, which is the closest unicellular sister to land plants, is the best-characterized of the charophycean green algae with respect to the process of sexual reproduction. To elucidate the molecular mechanism of intercellular communication during sexual reproduction, we created a normalized cDNA library from mixed cells of the sexual and the vegetative phases, and generated a cDNA microarray. In total, 3,236 ESTs, which were classified into 1,615 nonredundant groups, were generated for cDNA microarray construction. Candidate genes for key factors involved in fertilization, such as those that encode putative receptor-like protein kinases, leucine-rich-repeat receptor-like proteins, and sex pheromone homologues, were up-regulated during sexual reproduction and/or by the addition of the purified sex pheromones, and the expression patterns of these genes were confirmed by quantitative real-time PCR analysis. This first transcriptome profile of *Closterium* will provide critical clues as to the mechanism and evolution of intercellular communication between the egg and sperm cells of land plants.
Fertilization entails an intimate relationship between egg and sperm cells, which interact with each other at the cellular level. Along with the development of male and female gametophytes in plants, fertilization has been studied in many plant species (Huang and Sheridan, 1994; Heslop-Harrison et al., 1999; Faure et al., 2002). In addition to the pollination process, a long-lasting period of interaction between the diploid pistil and the haploid pollen tube is indispensable for successful fertilization in flowering plants. This interaction phase may be described as the progamic phase, which starts with the landing of the pollen grain on the stigmatic surface and ends with syngamy, which is the fusion of the sexual cells. The mechanism that directs the pollen tube through the pistil to the female gametophyte has been studied for a long time. The involvement of female tissues in defining a path for the pollen tube to the female gametophyte has been reported. On the other hand, the existence of pistil-produced chemotropic substances, i.e., chemoattractive pheromones for the male gametophyte, has been strongly supported by several independent studies. Although comprehensive reviews have been published (Higashiyama et al., 2003; Sanchez et al., 2004; Weterings and Russell, 2004), the intra- and intercellular events that occur during fertilization have not been elucidated fully at the molecular level.

Several studies have reported on the sexual interactions and sexuality of *Chlamydomonas reinhardtii*, which is a freshwater green alga of the Chlorophyceae (Ferris et al., 2002; Pan et al., 2003; Ferris et al., 2005). In addition, EST and whole genome projects are under way (Asamizu et al., 1999; Asamizu et al., 2000; Shrager et al., 2003). Using cDNA macroarrays, the transcriptional program for gametogenesis and the regulatory networks of gene expression during sexual differentiation have been analyzed critically (Abe et al., 2004; Abe et al., 2005).

Charophycean green algae, which are most closely related to land plants, form a relevant monophyly with land plants. Thus, charophyceans and land plants share many distinctive characteristics with respect to cellular structures and metabolism, and are
evolutionarily distant from other major green algae, i.e., the Chlorophyceae and Ulvophyceae (Graham and Wilcox, 2000; Karol et al., 2001). Charophyceans comprise five lineages (orders) of freshwater green algae: Charales, Coleochaetales, Zygmematales, Klebsormidiales, and Chlorokybales. The desmid *Closterium*, which belongs to Zygmematale, is the most successfully studied unicellular charophycean plant in terms of the maintenance of strains and sexual reproduction (Ichimura, 1971; Akatsuka et al., 2003; Fukumoto et al., 2003).

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. A possible mechanism for the sexual reproduction of *Closterium peracerosum-strigosum-littorale* complex (*C. pslc*) has been reported (Sekimoto, 2000). As the first step to understanding the molecular mechanism of *C. pslc*, 1190 5'-end expressed sequence tags (ESTs), which included 760 unique sequences, were established from cells in the sexual reproduction process (Sekimoto et al., 2003).

In this paper, we describe the first expression profile obtained using the charophycean green alga microarray, including 3,064 ESTs (2,047 newly prepared clones), which will be used to increase our understanding of the molecular mechanism of intercellular communication during the fertilization process in plants.

**RESULTS AND DISCUSSION**

**Construction of the cDNA Microarray**

The two cDNA libraries (1.6×10⁷ and 6.5×10⁶ clones) prepared previously from cells at various stages of sexual reproduction were used as the primary materials (Sekimoto et al., 2003). In addition, a cDNA library (4.9×10⁶ clones) derived from a mixture of vegetative mt⁺ and mt⁻ cells was constructed. A normalized cDNA library that contained 1.7×10⁵ independent clones was established from a mixture of these three cDNA libraries, with the aim of reducing
redundancy. To analyze both quantitatively and qualitatively genes related to the process of sexual reproduction of *C. psilc*, 2,304 PCR-amplified cDNA inserts were isolated from the normalized cDNA library. In addition to these cDNA inserts, 760 cDNA inserts, which were derived from previously determined nonredundant EST groups, were prepared (Sekimoto et al., 2003). These 3,064 cDNAs were spotted twice (left- and right-hand sides) onto each slide, to increase the reliability of the microarray data.

**Features of ESTs from Sexual and Vegetative *Closterium* Cells**

While spotting the cDNA inserts, single-pass sequencing from the 5'-end was performed on the 2,304 uncharacterized cDNAs. After the removal of vector-derived sequences and ambiguous sequences, we obtained sequence information for 2,046 cDNAs. In addition to the previously obtained 1,190 ESTs (accession numbers AU294770 to AU295959), 3,236 ESTs were established in all (Supplemental Table I). Clones that showed identity of >98% over stretches of >100 bp were grouped together, and the ESTs were clustered into 1,615 nonredundant sequences, though the real number of independent genes should be <1,615 due to nonoverlapping fragments derived from the same genes.

The EST sequences were compared with the public nonredundant protein sequence databases using the BLASTX program, and E<1.0e\(^{-5}\) was set as the level of significance. Thus, 1,045 of the 1,615 nonredundant sequences showed similarity to previously registered genes in the public databases. The source group with the highest similarity was land plants, including *Arabidopsis thaliana*.

The search results, which include the names of the proteins putatively encoded by EST clones as well as the accession numbers of the ESTs (BW646625 to BW648670), are shown in Supplemental Table I.
cDNA Microarray Analysis of Gene Expression

To monitor gene expression patterns during the sexual reproduction of *C. psle*, Cy5-labeled cDNA populations were prepared from cells that were incubated in MI medium with the opposite mating type for 2, 8, 24, and 72 h (mixing culture). As controls, Cy5-labeled cDNA populations from the respective mating types, incubated separately for 8 h in MI medium at a low cell density (mt⁺-L and mt⁻-L), were also prepared. These populations were independently hybridized to the microarray. To determine the changes in gene expression levels during these processes, the expression levels of the cells just before mixing (mt⁺-0, mt⁻-0) were also examined. After scanning and quantification, normalization was performed for each hybridization (Supplemental Table II).

In order to identify genes that are specifically expressed during sexual reproduction, cDNA spots were selected that showed a fivefold increase in expression over the time-course of sexual reproduction, as compared to the mt⁺-L or mt⁻-L cells. Those cDNA spots that were differentially expressed in mt⁺ cells, compared to mt⁻ cells, were also chosen as sex-specific genes and vice versa. To analyze pheromone-responsive genes, cDNA spots, the expression levels of which were elevated fourfold by the addition of PR-IP or PR-IP Inducer, were selected. In all, 292 cDNA spots were identified. To obtain sequence information for the selected cDNAs, the 5'-ends of which showed no sequence similarities, single-pass sequencing was carried out from the 3'-ends. The sequences of these 292 cDNA could be classified into 88 nonredundant gene clusters, and the average values of the respective gene clusters were used in the final dataset (Table I; Supplemental Table III).

Clustering of Sexual Reproduction-related Genes with Respect to Expression Patterns

The 88 gene clusters were subdivided into groups with respect to similar expression patterns during conjugation, sex-specificity, and responses to sex pheromone treatment. We classified 13 and 25 gene clusters in which gene expression was observed preferentially in
of the sexes (mt⁺-L and mt⁻-L) as groups A and B, respectively. Of the remaining 50 gene clusters, 44 clusters whose expression was elevated during conjugation were classified into group C. In addition, 25 and 16 gene clusters that were expressed in response to PR-IP Inducer and PR-IP were classified into groups D and E, respectively. As most of the pheromone-responsive genes showed elevated expression levels during conjugation, these genes were placed into subgroups C-D, C-E, D-E, and C-D-E (Fig. 1).

**Genes Expressed Specifically in One of the Mating Types**

Among the 13 gene clusters in group A, eight showed similarities with known proteins. Two of these clusters (cluster ID: 97 and 247) showed similarities with aquaporins, which represent an ancient family of channel proteins that transport water and certain neutral solutes across biological membranes (Chaumont et al., 2001), and these clusters were termed \( \text{CpAQP1} \) and \( \text{CpAQP2} \), respectively. The deduced amino acid sequences of \( \text{CpAQP1} \) and \( \text{CpAQP2} \) showed 93.1% identity with each other and contained Asn-Pro-Ala motifs, which were conserved within the family. During sexual reproduction, condensation of the cytoplasm and release of protoplasts from the cell wall are observed (Sekimoto et al., 1990). For these processes, the content of water and/or some solutes in cells must undergo change. These proteins may play a role in these processes. In higher plants, aquaporins comprise four different groups of highly divergent proteins, being present in the tonoplast, plasma membrane, and possibly in other internal membranes. Given that \( \text{CpAQP1} \) and \( \text{CpAQP2} \) comprised a monophyletic clade, and were separate from genes derived from other land plant lineages (data not shown), their actual roles in mt⁺ cells as well as their cellular localizations remain unclear.

Genes with significant levels of similarity to carbonate dehydratase (ID: 106 and 1043), carbonate anhydrase (ID: 245), and glyceraldehyde-3-phosphate dehydrogenase (ID: 201) were also identified in group A. These genes are known to have roles in cellular
metabolism, although specific roles in mt⁺ cells remain to be analyzed. Cluster 1441 showed similarity with the heparanase-like protein from Rattus norvegicus. The deduced proteins from Closterium and Rattus contain the tandem repeat sequences (P/S)AD(N/S)DNTAV(V/I) and D(S/H)D(V/D)SSGPVDS, which are not significantly similar to each other. Since the apparent sequence similarity may be attributed to the repeated structures, a functional relationship cannot be deduced.

Among the genes that were expressed specifically in mt⁻ cells (group B), the expression levels of the genes that encode PR-IP Inducer (ID: 1) and the homologue (ID: 179) were found to be most critically restricted in the mt⁻ cells. PR-IP Inducer has been identified as a sex pheromone that is released from mt⁻ cells and induces PR-IP production in mt⁺ cells during the sexual reproduction of C. psilc. Recently, recombinant PR-IP Inducer produced by yeast cells has been shown to induce sexual cell division in mt⁺ cells, in addition to its PR-IP-inducing activity (Tsuchikane et al., 2005). Production of the recombinant protein is under way, to facilitate evaluation of the biological functions of the PR-IP Inducer-like protein. In addition, seven gene clusters showed significant similarities to known proteins, such as gamma-tocopherol methyltransferase (ID: 216), α-tubulin (ID: 219), protein kinase (ID: 240), epimerase-like protein (ID: 397), PREG-like protein (ID: 1261), glycosyltransferase family protein (ID: 1279), and ubiquitin-specific protease 2 (ID: 1374). These sex-regulated genes from groups A and B are useful in the identification of differences between the sexes, and in the elucidation of the sex determination mechanisms of C. psilc.

Genes Expressed during Sexual Reproduction but Not in Response to Pheromones

Among the remaining 50 gene clusters, 11 showed high expression levels during sexual reproduction but were not remarkably responsive to the pheromone treatment. Five genes showed similarities to known proteins, which included glycine dehydrogenase (ID: 1226), the 22-kDa protein of Photosystem II (ID: 223), hypothetical proteins (ID: 988),
putative pectin acetyltransferase (ID: 988), and low-CO\textsubscript{2}-inducible proteins (LCIB and/or LCIC) from 
\textit{Chlamydomonas reinhardtii} (ID: 952 and 1205). With the exception of the pheromone-encoding genes reported by us, these are the first genes implicated in the sexual reproduction of \textit{Closterium}; furthermore, they will be useful in characterizing the metabolic changes that occur during the process of reproduction.

**PR-IP Inducer-responsive Genes Specifically Expressed in Mt\textsuperscript{+} Cells**

Twenty-five gene clusters responded to the addition of PR-IP Inducer. Of these, 20 gene clusters were further up-regulated during sexual reproduction and classified into subgroup C-D. In this group, gene cluster 104 showed significant similarity to the receptor-like protein kinase and was named \textit{CpRLK1}. The conserved regions of the kinase domain were found in the deduced amino acid sequence, which suggests that the gene is functional, although the full-length cDNA sequence is not currently available. In plants, many genes encode receptor-like protein kinases (Shiu and Bleecker, 2001), although the functions of most of these genes are unknown. In addition, information regarding their ligands is rather limited (Kachroo et al., 2001; Takayama et al., 2001; Wang et al., 2001; Matsubayashi et al., 2002; Scheer and Ryan, 2002). In the case of the \textit{CpRLK1} protein, the ligand is unknown, although PR-IP Inducer is a candidate. A receptor for PR-IP Inducer should be expressed in \textit{mt}\textsuperscript{+} cells but not in \textit{mt}\textsuperscript{-} cells. It is reasonable to assume that the expression of the gene that encodes this type of receptor is activated after acquisition of the ligand itself during sexual reproduction. To facilitate characterization of the gene product, cloning of the full-length cDNA is under way.

Gene clusters that encode the 19-kDa and 42-kDa subunits of PR-IP (ID: 76 and 3), which is a sex pheromone produced in \textit{mt}\textsuperscript{+} cells, were classified into subgroup C-D; in addition, our results corroborate a previous report that showed the gene expression patterns (Sekimoto et al., 1994). Furthermore, genes that encode homologues of the 19-kDa and 42-
kDa subunits (ID: 9 and 239) were also found to be involved, which suggests that the gene products also play roles in the progress of sexual reproduction, presumably as pheromones of unknown function.

One of the remaining genes showed significant similarity with phytochrome A repressor proteins (ID: 1454). Indeed, light is indispensable for the sexual reproduction of *Closterium*, although the light requirement for mating activation has been suggested as being primarily dependent on photosynthesis (Ueno and Sasaki, 1978; Kato et al., 1983; Sekimoto and Fujii, 1992). Some photo-sensing molecules and a regulatory mechanism should be involved in the regulation of the progression of sexual reproduction (Sekimoto and Fujii, 1992). The products of two genes showed high levels of sequence similarity to the glutathione S-transferase of *Nostoc punctiforme* (ID: 502) and an unknown protein of *Arabidopsis thaliana* (819). The remaining 12 genes did not show any significant similarities to known proteins.

**PR-IP-responsive Genes Expressed Specifically in Mt⁺ Cells**

In subgroup C-E, 11 gene clusters were found. Among these, we identified cDNA clone 136, the product of which showed high-level similarity to leucine-rich repeats (LRR)-containing transmembrane protein kinase. Several leucine-rich repeats and a transmembrane domain were found at the 5’-end and 3’-end, respectively, while conserved regions for the kinase domain could not be found at the 3’-end (data not shown). Therefore, this cDNA clone was named *CpRLP1* (receptor-like protein-1). The primary function of the LRR motifs is to provide a versatile structural framework for the formation of protein-protein interactions (Kobe and Kajava, 2001). Since *CpRLP1* was expressed specifically in mt⁺ cells, and its expression was stimulated by the addition of PR-IP, the primary function of the *CpRLP1* protein appears to be related to the acquisition of PR-IP. *In vivo* binding assays have demonstrated the presence of a receptor for the 19-kDa subunit of PR-IP and its appearance...
during sexual differentiation (Sekimoto et al., 1993). However, molecular biological information for this molecule is lacking. As in the case of the CLV2 protein of Arabidopsis thaliana (Jeong et al., 1999), the CpRLP1 protein may form a heterodimer with another protein, such as a receptor-like protein kinase, to transduce the extracellular signal of PR-IP to the intracellular compartment.

Two genes (ID: 609 and 1283) of subgroup C-E encoded homologues of luminal-binding proteins (BiP) and were named CpBiP1 and CpBiP2, respectively. BiP is a member of the Hsp70 family and acts as a molecular chaperone that aids in the folding and assembly of proteins routed through the endoplasmic reticulum (ER) (Denecke et al., 1991). Although BiP is present constitutively during normal growth, rapid induction occurs under conditions that cause the accumulation of abnormal proteins in the lumen of the ER, and BiP accomplishes its function in the lumen (Wrobel et al., 1997). During sexual reproduction, cells have to change from the vegetative to the sexual state, with consequent de novo protein synthesis and protein degradation. The CpBiP proteins may promote protein folding and assembly and may dissolve protein aggregates that are formed during these processes.

Among the other eight genes of subgroup C-E, one gene (ID: 285) did not show any sequence homology to known proteins. At present, the roles of these genes in sexual interaction remain unclear.

**Genes Expressed in Response to Both Pheromones**

Two genes were up-regulated not only during sexual reproduction but also in response to both pheromones (subgroup C-D-E). One of these genes (ID: 1484) showed weak sequence similarity to the hypothetical protein of Dictyostelium discoideum, while the other (ID: 1456) showed a high level of similarity to putative glyoxalase II (AtGLX2-3; U74610). The latter gene was named CpGLX2-1. Glyoxalase II is part of the glutathione-dependent glyoxalase detoxification system and is thought to be involved in cell proliferation. It belongs to the
metallo-β-lactamase superfamily (Crowder et al., 1997; Maiti et al., 1997; Zang et al., 2001). A β-lactamase-like domain and a metal-binding domain (T-H-X-H-X-D-H) were conserved in the deduced CpGLX2-1 protein. In Arabidopsis thaliana, the presence of at least four glyoxalase II isozymes has been reported (Maiti et al., 1997). AtGLX2-3 has been suggested as encoding a mitochondrial isozyme, although biochemical and physiological characterizations have not been reported. Analysis of the deduced CpGLX2-1 protein using PSORT (Nakai and Kanehisa, 1992) predicted that, unlike the AtGLX2-3 protein, CpGLX2-1 localizes to microbodies (accuracy rate of 0.64) or to the cytoplasm (accuracy rate of 0.45).

Real-time PCR Analysis Clustering of Sexual Reproduction-related Genes with Respect to Expression Patterns

To confirm the expression patterns obtained in our microarray analyses and to check the reliability of the data, quantitative real-time PCR was performed using TaqMan probes for eight representative genes (Supplemental Table IV). The expression levels of six conjugation-related genes [CpRLP1, CpRLK1, the gene encoding the 19-kDa subunit of PR-IP (Cp19KSU), CpGLX2-1, and genes showing no homology (tentatively named Cp-01 and Cp-48)] were elevated during sexual reproduction (Fig. 2). The expression levels of CpRLK1, Cp19KSU, and Cp-01 in mt+ cells were stimulated by the addition of PR-IP Inducer, whereas those of CpRLP1 and Cp-48 in mt− cells were also stimulated by the addition of PR-IP (Fig. 3A, B). In the case of CpGLX2-1, the stimulatory effects of both sex pheromones in opponent cells were also confirmed (Fig. 3C). In the case of two sex-specific genes (Cp-41 and the PR-IP-inducer gene CpPI), the expression of Cp-41 was restricted in mt+ cells, whereas the accumulation of CpPI mRNA was limited in mt− cells, especially at low cell density in nitrogen-depleted medium (Fig. 4). These results are concordant with the expression patterns obtained from the microarray analyses, and indicate that the microarray data presented here are highly reliable.
CONCLUSIONS AND PERSPECTIVES

In this study, we generated an additional 2,046 ESTs. The resulting 1,615 nonredundant ESTs, which include the 760 previously characterized nonredundant clones, are available for downstream experiments and in silico analyses. Subsequently, the cDNA microarrays, on which 3,064 ESTs were spotted, were constructed to reveal comprehensive gene expression profiles during the sexual reproduction process (Supplemental Table II). According to the expression patterns, the genes spotted on the glasses could be classified into several groups. Thirty-eight mating-type-specific genes and 50 conjugation-related and/or pheromone-responsive genes were discovered, none of which have previously been deposited in the public databases, with the exception of the previously reported sex pheromone genes, which encode two PR-IP subunits and the PR-IP inducer. In addition, although many of the deduced amino acid sequences of the ESTs on the array have no similarity to known proteins, a number of interesting ESTs, such as those related to intercellular communication, receptor-like protein kinase (CpRLK1), and leucine-rich-repeat-containing receptor-like protein (CpRLP1), were unearthed by data mining. In Closterium, intercellular communication between two mating types via pheromonal substances is essential for successful conjugation (Tsuchikane et al., 2003; Tsuchikane et al., 2005). To date, direct information on the relationships between sex pheromones and these molecules has been lacking. Cloning of the full-length cDNAs for these receptor candidates and testing of the binding activities between the extracellular domains and sex pheromones will shed light on the molecular mechanisms of intercellular communication during the sexual reproduction of Closterium.

In higher plants, the fertilization process occurs in the ovules, and thus it is not easy to analyze the cellular responses of gametes in vivo. In addition, it is difficult to isolate sufficient sperm and eggs from the plants for in vitro molecular investigations of fertilization. However, it is relatively easy to induce sexual reproduction in C. pslc, and some of the sexually related
genes in *C. pslc* showed similarities to previously deposited genes from land plants, including hypothetical genes and/or genes whose functions were unknown (Supplemental Table III). It would be important to investigate whether such homologous genes are also up-regulated in sexual cells. If any show a promising expression pattern, phenotypic analyses by reverse genetics should be applied. Because charophycean green algae are the closest relatives to land plants, the expression profiles generated from this array, and some genes detected by these analyses, should help reveal the molecular mechanisms of gametogenesis and intercellular communication during fertilization in land plants.

The *Closterium* microarray resource is a potentially useful and beneficial tool for the exploration of genes that are regulated in response to environmental changes, as well as sex-related phenomena. We can easily monitor gene expression changes caused by environmental modifications without influences from other tissues and organs, since *Closterium* is a unicellular plant. In addition, *Closterium* has an experimental advantage that results from its evolutionary position on “the tree of life.” This unicellular plant is most closely related to land plants, and its cellular features and metabolism are more similar to those of land plants than those of the “green yeast” *Chlamydomonas* (Wilcox and Graham, 2000). Recently, we isolated the MADS-box gene (*CpMADS1*) from *Closterium pslc* and implicated it in sexual differentiation (Tanabe et al., 2005). Loss-of-function experiments and this microarray technique are indispensable for studies concerned with unveiling the impact on critical phenomena in plants of the genetic network that is under the regulation of the MADS-box protein and other key regulators. The phenotypic profiles prospectively obtained from transformation experiments with *Closterium* cells, as well as the transcriptome resources, will provide insights into intercellular communication and the evolution of land plants.

**MATERIALS AND METHODS**
Plant Materials, RNA Isolation, and Construction of cDNA Libraries

The strains of heterothallic *Closterium peracerosum-strigosum-littorale* complex (*C. pslc*) were NIES-67 (mt+) and NIES-68 (mt’), which were obtained from the National Institute for Environmental Studies, Ibaraki, Japan. The respective vegetative cells (mt+_V and mt’_V) were obtained from cultures grown in nitrogen-supplemented medium (C medium; http://www.nies.go.jp/biology/mcc/home.htm), as previously described (Sekimoto et al., 1990).

The sexual reproduction of *C. pslc* was induced as follows. Vegetatively growing cells of the two mating types were harvested, washed three times with nitrogen-depleted medium (MI medium; Ichimura, 1971), and incubated separately in MI medium (3.0×10^5 cells/mL) under continuous light for 24 h (high-density pre-culture; mt+ _0, mt’ _0). The cells of both mating types (5.4×10^5 each) were mixed in 75 mL fresh MI medium (7.2×10^3 cells/mL) in 300-mL Erlenmeyer flasks, and incubated under light (low cell density culture). At various time intervals (2, 8, 24, and 72 h), the cells were harvested, frozen in liquid nitrogen, and stored at −80°C (mix_2 h, mix_8 h, mix_24 h, and mix_72 h, respectively). As controls, cells of the respective mating types were incubated in MI medium at a low cell density (mt+_L and mt’_L; 7.2×10^3 cells/mL of each) for 8 h without mixing, then harvested and stored. In addition, mt’ cells and mt+ cells that had been incubated for 8 h in MI medium (7.2×10^3 cells/mL) that contained PR-IP and PR-IP Inducer at final concentrations of 4.8×10^{-9} M and 3.0×10^{-8} M, respectively, were harvested and stored (listed in the Tables as PR-IP and Inducer).

Poly(A)^+ RNA was isolated using the µMACS mRNA Isolation Kit (Miltenyi Biotec, Bergisch, Gladbach, Germany) or the PolyATtract mRNA Isolation System (Promega, Madison, WI, USA) following treatment with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), in accordance with supplier instructions.

The cDNA library was prepared from poly(A)^+ RNA that was isolated from a mixture
of vegetative cells (mt+V and mt_V) and cells at various stages of sexual reproduction (mt+_0, mt-_0, mix_2 h, mix_8 h, mix_24 h, and mix_72 h) using a cDNA library synthesis kit (Superscript Plasmid System; Invitrogen), according to supplier instructions. This primary cDNA library and two previously prepared cDNA libraries (Sekimoto et al., 2003) derived from cells in the various stages of sexual reproduction were mixed. Normalization was performed as previously described (Sekimoto et al., 2003), and used to derive EST sequence information.

Sequence Analysis

The EST sequences were determined using the multi-capillary automated DNA sequencers RISA-384 (Shimadzu Corporation, Kyoto, Japan) and CEQ 2000XL (Beckman Coulter, Fullerton, CA, USA). Both vector-derived and ambiguous sequences were removed from the collected EST sequences by computer-aided analyses. Each sequence was then subjected to similarity searching against the NCBI nonredundant protein database (nr) using the BLASTX algorithm. Sequence similarities were considered significant when the expected value was <1.0e–5 at the amino acid sequence level. EST redundancy was checked using an alignment program (BLAST) with a dataset of itself (Altschul et al., 1990). Clones that showed >98% identity for stretches of more than 100 bp were grouped together. The identified sequences have been deposited in the DNA Database in Japan (accession numbers BW646625 to BW648670).

Preparation and Analysis of the cDNA Microarray

The inserts of the cDNA clones were amplified by polymerase chain reaction (PCR) using T7 and SP6 universal primers that were complementary to the vector sequences flanking both sides of the cDNA insert. The PCR products were purified using 96-well MultiScreen PCR plates (Millipore, Bedford, MA, USA). Each purified cDNA insert was
mixed with reagent D (Amersham Biosciences, Piscataway, NJ, USA), and each cDNA clone was spotted in duplicate on aluminum-coated and DMSO-optimized glass slides using the Array Spotter Generation III (Amersham Biosciences). Labeling, hybridization, and scanning were performed as previously described (Endo et al., 2002). The fluorescence intensity for each spot was captured and quantified using MICROARRAY SUITE (Scanalitics, Fairfax, VA, USA). Because each cDNA clone was spotted in duplicate (right- and left-hand sides), normalization was performed against the respective sides of the same glass slide, as described below. The 50th percentiles of all values obtained from the respective sides were used as the synthetic positive controls for each cDNA, and the value for each cDNA was divided by these synthetic positive controls. These results were then filtered to eliminate those cDNAs with ratios between the right and left position of <1/3 or >3, and those cDNAs with values before normalization of <50 in all hybridizations. The average value of the duplicated spots was used as the normalized value for each cDNA for each hybridization. After normalization, cDNAs with a greater than fivefold increase in expression during conjugation, as compared with mt+ or mt- cells cultured in MI medium at low cell density without mixing (Mt+_L or Mt-_L), were chosen. We also selected cDNAs that showed a fourfold difference in expression between the mt+ and mt- cells or a fourfold increase in expression following the addition of purified sex pheromones (PR-IP and PR-IP Inducer). The reproducibility of the expression analysis results was confirmed in two biologically independent experiments.

**Real-time PCR**

Real-time PCR analyses were performed with the ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions. Primers and probes for the target sequences were designed using the Primer Express software (Applied Biosystems). The cDNA was synthesized from total RNA using the TaKaRa RNA LA PCR Kit (AMV) ver. 1.1 (Takara Bio, Ohtsu, Shiga, Japan) and random primers, according to the
manufacturer’s instructions. The primers and probes for each gene are shown in Supplemental Table IV. PCR was performed for 43 cycles at 95°C for 15 s and at 60°C for 1 min. The signals were detected in the ABI Prism 7000 as fluorescent emissions generated by the dissociation of fluorescent chemicals from the TaqMan probes. The transcript levels were determined by the slope of the curve generated by amplification of serially diluted plasmids that carried the respective genes. For the cycle in which all the signals were amplified exponentially, the signals were converted into numeric values of 18S rRNA using the pre-developed TaqMan assay reagent for eukaryotic 18S rRNA (Applied Biosystems) to normalize all signals.

Statistical analysis was performed according to standard procedures, as indicated. The reproducibility of the expression analysis results was confirmed in two independent experiments, and standard errors are shown.

ACKNOWLEDGMENTS

The authors wish to thank Ms. Ayumi Ihara and Ms. Sayaka Mikami for technical assistance.

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FIGURE LEGENDS

Figure 1. Overlapping of conjugation-related and pheromone-responsive genes. The numbers of genes with elevated levels of expression during conjugation (C), after treatment with PR-IP Inducer (D), or after treatment with PR-IP (E), are indicated.

Figure 2. Expression patterns of six representative conjugation-related genes from Closterium peracerosum-strigosum-littorale complex, as analyzed by real-time PCR. Complementary DNAs (cDNAs) were prepared from mating-induced cultures at the indicated times (2, 4, 8, 24, and 72 h). cDNAs were also prepared from the respective mating types just before mating induction (mt+₀h, mt-_₀h). The relative real-time PCR values are shown as the means from
two independent experiments, with normalization to the expression of 18S rRNA. The expression level of each gene from an individual cDNA is indicated as the relative abundance; the maximum value for the expression of each gene during conjugation is designated as 100.

**Figure 3.** Effects of sex pheromones on the expression of six representative conjugation-related genes of *Closterium*. The cDNAs were prepared from cultures that were treated with sex pheromones at the indicated final concentrations. The relative real-time PCR values are shown as the means from two independent experiments, with normalization to the expression of 18S rRNA. The expression level of each gene from an individual cDNA is indicated as the relative abundance; the mean signal values detected for mt⁺ or mt⁻ cells incubated in sex pheromone-free medium are assigned the value of 1.0. (A) Effect of PR-IP Inducer on the expression of three conjugation-related genes (*CpRLK1*, *Cp19KSU*, and *Cp-01*) in mt⁺ cells. (B) Effect of PR-IP on the expression of two conjugation-related genes (*CpRLP1* and *Cp-48*) in mt⁻ cells. (C) Effects of sex pheromones on the expression of the *CpGLX2-1* gene. The left panel indicates the effect of PR-IP Inducer on mt⁺ cells. The right panel indicates the effect of PR-IP on mt⁻ cells.

**Figure 4.** Sex-specific expression of the *Cp-4I* and *CpPI* genes under various culture conditions. The cDNAs (mt⁺_V, mt⁻_V, mt⁺_0, mt⁻_0, mt⁺_L, and mt⁻_L) were prepared from the respective cultures noted in the Figure. The various culture conditions are described in the Materials and Methods section. The relative real-time PCR values are shown as the means from two independent experiments, with normalization to the expression of 18S rRNA. The expression level of each gene from an individual cDNA is indicated as the relative abundance; the maximum value for the expression of each gene among cultures is designated as 100.
Table I. Representative conjugation-related, sex pheromone-responsive, or sex-specific genes identified by cDNA microarray analysis

<table>
<thead>
<tr>
<th>Cluster ID</th>
<th>Description</th>
<th>Gene Name</th>
<th>mix / mt+_Lb</th>
<th>mix / mt-_Lb</th>
<th>Inducer / mt+_Lb</th>
<th>PR-IP/ mt+_Lb</th>
<th>mt+_Lb / mt-_Vb</th>
<th>mt-_Lb / mt-_Vb</th>
<th>group</th>
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<tr>
<td>76</td>
<td>19-kDa subunit of PR-IP</td>
<td>Cp19KSU</td>
<td>5.46</td>
<td>10.63</td>
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<td>97</td>
<td>aquaporin-like protein</td>
<td>CpAQP1</td>
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<td>0.56</td>
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<td>6.19</td>
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<td>1,456</td>
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<td>2.14</td>
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<td>0.58</td>
<td>0.39</td>
<td>6.85</td>
<td>3.35</td>
<td>3.35</td>
</tr>
</tbody>
</table>

1IDs for 1,615 nonredundant sequences after clustering.
2Expression ratios of cells in mating to mt' cells, at low cell density in nitrogen-depleted medium.
3Expression ratios of cells in mating to mt' cells, at low cell density in nitrogen-depleted medium.
4Incubation time for induction of mating (conjugation).
5Expression ratios of mt' cells to mt' cells, at low cell density in nitrogen-depleted medium.
6Expression ratios of PR-IP Inducer-treated mt' cells to pheromone-free mt' cells, at low cell density in nitrogen-depleted medium.
7Expression ratios of PR-IP-treated mt' cells to pheromone-free mt' cells, at low cell density in nitrogen-depleted medium.
8Expression ratios of mt' cells incubated in nitrogen-depleted medium to vegetative mt' cells.
9Groups classified in respect to similar expression patterns during conjugation, sex-specificity, and responses to sex pheromone treatment.
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