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Genes Specifically Expressed in Sexually Differentiated Female Spheroids of *Volvox carteri*

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***Volvox carteri* is a multicellular green alga with only two cell types, somatic cells and reproductive cells. Phylogenetic analysis suggests that this organism has evolved from a *Chlamydomonas*-like unicellular ancestor along with multicellularity, cellular differentiation, and a change in the mode of sexual reproduction from isogamy to oogamy. To examine the mechanism of sexual differentiation and the evolution of oogamy, we isolated 6 different cDNA sequences specifically expressed in sexually differentiated female spheroids. The genes for the cDNAs were designated *SEF*1 to *SEF*6. The time course of accumulation of each mRNA was shown to be distinct. The expression of some of these genes was not significantly affected when the sexual inducer was removed after the induction of sexual development. Sequence analysis indicates that *SEF*5 and *SEF*6 encode pherophorin-related proteins. Of these, SEF5 has the unique structural feature of a polyproline stretch in the C-terminal domain in addition to the one found in the central region.**

Key words: extracellular matrix, oogamy, pherophorin, sexual differentiation, *Volvox*.

Abbreviations: ECM, extracellular matrix; RACE, rapid amplification of cDNA ends; RT, reverse transcription.

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Volvox carteri is a multicellular organism with only two cell types, somatic cells and reproductive cells. Asexual spheroids of both male and female strains contain about 2,000–4,000 small biflagellate somatic cells that are morphologically similar to *Chlamydomonas* and about 16 larger immotile reproductive cells called gonidia that lie below the somatic cell sheet. Volvocaceae species are considered to have evolved from *Chlamydomonas*-like unicellular organism(s) along with multicellularity, cellular differentiation, and a change in sexual reproductive mode from isogamy to oogamy (*1*, *2*).

While *V. carteri* reproduces asexually in its usual life cycle, it reproduces sexually in the presence of a sex-inducing pheromone (inducer), that is a 32-kDa glycoprotein (Fig. 1) (*3–6*). The sexual reproduction of *V. carteri* is quite different from that of *Chlamydomonas*. First, *V. carteri* is oogamous; the sexually differentiated female and male spheroids contain immotile eggs and swimming sperm packets, respectively. On the other hand, *C. reinhardtii* is isogamous and its gametes of opposite mating type (mt^+ and mt^-) are morphologically indistinguishable. Second, vegetative cells of *C. reinhardtii* can differentiate directly into gametes under nitrogen starvation and blue-light irradiation (*7–12*). In contrast, the reproductive cells of *V. carteri* do not differentiate directly into eggs or sperm when exposed to the sexual inducer; sexually induced spheroids modify the developmental programs of the gonidia resulting in the production of sexual progeny with eggs or sperm packets in the next generation. Third, the egg cells reside inside the somatic cell sheet and extracellular matrix (ECM) in *V. carteri* so that sperm packets first come into contact with somatic cells or ECM and not with the egg cells. In contrast, the gametes of *C. reinhardtii* directly recognize the gametes of opposite mating types using agglutinins on their flagella (*13–15*). These differences between closely related organisms are beneficial in studying the evolution of the mode of sexual reproduction.

The sexual development of *V. carteri* has been investigated by light- and electron-microscopy (*3*, *16*, *17*), and some studies on the early responses of sexual spheroids to the sexual inducer have been conducted (*18–21*). However, little is known about the underlying molecular events. To investigate differences in gene expression between asexual and sexual spheroids, we attempted to isolate genes that are up-regulated in sexually differentiated spheroids by using suppression subtractive hybridization (SSH) (*22*). From a subtracted cDNA library, we identified 6 genes that are expressed in sexual female spheroids, but are absent or barely detectable in vegetatively growing spheroids. Sequence analyses revealed that two of these genes encode pherophorin-related proteins. Pherophorins are defined as having an N-terminal domain, central polyproline stretch, and C-terminal domain with homology to a sex-inducing pheromone (*23*, *24*); they are ECM proteins that form meshwork structures constituting ECM. Thus, the pherophorin-related proteins that we identified might be involved in the remodeling of ECM during the sexual differentiation of *V. carteri*.

MATERIALS AND METHODS

Volvox *Strain and Culture Conditions*—Strain EVE isolated from *Volvox carteri* f. *nagariensis* Iyengar, strain

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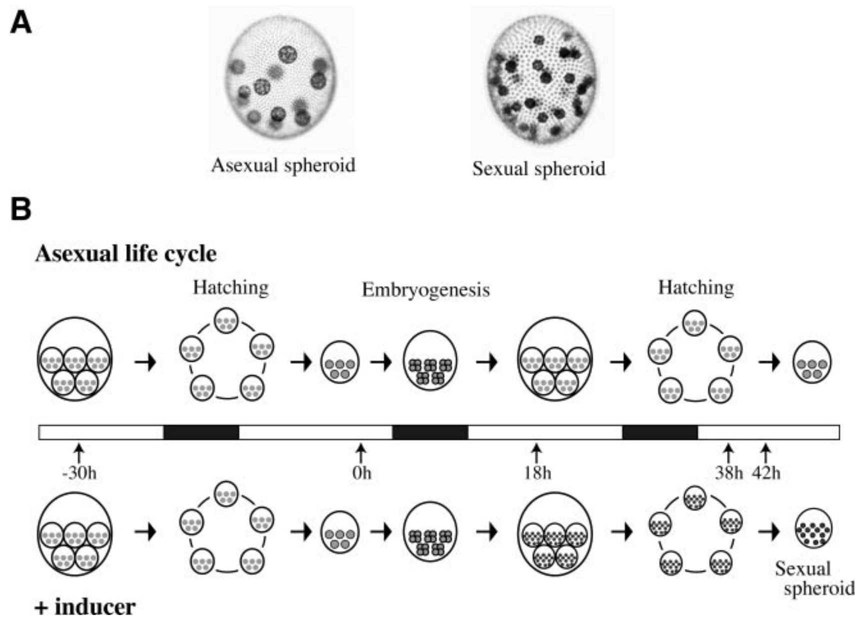

Fig. 1. **The asexual and sexual life cycles of *V. carteri*.** (A) A mature asexual spheroid (left) and a mature sexual female spheroid (right). Eggs in sexual spheroids are larger in number, and have a denser appearance. (B) The life cycle is synchronized by a 16 h light (open boxes)/8 h dark (filled boxes) cycle. In the asexual life cycle, gonidia start cell-cleavage to produce the next generation every 48 h. The time point when cell-cleavage is initiated is defined as 0 h. The sexual inducer was added 30 h before the initiation of cell-cleavage. The developmental states of the asexual and sexual life cycles are illustrated above and below, respectively.

UTEX HK10 (female), was obtained from D.L. Kirk (*25*). Synchronous cultures were maintained in Standard *Volvox* Medium (SVM) at 30°C under a 16 h light–8 h dark (10,000 lux) cycle. The SVM used in this study was modified from that described in Kirk and Kirk (*26*) to include 1.68 mM 2-[*4*-(2-hydroxyethyl)-*1*-piperazinyl]ethanesufonic acid (HEPES) in place of glycylglycine.

Induction of Sexual Development in V. carteri—An asexually growing culture was subjected to heat-shock to induce the production of sexual pheromone and then filtrated. The time and duration of heat-shock were as described (*27*). The filtrate was used to induce the sexual development of spheroids in another culture 30 h before the initiation of cell cleavage of the gonidia (*27*).

Construction of a Subtracted cDNA Library and Screening by Differential Hybridization—Total RNA was isolated from both sexually differentiated and asexually growing female spheroids as described by Kirk and Kirk (*28*). Poly(A)$^+$ RNA was purified using Oligotex-dT30 beads (Takara). SSH was performed on the cDNA of sexually differentiated female spheroids and the cDNA of asexually growing female spheroids using a PCR-select cDNA subtraction kit (Clontech) according to the manufacturer's instructions. The PCR product was digested with *Eag*I, purified with a QIAquick PCR Purification kit (QIAGEN), ligated into pBluescript II SK$^+$, and used to transform *Escherichia coli* JM109. The cDNA inserts from a total of 648 recombinant clones were amplified by PCR using ExTaq DNA polymerase (Takara) with oligonucleotide primers (NA1, 5′-GGA TCC ACT AGT TCT AGA GCG G, and NA2, 5′-TTG GAG CTC CAC CGC GGT GGC G). The PCR products were denatured in 0.3 N NaOH, dot-blotted onto HybondN$^+$ membranes (Amersham Pharmacia Biotech), and then hybridized with forward or reverse subtracted cDNA probes. Hybridization signals were detected with the Gene Images AlkPhos Direct Labeling System (Amersham Pharmacia Biotech).

Semi-Quantitative RT-PCR—Total RNA was isolated from both induced and vegetative spheroids at 6 h intervals. Reverse transcription (RT) was performed using ReverTra Ace (Toyobo). The PCR cycle was 94°C for 30 s, 58°C for 30 s and 72°C for 30 s. The number of the PCR cycles was determined for each case so that the amount of the PCR product corresponded to the amount of template in the linear amplification process. The numbers of reaction cycles were as follows: 24 cycles for *SEF*5, 28 cycles for *SEF*1-3 and 30 cycles for *SEF*4 and *SEF*6. Reaction products were electrophoresed in agarose gels. Band intensities were quantitated using a Fluor-S MultiImager (Bio-Rad) after staining with ethidium bromide.

Oligonucleotides—Oligonucleotides for semi-quantitative RT-PCR were SE11: 5′-CGT CTA AAG GCC ATC AAT GCT ATC, SE12: 5′-ACC TCC TCC GTT GCC ATC ATC ATG, SE21: 5′-CCG ACC CGT TCG GCC ATG AAG, SE22: 5′-GTA CCC TAC AAA ACA CCG TCT CG, SE31: 5′-GTA CTG GAG AGT GTT GTG TTG CTC, SE32: 5′-TGT CGC TGC TCG CTT GGG ATC GAC, SE41: 5′-GCT GCG TTC CGT TAG TTG CCG CCG, SE42: 5′-CTA CCC GTG TCA GAC GTG GAA TCG, SE51: 5′-CTG ATT GGT GCC TGC CAG CAG AAC, SE52: 5′-CTC TCT TGT GGA CAT CAT AGA TTG, SE61: 5′-TAC GTG TGT TCT CCT ACA GTC ACC, SE62: 5′-GCC GCC TTC GTT TAA GGG ACC GTG, S18u: 5′-ATG GGC TCT CTG GTC CAC GGC, and S18r, 5′-CGG ATC TTC TTC AGG CGC TCC. Oligonucleotides for RACE were SE53: 5′-GGA GTA TGA CTA TGA TCC TTC AC, SE54: 5′-CGG TAT GGA GGT TTG TGG CCA ATC and SE55, 5′-CAG GCA ACT GCT AGA TAC GTT GAC.

Separation of Somatic Cells and Egg Cells—Each cell type was separated as described (*19*). The egg cells were separated from the somatic cells by filtration through a 5-μm mesh nylon screen attached to a Magnetic Filter Funnel (Gelman Sciences).

Removal of Sexual Inducer—A culture containing the sexual inducer was filtrated 18 h after the initiation of embryogenesis. The spheroids were washed 5 times with

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fresh SVM and transferred to fresh SVM or SVM containing concanavalin A (2.5 μg/ml, Sigma).

Cloning and Sequence Analysis of cDNA—Rapid amplification of cDNA ends (RACE) was performed with a FirstChoice™ RLM-RACE kit (Ambion) and 5′ RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen), to obtain 5′- and 3′-regions of cDNA. Reverse transcription was performed with *Bca* PLUS RTase (Takara). Total RNA from somatic cells of sexually differentiated spheroids was used as a template. The cDNA was cloned into pBluescript II SK$^+$. Nucleotide sequences were determined with the *Bca*BEST dideoxy sequencing kit (Takara) or the Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) using an automated DNA sequencer (ALF express II, Amersham Pharmacia Biotech). Clones from at least two independent reactions were analyzed to determine the nucleotide sequences. The N-terminal signal sequence was predicted according to Nielsen *et al*. (*29*). CLUSTALW was used for the alignment of the amino acid sequences of pherophorin proteins (*30*).

Nucleotide Sequence Accession Numbers—The nucleotide sequence of the cDNA from *SEF*1, *SEF*2, *SEF*3, *SEF*4, *SEF*5 and *SEF*6 are available in the GenBank database under accession numbers AB104489, AB104490, AB104491, AB104492, AB104494 and AB104493, respectively.

RESULTS

Construction of a Subtracted cDNA Library and Screening of Differentially Expressed cDNAs—Since eggs of *V. carteri* are fertile by the time the sexual progenies hatch, the poly(A)$^+$ RNA used for constructing a cDNA library was prepared from young sexual spheroids that had just hatched out of their parental spheroids. This time point corresponds to 38 h after the initiation of sexual embryogenesis (see Fig. 1B). From the subtracted cDNA library enriched with cDNAs that are differentially expressed in the sexual spheroids, 648 clones were randomly chosen and their inserts were subjected to differential screening by dot-hybridization. Out of 648 clones, 103 showed hybridization signals only with forward subtracted cDNA probe. Most of the candidate clones were found to be redundant by sequence analysis, so the candidate clones were further analyzed by reciprocal hybridization; those clones that hybridized to the already sequenced clones were not subjected to further analyses. RT-PCR analysis with a primer set specific to each group showed that 11 different groups of clones were specifically expressed in sexual spheroids. Since reciprocal RT-PCR analysis showed that certain clones were derived from the transcript of the same gene, the groups were re-classified into 6 groups, with the genes encoding them named *SEF*1 to *SEF*6 for sexual female-specific expression.

Sexual Spheroid-Specific Expression of the SEF Genes—The expression patterns of the 6 genes were investigated by semi-quantitative RT-PCR by using the total RNA from sexual and asexual spheroids. The mRNA of ribosomal protein S18 was used as an internal standard as described before (*31–33*). Figure 2 shows that the *SEF* genes are expressed at significantly higher levels in sexually-induced spheroids than in asexual ones. Apparently, transcripts of some of the *SEF* genes accumulate in two phases. Transcripts of *SEF*1, *SEF*2, *SEF*4 and *SEF*5 accumulate with the first peak at 12–18 h, and after transient reduction at 24 h, a second increase follows. In contrast, *SEF*3 is not expressed in the early phase; it accumulates after 30 h when the daughter spheroids mature. The expression pattern of *SEF*6 is quite different from the rest of the *SEF* genes. It is highly expressed even at the initiation of the embryogenesis of sexual spheroids (see the 0 h time point in Fig. 2). This might correspond to the rapid induction of *SEF*6 in response to the sexual inducer as shown later. In addition, the expression profile of *SEF*6 seems to follow periodical cycles during the development of sexual spheroids (Fig. 2).

Cell type-Specificity of the Expression of SEF Genes—To identify the cell types in which the *SEF* genes are expressed, we separated somatic cells and eggs in sexual spheroids 42 h after the initiation of embryogenesis. By this time, all sexual spheroids had hatched (Fig. 1B). The expression of each gene was examined by RT-PCR using the total RNA isolated from somatic cells and eggs as templates (Fig. 3). *SEF*1, *SEF*2 and *SEF*3 were found to be expressed mainly in egg cells, while considerable levels of the *SEF*1 transcript were detected in somatic cells. In contrast, *SEF*5 and *SEF*6 were mainly expressed in somatic cells. *SEF*4 was expressed in both somatic cells and eggs. (Note: somatic cells used in this analysis were derived from parental spheroids and sexual progenies. It was not possible to separate them according to their size difference.)

Effect of the Removal of the Sexual Inducer on the Expression of SEF Genes during Maturation of Sexual Spheroids—Parental spheroids must be exposed continuously to the sexual inducer for an appropriate period of time prior to the embryogenesis of daughter spheroids to initiate sexual development. Once cell-cleavage of the gonidia in the parental spheroid has started, the resulting progenies become morphologically sexual. However, it takes about a day until they can come into contact with the sperm packets because the sexual progenies stay inside the parental spheroids. The sexual inducer was removed during the maturation stage of the sexual spheroids to see whether its presence is required for the expression of *SEF* genes after the completion of morphogenesis in the parental spheroids. The spheroids were washed with inducer-free medium 18 h after the initiation of sexual embryogenesis when daughter spheroids had been given sufficient time to develop (the 18 h time point in Fig. 1B). The washed spheroids were cultured in inducer-free medium or medium containing concanavalin A (Con A), a known inhibitor of the inducer (*27*, *34*). Total RNA was isolated 24 h after the removal of the inducer (the 42 h time point in Fig. 1B). As shown in Fig. 4, the amounts of *SEF*3, *SEF*4, *SEF*5 and *SEF*6 transcripts in the spheroids grown in the inducer-free medium are comparable to those in spheroids grown in the presence of the inducer. In addition, *SEF*1 and *SEF*2 mRNAs were present at significant levels in the inducer-free medium. Similar results were obtained for spheroids cultured in inducer-free medium containing Con A (Fig. 4). These results show that the inducer is not required to maintain the expression of these *SEF* genes after the daughter spheroids have developed, but it does help some of the genes achieve maximal levels of expression.

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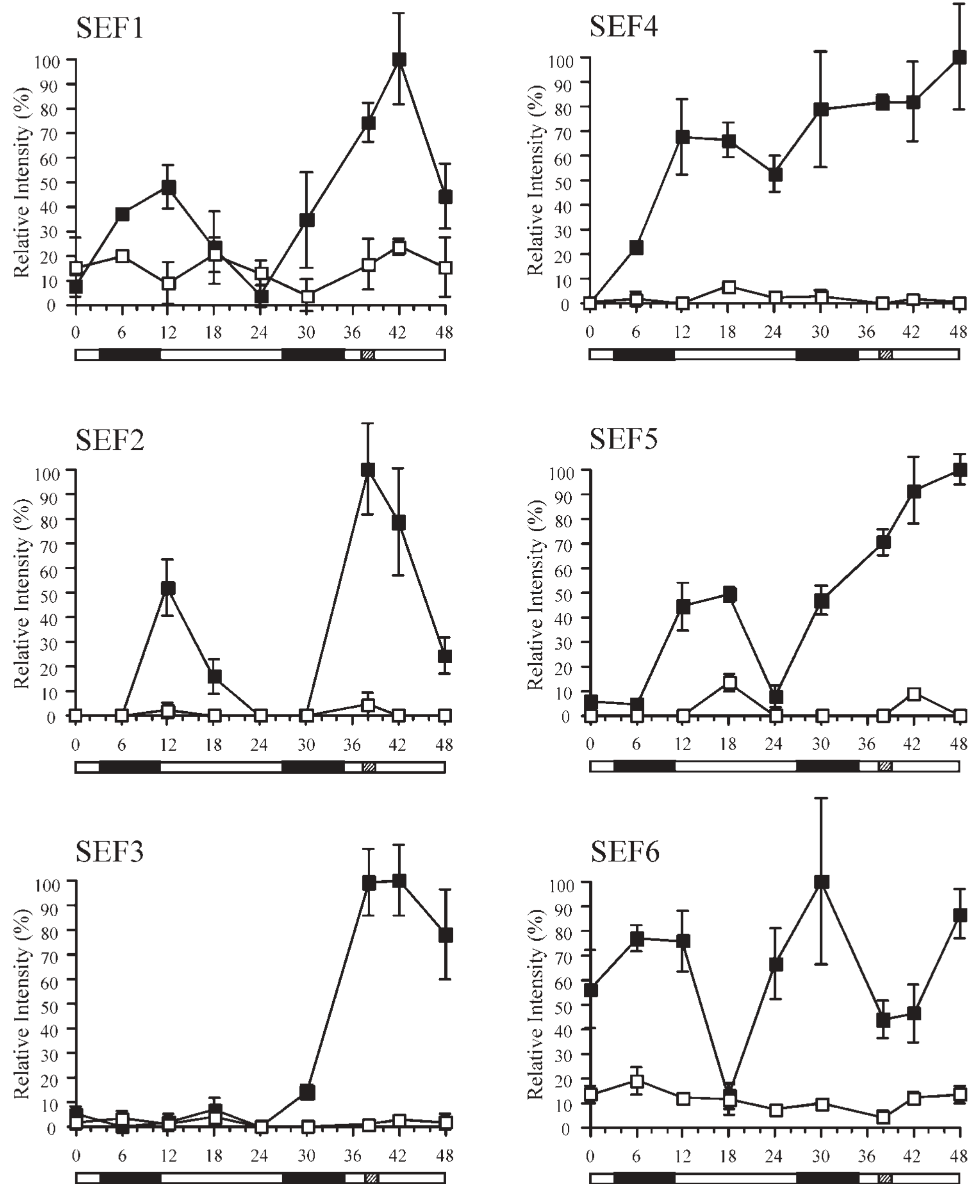

Fig. 2. **Time course of *SEF* gene expression determined by semi-quantitative RT-PCR.** The cDNA for each *SEF* gene was amplified by RT-PCR, electrophoresed in an agarose gel, and the band intensities were quantitated. Values were normalized using the amplification product of ribosomal protein S18 as an internal standard. Intensities relative to the maximally expressed level for each cDNA are plotted (filled squares: sexually induced spheroids; open squares: vegetative spheroids). Values are mean ± SD of four reactions. The time point of the initiation of cell-cleavage of sexual spheroids is shown as 0 h. Open and filled areas in the box below each panel indicate the light and dark periods, respectively. Shaded areas in the boxes indicate the time points when sexual spheroids hatched.

SEF*5 and* SEF*6 Encode Pherophorin-Related Proteins*—Sequence analysis of the *SEF* cDNA fragments initially isolated from the subtracted cDNA library indicated that *SEF*5 encodes a protein of the pherophorin family. While performing RACE to obtain full-length cDNA for *SEF*5, we identified 3 different clones whose polyadenylation sites were differed slightly from one another (Fig. 5). The longest cDNA was 3,200 bp in length. This cDNA sequence contains 3 putative polyadenylation signals specific to *V. carteri*, UGUAA (*35*, *36*), near the poly(A) tail. The deduced SEF5 protein precursor comprising 606 amino acids with a putative signal peptide of 24 amino acids possesses a polyproline stretch in the pheromone-like domain at the C-terminus in addition to the polyproline stretch in the central region (Figs. 5 and 6). The amino acid sequence of the N-terminal domain of SEF5 without the signal peptide resembles those of pherophorin III (61% identity) and pherophorin II (61% identity). The amino acid sequence of the C-terminal domain also resembles those of pherophorin II (45% identity) and pherophorin III (45% identity). These results indicate that *SEF*5 encodes a new member of the pherophorin family with a unique polyproline stretch in the C-terminal domain (Fig. 6).

In addition to *SEF*5, the *SEF*6 cDNA fragment isolated by 3′-RACE analysis showed similarity to a pherophorin. However, the 5′-terminal cDNA fragment for this gene could not be obtained after several attempts of 5′-RACE. This is probably because it contains a long GC-rich polyproline region. This situation was previously described for aother pherophorin family protein, pherophorin-S, and DZ-HRGP in *V. carteri* (*20*, *37*). The deduced amino acid sequence of the C-terminal region of *SEF*6 is different from those of any known pherophorin, but it shows similarity to the C-terminal pheromone-like domain of pherophorin family proteins (34% identity and 56% similarity to the

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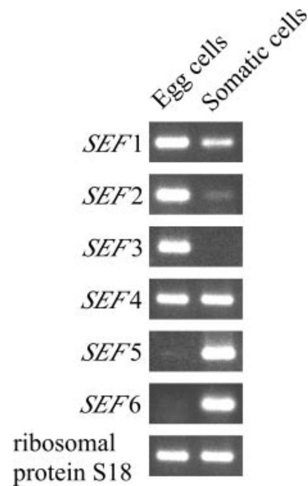

Fig. 3. **Cell-type specificity of *SEF* gene expression.** Total RNA was isolated from eggs and somatic cells 42 h after the initiation of embryogenesis. RT-PCR products for each *SEF* gene were analyzed by agarose gel electrophoresis. The RT-PCR products of ribosomal protein S18 were used as an internal control.

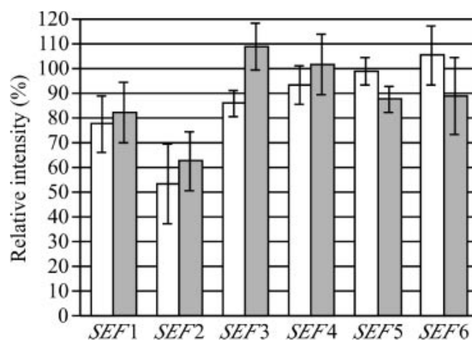

Fig. 4. **Effect of the removal of the sexual inducer on *SEF* gene expression.** The sexual inducer was washed out at 18 h after the initiation of embryogenesis, and the spheroids were transferred to medium with or without Con A (2.5 μg/ml); after 24 h, total RNA was recovered. The amounts of gene transcripts were determined by semi-quantitative RT-PCR. Values are mean ± SD of four reactions. Each bar shows the band intensity relative to that for spheroids grown in the presence of the inducer. Open bars: spheroids transferred to medium without Con A. Shaded bars: spheroids transferred to medium with Con A.

C-terminal region of pherophorin II), indicating that *SEF*6 encodes a pherophorin family protein.

The cDNA fragments of *SEF*1, *SEF*2, *SEF*3 and *SEF*4 did not show any significant similarity to known genes in a BLAST search. Although putative upstream and downstream sequences of *SEF*1, *SEF*2, *SEF*3 and *SEF*4 cDNA fragments were obtained from the JGI website (http://genome.jgi-psf.org/chlre2/chlre2.home.html), none showed any significant similarity to known genes.

Early Response of SEF*5 and* SEF*6 to the Sexual Inducer*—There have been reports that the expressions of some members of the pherophorin family are rapidly induced in vegetatively growing spheroids in response to the sexual inducer (*37–39*). The early response of *SEF*5 and *SEF*6 to the inducer was examined by exposing vegetatively growing spheroids to the inducer for 90 min. As shown in Fig. 7, no expression of *SEF*5 was seen up to 90 min after the application, while the expression of *SEF*6 occurred rapidly after application of the inducer. Thus, while both *SEF*5 and *SEF*6 encode pherophorin-related proteins, their responses to the inducer differ from one another.

DISCUSSION

The transcripts of 6 *SEF* genes are absent or barely detectable in vegetatively growing spheroids but accumulate in sexual spheroids (Fig. 2). As summarized in Table 1, the expression profile of each *SEF* gene differs somehow from those of the others. Of the 6 genes, *SEF*3 exhibites the simplest expression profile; its transcript is barely detectable during early development, and after completing embryogenesis it gradually accumulates to achieve maximal accumulation after hatching (Fig. 2). In contrast, *SEF*1, *SEF*2, *SEF*4, and *SEF*5 exhibit biphasic expression profiles. The transcripts of these genes accumulate during early development, and then their levels decrease transiently after completing embryogenesis at about the 24 h time point (Fig. 2). After the transient decrease, their levels increase again to achieve maximal accumulation after hatching (Fig. 2). Interestingly, it has been reported that during the asexual life cycle, the expressions of most maturation-abundant genes show a similar transient reduction before maximal expression (*40*). Thus, it seems possible that a similar developmental regulatory mechanism affects the transient decrease in both sexual and asexual spheroids. In addition to developmental regulation, another factor that may affect the expression of these genes is light/dark conditions, since the transient decrease occurs during the light period under our experimental conditions. Although further study under different light/dark conditions is needed to examine the fine regulatory mechanisms of these genes, it is obvious that the expression of these genes is developmentally regulated since maximal accumulation is achieved only after the maturation of sexual spheroids. In contrast to *SEF1*, *SEF2*, *SEF3*, *SEF4* and *SEF5*, which are expressed after the initiation of embryogenesis of the sexual spheroids, the *SEF*6 transcript accumulates before the initiation of embryogenesis (see time point 0 in Fig. 2), reflecting a rapid induction of this gene after the application of the sexual inducer to asexual spheroids (Fig. 7). The transcript level of this gene fluctuates during development of the sexual spheroid (Fig. 2).

The amounts of transcripts of some *SEF* genes decrease significantly after hatching of the sexual spheroids. As shown in Fig. 2, the transcripts of *SEF*1, *SEF*2 and *SEF*3 decreased to 44%, 24% and 78% of the maximal level, respectively, at the 48 h time point when the eggs were still fertile (*36*). These genes are expressed predominantly in egg cells (Fig. 3), and thus they may play specific roles in fertilization. Although egg cells remain fertile for several days after hatching, they redifferentiate into asexual gonidia even in the presence of the inducer if they do not encounter sperm packets (*3*). It is tempting to speculate that the decrease in the levels of the *SEF*1, *SEF*2, and *SEF*3 transcripts is related to the redifferentiation of eggs into asexual gonidia, since the products of these genes would gradually decrease after hatching.

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1    ATACTTGGTTCACTCACTTC AAGTTGAGATGGAAAAGAGG ATAATTCATTTCCTCTTCTT GTCAACGCTGGCGGTCGGGT GGGCCATTGGTGCGGATGCC
                                 M E K R   I I H F L F L   S T L A V G W   A I G A D A ▲
101  CAGGAGTATGACTATGATCC TTCACGCGTCCAGACTGGTA CAACACCAGATTTTCCCTAC CGCAAATGCAACACGACGAA TGGCCCTTATCGGCTGAATC
     Q E Y D Y D P   S R V Q T G T   T P D F P Y   R K C N T T N   G P Y R L N P
201  CGGTATGGAGGTTTGTGGCC AATCGGATCTATTGCTTTAC TATCCAGGTTAAGCTGGATC CCCTTTCCTGTTCTGGCCCC TGCTGCAACGCTGACCTGCA
      V W R F V A   N R I Y C F T   I Q V K L D P   L S C S G P   C C N A D L H
301  CAAAATAGAGTTCAACGTAT CTAGCAGCTGCCTGGTCGCC GGGGCCTCCGTCATCGCCAG TGTGAATGGCAGAAAAACCA TGATCGCGCCGGCATTTGAC
      K I E F N V S   S S C L V A   G A S V I A S   V N G R K T M   I A P A F D
401  AGGCCGGCAACTGGCCCGCC TGGCTCGGCCGTTCTGCGAA TCACCAACCTGGGCCTCACC ACGGAGACCGCCCAGAATGC AGAGCTGTGCCTCGTCCTCA
     R P A T G P P   G S A V L R I   T N L G L T   T E T A Q N A   E L C L V L R
501  GAACCAACCGCGGCGGCCAG GGCTGCACAACCCTGCAACA GCTGTGCACGTCGGCCGGTC TCCCCGCAGGCACCTGTACG GTGGCGACGTATGACGCAGC
      T N R G G Q   G C T T L Q Q   L C T S A G L   P A G T C T   V A T Y D A A
601  CTGCGACTGCTGCCCACAAA ATATCGTTGTTGAGCCACAG CCACCACCGCCGCCACCGCC GCCACCGCCACCGTCGCCAC CACCGCCGCCACCGCCGCCA
      C D C C P Q N   I V V E P Q   P P P P P P P   P P P P S P P   P P P P P P
701  CCGCCACCGTCGCCACCACC GCCGCCACCGCCGCCACCGC CACCGTCGCCACCACCGCCG CCACCGCCGCCACCGCCACC GTCGCCACCACCGCCGCCAC
     P P P S P P P   P P P P P P P   P S P P P P   P P P P P P P   S P P P P P P
801  CGCCACCGTCGCCATCGCCG CCGCCGCCGCCACCGTCGCC ATCGCCGCCGCCGCCACCCC CACCTCCATCACCCCCACCG GCACCCCCTCCATTTGTGCC
      P P S P S P   P P P P P S P   S P P P P P P   P P S P P P   A P P P F V P
901  TGGCTTTTTCGATTGCGAAC TCTGTTTCGCTGCAGAGCTG ACTCCACCGACTGATTCCGC ACCCTACCGTTTCGACAACG CCACCTGCACCGCCTTCCAA
      G F F D C E L   C F A A E L   T P P T D S A   P Y R F D N A T C T A F Q
1001 ACGGCTGTCTCCGAAGACAT GAACAGCATACTTCAAAATT TCAACGTCACCATCAAGAGC CCATATCCAAATAGTACCTC ATGCACTGGCACGCAGGCCA
     T A V S E D M   N S I L Q N F   N V T I K S   P Y P N S T S   C T G T Q A I
1101 TTGTGTGCGGCAAATTCCTT AAAGGCGATCTCGCCTTGCT TGAGCAAACAGTCCGCAGCG AAATCTACCAAAAGGTTGTC AAATGGCTCGCGATCATCAC
      V C G K F L   K G D L A L L   E Q T V R S E   I Y Q K V V   K W L A I I T
1201 GGGTGAAGGTGAATGCAAGC CGCAGCTGTCGGGGTACGAG GTGACGGTCAAAACGCTCCC CGGCGCTTCTCCGGCCTGCA TCGATGTGGAGGCGGCTGTT
      G E G E C K P   Q L S G Y E   V T V K T L P   G A S P A C I   D V E A A V
1301 CTTTGCCTCATTTACGATCC TCCTCCTCCTCCTCCTCCAC TTCCTCCACTTCCTTTCCCC AACTGCACGTGCAACACAAC GCAAAACATCATGCCGTACA
     L C L I Y D P   P P P P P P L   P P L P F P   N C T C N T T   Q N I M P Y K
1401 AGTTATCTCCCACGTGGTAC ACGACCAACTCGAGCCGTAG CTACGCCTCGGAGTACTGCT TCACGCTTAGTGTGCTGCCA CCTGAACTCGTGGTGCCGAG
      L S P T W Y   T T N S S R S   Y A S E Y C F   T L S V L P   P E L V V P S
1501 CACCTGCATGAGCAAGAACG ACACACTCGCGAAACTCGAA TGGTATATAGATGAAAGGAT GAGGCCGGCGGTCAAGGGCT TCACCCTATATCCAGCCGTG
      T C M S K N D   T L A K L E   W Y I D E R M   R P A V K G F   T L Y P A V
1601 GGTCCGAGTAGAACAGTCTC CCCAAGCTGGGGTGCCACAG GAGTCCAGACACTGAAAGTT AACCTCAACTGGAATGCGAC GATGGCCAACGGCGGAAAGG
     G P S R T V S   P S W G A T G   V Q T L K V   N L N W N A T   M A N G G K V
1701 TCTGCATAGCTGTTGCCAAG CCGTACAGGTTGGAGGACCT GTGCCTGGGGCCCAGAGGCA CCGCAAGCATTGGGAAATGT TACGCCAGCATTTTCAACAG
      C I A V A K   P Y R L E D L   C L G P R G T   A S I G K C   Y A S I F N R
1801 GGACAGCTCGGACTACTGCT GTCCCACCTACCTCACTAGT CCGCAGTAGAGGAGCCCGCA TGGTTGCTGGCGGCCTCAGT GAGGCACACGCGCGCGTGTG
      D S S D Y C C   P T Y L T S   P Q *
1901 TGTGTGTTTGTGTATGCGTG TGTGTATTGCGTTGACGGAG CGCTGGCTGCTGAAACTCCG CCCAAGCAGCTGCCTGCAGG CAGGTTTCCAACCTTCCGCG
2001 AGTGAGGGGGGTTGTTGAGA CGACGACTTGACGGTCGGTG TGAACCGGGACCTGTCACTT AGCATGCAGCTGCTGCTGAT GAGAGCACCAGCTGGAAGCA
2101 GTGGTGGGTATTTGGTGCAC ATAAAACCACCATTACTGCT GCTTGCTTCAGGTTAATTTC TTATATGGCGTATTTGATAG TCTGAAAACAAATCAGTGAT
2201 TCCACTCTAAAGATTAGGGA GTTGTACGTGTCGCCTTTAC AAGGGATGGAGTTGGGGGTT GTGTGGGGTTGGGCGGAAAT GAGCGAGCATGCCGTCGAGC
2301 TATGTTTCGTTCTGCTGCTG ATCACTGGGGCGGTGAAGAC GTCCGTCCGTTTTGTAGATG TTTACACGCGCCTCGCTCAG AACGTAATGGTGAGGTTTCA
2401 ATGGTTAGGGTATCGGCCGT CCGCCGCTCTCTTGTGGACA TCATAGATTGATTTCATACA GTCTCCATAGCTGTTTCTTG GCATATATTGGCATAACTTA
2501 CTTGTTTTATAGCATTGCAT GGGCCGCCATCGTGCTGGCC AGTAGCTTGTCCGGCTTGGT TGTGATCGGGTGGTTGATAA TAGGTATCTATGCATCTAAC
2601 CCTCAATATAGGATGTGTTC TGCTGGCAGGCACCAATCAG TACACGACTGCTTTCACGTT AATGTTATACAGTAAATTCA TGTATAGTACGTATTTTTGG
2701 CAATGCCAGTCTGGGGGCCA GTCTGTTTACGGACTTCATC ATCGTATCCCACCATTGACT GATTCTAGGCCGAGATGATG GCCAGGAAGCAGCACAAACA
2801 TTCCCCGGCCATGCCTTGTG GTGGCCACGTCATGCATGCT CAATTAGGCAAGGGGGTGAC GGCCGGGAGGCAAGGAATTC CTTCGAAGGTGCAGTGTCTG
2901 AAAGGAGCACAGCAGTCCCT GCGTGTATTGCCAAACCTGT GCACTTTGACACGACTCAAG GTTGATTTGACCCTATCCTG GCCCCCGGTTTGGGAAGCAC
3001 TTTCACGGGGCACAGGACTT CCGACCACGCATGCTTTCCC GTCGTTTCGCCTGCTGTCAT GTGGCTAGCGTTCCTGACTG CTGAAGCTGCCAGAAGCCCA
3101 AGGTTCGGCAATTCGTTGTA ATATGTAACGCAGATGCCAA TTGGGTGGTCTGGAATCTAT GTGAGTGCTGTAACACGCGG CCGGTTGCCAAAAAAAAAAA
```

Fig. 5. **Nucleotide sequence and deduced amino acid sequence of the *SEF*5 cDNA.** Nucleotide sequence of the longest cDNA is shown. Arrows and underlines indicate alternative polyadenylation sites and putative polyadenylation signals, respectively. The arrowhead shows the predicted cleavage site of the signal peptide. Shaded and open boxes show polyproline stretches and putative *N*-glycosylation sites, respectively.

Sequence analysis indicated that *SEF*5 and *SEF*6 encode pherophorin family proteins. The expression pattern of *SEF*5 is unique among pherophorin family proteins because it is not induced simply by adding the inducer, but is induced after the initiation of sexual development (Figs. 2 and 7). This expression pattern suggests that it has a specific role in sexually differentiated spheroids. Interestingly, the SEF5 protein has a remarkable structural feature. Although the pherophorins described so far consist of two globular domains separated by a central polyproline stretch, SEF5 has an additional polyproline stretch in the C-terminal pheromone-like domain (Figs. 5 and 6). It has recently been demonstrated that the isolated polyproline domains of pherophorin-DZ1 and -DZ2 exhibit rod-like

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Ph I     332  TCLEGTATTTCK----------PGNPDFPKCECETKPAATRFAALPTLTQEPGRPSNRTNSTLYCFT
Ph S     424  TCLDLSGSTSCN----------AAATPVPSSTCNMAKGTLPFLVSTRYMVN----SWSANSTEYCFQ
Ph DZ1   836  LCLPLLSSVSCN----------LPFTPFPNCTCNTTQGILPFTVSPRFTTG----NRTATTTEYCFT
Ph II    307  QCLDLSQSASCF----------LPGVPFPNCTCNTTQGVMPFTVSPTWYAQPANVRWGRNVTEYCFT
Ph III   307  QCLSLSQSTSCF----------LPGVPFPNCTCNTTQGVMPFVVSPTWYAQPANAHWGRNVTEYCFT
SEF5     416  ACIDVEAAVLCLIYDPPPPPPPLPPLPFPNCTCNTTQNIMPYKLSPTWYTTNSSRSYA---SEYCFT

Ph I     389  LQVVAP-LNPNGLCGNTTTLLKAELWGNDIPTQRRKILALAFKAAGASSPLRYLSPSWGSAGEQTLK
Ph S     477  VNVLDASQVVPGGCSGDLSQLGEVQLYARRNLSASVHAVRIYPSVG---SSSIVTPSWTAIGGAYLF
Ph DZ1   889  VNTIPPSQIMPNTCGSANDTLTKIEWYANQNMSSWVAGINLYPASG---PFVKRSSSWGAAGTNSLK
Ph II    364  VNTLQPSQVVPSTCYNANDALAKIEWYASDAFRSAVKGFTVYPAGG---SNKTIADSWGATGTDTLK
Ph III   364  VNTLPPSQVVPSSCYDPNDALAKIEFYAHDMYRPAVKGFSVYPATNSTTSRTFISDSWGALGVDTLK
SEF5     480  LSVLPPELVVPSTCMSKNDTLAKLEWYIDERMRPAVKGFTLYPAVG---PSRTVSPSWGATGVQTLK

Ph I     455  VSGLNWDASQADGAKICMELSNDTNLKTFCNT-----GQDTCWINLFSPDKQ--CCPLFAASLTP-
Ph S     541  NVPLHWSISQAQDASVCIELDNTKTMLDLCLG-----FPGQCYVSTVNTNRD--CCPSYRTALGLL
Ph DZ1   953  ATPINWSIAQASGARVCVEVKNPKTMSDLCLG-----IASQCFLSTFNSNKD--CCPIYGSGFA--
Ph II    428  VN-LNWNLLQANGGKVCVAIQNPFTMGDICKG-----ALGQCYASIFNRDNSDYCCPIYRTGP---
Ph III   431  VS-LNWTDEQANGGLVCVAIQNPITMSDVCKGG----IGEQCYVSIFNRDNSDYCCPIYRTGP---
SEF5     544  VN-LNWNATMANGGKVCIAVAKPYRLEDLCLGPRGTASIGKCYASIFNRDSSDYCCPTYLTSPQ--
```

Fig. 6. **Alignment of the C-terminal pheromone-like domains of *SEF*5 and other pherophorin proteins.** Ph I: pherophorin I (*37*), PhII: pherophorin II (*37*), Ph III: pherophorin III (*42*), Ph S: pherophorin-S (*38*), and Ph DZ1: pherophorin-DZ1 (*39*).

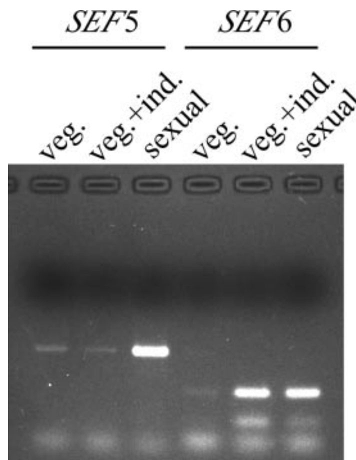

Fig. 7. **Early response of *SEF*5 to the sexual inducer**. Total RNA was extracted from a portion of the culture of vegetatively growing spheroids at 30 h before the initiation of cell-cleavage of the gonidia ("veg."). The inducer was added to the remaining portion of the culture, and total RNA was isolated at 90 min ("veg. + ind.") or 72 h ("sexual") after the addition of the inducer. The sexual spheroids ("sexual") bear eggs at this stage. The RT-PCR products for *SEF*5 and *SEF*6 transcripts were analyzed by agarose gel electrophoresis.

Table 1. **Summary of the expression profiles of *SEF* genes.**

Gene	Expression profile	Cell-type specificity		Decrease at 48 h time point
		Egg	Somatic cell	
*SEF*1	Biphasic accumulation	+	+/–	+
*SEF*2	Biphasic accumulation	+	–	+
*SEF*3	Late accumulation	+	–	+
*SEF*4	Biphasic accumulation	+	+	–
*SEF*5	Biphasic accumulation	–	+	–
*SEF*6	Fluctuates	–	+	–

structures under electron microscopy, and that highly-purified intact pherophorin proteins form an insoluble fibrous network in an autocatalytic reaction *in vitro* (*39*). The ECM of *V. carteri* has been considered to contain these pherophorin-based substructures. The expression of the SEF5 protein with its unique structural feature might result in the structural rearrangement of the ECM in sexual spheroids. Another interesting finding has been reported regarding *GAS*28; this gene encodes a protein with homology to pherophorin that is specifically induced during gametogenesis in *C. reinhardtii*, a close relative of *Volvox* (*41*). Thus, reorganization of ECM during sexual development might take place in both *Chlamydomonas* and *Volvox*.

In this study, we were able to identify 6 genes that are up-regulated in the sexual female spheroids of *V. carteri*. A variety of genes that are expressed during gametogenesis or zygote formation have been isolated from *C. reinhardtii*. Further studies on genes involved in the sexual differentiation of *C. reinhardtii* and *V. carteri* will provide vital clues for elucidating the evolution of sexual reproduction from isogamy to oogamy.

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