LIBRARY
JAN 1 1 2010
SAINT MARY'S UNIVERSITY
HALIFAX, CANADA

SYMBIOSIS (2007) 43, 57–64 ©2007 Balaban, Philadelphia/Rehovot ISSN 0334-5114

Transfer, integration and expr ssion of functional nuclear genes between multicellular species

Sidney K. Pierce*, Nicholas E. Curtis, Jeffery J. Hanten, Susan L. Boerner, and Julie A. Schwartz

Department of Biology, SCA 110, University of South Florida, 4202 E. Fowler Ave., Tampa, FL 33620, USA, Email. pierce@cas.usf.edu

(Received December 22, 2006; Accepted April 11, 2007)

Abstract

The sea slug, *Elysia chlorotica*, is a deep green color due to the presence of intracellular, symbiotic chloroplasts captured from algae during feeding. Several chloroplast proteins, including some that are nuclear-encoded such as FCP, LHC 1 and LHC 2, are synthesized while the plastids reside in the host cytoplasm, for as long as 9 months. Using PCR, we have identified the sequences for *fcp*, *Lhcv 1* and *Lhvc 2* in genomic DNA and mRNA from adult slugs, and from pre-hatched veliger larvae. These results show that the algal nuclear genes are present in the animal cell, are transcribed and are transmitted to the offspring. This is the first demonstration of transfer of functional, inheritable genes between multicellular organisms.

Keywords: Gene transfer, chloroplast symbiosis, sacoglossa, chromophyta

1. Introduction

Transfers of functional genes between prokaryotes, between prokaryotes and unicellular eukaryotes and also between organelles and nuclei are well established phenomena (Delwiche, 1999; Jain et al., 2002; Andersson et al., 2003). While similar transfers between multicellular organisms are the theoretical basis of gene therapy, genetic engineering and also could be a process of major evolutionary significance, no direct evidence for the transfer of functional genes between multicellular species has been found. The potential from uncovering the mechanism underlying the transfer of a functional gene between multicellular organisms is so significant that the editors of *Science* listed it as one of 125 important questions that should be addressed in the next 25 years (Kennedy and Norman, 2005). We report below the discovery of the transfer of three functional, nuclear-encoded genes between multicellular organisms from two Kingdoms. The nucleotide sequences for the genes are present in the genomic DNA and mRNA, and the proteins they code for are synthesized in, and are functional in, the host cell.

We have been studying *Elysia chlorotica* (Gould), a sacoglossan sea slug, that has a special type of symbiosis, called chloroplast symbiosis or kleptoplasty, with chloroplasts from the chromophytic alga, *Vaucheria litorea* (C. Agardh). The slug slits open the coenocytic *V. litorea* filaments, sucks out the contents and certain cells that line the digestive diverticula take up and incorporate the chloroplasts. Once inside the host cell, the isolated chloroplasts continue to photosynthesize for up to 9 months. Previously, we found that several chloroplast proteins are synthesized while the captured organelles reside in the host cell (Pierce et al., 1996; Hanten and Pierce, 2001). Some of these are proteins that are nuclear-encoded in the algal cell and, indeed, their synthesis in the host cell is inhibited by cycloheximide, also indicating nuclear-encoding (Hanten and Pierce, 2001; Pierce et al., 2003). If the gene for the chloroplast protein is present in the animal genome, lateral gene transfer must have occurred.

By immunolabelling techniques, we have previously identified several of the cycloheximide-inhibited proteins as fucoxanthin, chlorophyll binding protein (FCP) and several of the light harvesting complex polypeptides (LHC) (Hanten and Pierce, 2001). We have purified *V. litorea* FCP and determined both n-terminal and internal amino acid sequences (Pierce et al., 2003). Using the native FCP amino

*The author to whom correspondence should be sent.

acid sequence information and *Lhcv 1* and *Lhcv 2* nucleotide sequences from Genbank for primer design, genomic DNA and cDNA made from mRNA extracted from both *E. chlorotica* and *V. litorea* were tested using polymerase chain reactions (PCR) for the presence of *fcp* and *Lhcv 1-2*. In addition, genomic DNA extracted from pre-hatched *E. chlorotica* veliger larvae, which do not contain symbiotic plastids and have never fed, was also tested for the presence of the genes. The results are the first demonstration of the transfer of functional nuclear genes between multicellular organisms.

2. Materials and Methods

Animals and algae

Elysia chlorotica were collected from Martha's Vineyard, MA, shipped to Tampa and kept in aquaria containing artificial sea water (Instant Ocean, 1000 mosm) at 10°C on a 12/12 hr light/dark cycle. *Vaucheria litorea* was harvested from our laboratory culture, which we have maintained for several years. The starting material for this culture came from the same collecting site on Martha's Vineyard used for the slugs.

Genomic DNA purification

Genomic DNA was purified from adult *E. chlorotica* that had been starved for at least a month to ensure that the gut was empty, and also from pre-hatched *E. chlorotica* veliger larvae, using a Nucleon® genomic DNA extraction kit, PhytoPure® (Tepnel Lifecodes, Stamford, CT) per manufacturer's instructions. The larvae were obtained by allowing egg masses, which had been deposited in our aquaria, to develop for several days in 0.2 μm filtered sea water containing 5 μg/ml Rifampicin (West et al., 1984) at 20°C. The veligers were well developed at that point, but had not hatched, so they had never been directly exposed to sea water nor eaten anything. The purified genomic DNA was treated with RNase I_f (NEB, Ipswich, MA), and then precipitated with isopropanol. The precipitated DNA was washed with 75% ethanol, resuspended in nuclease free water, and quantified spectrophotometrically (260 nm).

Total RNA isolation/mRNA purification

Total RNA was isolated from *E. chlorotica* that had been starved for 3 months, by homogenizing them in Trizol® Reagent (Invitrogen, Carlsbad, CA), and centrifuging at 11,950 x g to remove cellular debris. The supernatant was extracted with chloroform (1:5 v/v) and then the samples were spun at 12,000 x g to separate aqueous and organic phases. The total RNA was precipitated from the aqueous phase by first adding isopropanol followed by an equal volume of a solution containing 0.8 M Na citrate and 1.2 M NaCl. The precipitated RNA pellet was washed with 75% ethanol, dried, and then resuspended in diethylpyrocarbonate (DEPC)-treated water. The total RNA was quantified spectrophotometrically (260 nm). mRNA was purified from total RNA using Dynabeads® (Dynal Biotech, Oslo, Norway) following manufacturer's instructions.

cDNA synthesis

cDNA was synthesized from the purified mRNA using PowerScript™Reverse Transcriptase (Clonetech, Mountain View, CA) and random nonamers as per manufacturer's instructions.

Primer design

Oligonucleotide sequence primers (Operon Bio-technologies, Huntsville, AL) were designed to specific regions of *V. litorea Lhcp 1* (AF336982) and *Lhcp 2* (AF336985) sequences (Table 1) using the NCBI database. Hereafter, we have labeled these two light harvesting

Table 1. Primer sequences used in the PCR reactions.

Gene	Primer sequence	Touchdown temperature range
fcp	Forward: 5' GCTGGATTCACTACTGATGGC 3' Reverse: 5' GGGTCGTCGTATCTAACCCAG 3'	65–55°C
Lhcv 1	Forward: 5' GTGGAAATAAAGCACGGA 3' Reverse: 5' CGCCCATTGTTAAGCTC 3'	60–50°C
Lhcv 2	Forward: 5' GCCATCAATCCTCACCCG 3' Reverse: 5' CCTTCCGTTCTTAATCTCTTT 3'	60–50°C
V. litorea ITS (Green et al., 2000)	Forward: 5' CCAACATATTCATCCTC 3' Reverse: 5' ATTGCACCATTGCTGGC 3'	52–40°C
Actin	Forward: 5' AGGGTGTCATGGTTGGTA 3' Reverse: 5' GATCCACATCTGCTGGAA 3'	60–50°C

complex genes from *V. litorea* as *Lhcv 1* and *Lhcv 2*, with the "v" standing for *V. litorea*. The nomenclature of the light harvesting complex proteins and their genes is confusing and often contradictory. This is not the place to debate the issue, but the "*Lhcp 1*" and "*2*" labels in the database seem incongruous with current light harvesting complex protein labeling procedures. We have used the nomenclatural system proposed by Jansson et al. (1999).

Additional oligonucleotide sequence primers were designed to specific regions of *V. litorea* FCP (Table 1) using native amino acid sequence (Pierce et al., 2003).

Touchdown PCR/agarose gel analysis

PCR reactions (25 μl) were performed using approximately 100 ng of genomic DNA or 75 ng of cDNA, 12.5 pmol of each primer, 0.25 mM dNTP mix (ID Labs, London, Ontario, Canada) and 1.25 units of IDProof™ DNA polymerase (ID Labs). Touchdown PCR was performed on both genomic DNA and cDNA samples using an annealing temperature range from 5°C above to 5°C below the melting temperature of the sequencing primers for 20 cycles dropping 1°C every other cycle (Table 1). Twenty additional cycles were run at the lowest annealing temperature condition. Each cycle consisted of 30 s at the denaturing temperature (94°C), 30 s at the annealing temperature, and 45 s at the extension temperature (72°C). PCR products were separated on a 1.2% agarose gel containing 0.02% ethidium bromide and visualized by UV illumination. It should be noted that in all the genomic DNA samples from both adults and veliger larvae, regardless of the method of DNA extraction, PCR amplification only occurred in 15–20% of the reactions using our methods. The reason for this inconsistent amplification is not clear, however, it seems likely that inhibition by muco-polysaccharides, which are always present to some degree, no matter the purification method due to the extreme mucogenesis of the slugs, may be the cause. Also, as the level of genomic DNA purity was increased by procedures such as cesium gradient treatment, a higher percentage of reactions successfully amplify, also suggesting the presence of some sort of inhibitor in the non-gradient treated samples. Low copy number might be another cause of the amplification pattern. For instance, cDNA made from purified mRNA, which would have a much higher copy number than genomic DNA, always amplified.

Cloning of PCR products/sequencing

PCR products were excised from the agarose gel and purified using the QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA). Purified DNA was then cloned using the TOPO TA cloning kit (Invitrogen, Grand Island, NY) as per manufacturer's instructions. Clones were sequenced commercially (Macrogen Sequencing Services, Seoul, S. Korea). Sequence data were analyzed by BLAST (http://ncbi.nlm.nih.gov/blast) searching.

Control experiments

Several controls were run to ensure that the results were not from contamination by *V. litorea* DNA. First, blank PCR reactions were run with each primer pair using the touchdown conditions described above, but without template. Second, DNA was extracted from a non-kleptoplastic slug, *Aplysia californica* (Cooper), as described above and tested with the same primer pairs in the same protocols. Third, *V. litorea* internal transcribed spacer regions (*ITS*) primers (Table 1) were tested on the algae, adult and larvae, and *A. californica* genomic DNA preparations with positive results found only with the algae DNA. Fourth, the genomic DNA extraction procedure and subsequent PCR reaction for *fcp* was done on larvae in another building using all new materials as well as equipment that had never been in our lab. Finally, all of the results were repeated on different preparations from 3 sets of animals or egg masses done weeks apart with fresh reagents by two researchers, all with the same results.

Algal and slug template quality as well as reagent quality were verified with primers designed to highly conserved regions of the actin gene (Table 1), which were determined from alignments of opisthobranch and chlorophyte sequences available in Genbank.

3. Results

The PCR reactions with the specific *fcp* primers, using *E. chlorotica* adult genomic DNA and cDNA, as well as veliger larvae genomic DNA, as templates all produced a 323 bp product (Figs. 1–3). The product sequences (Accession numbers: *fcp* adult genomic DNA-EF559310, cDNA-EF559312, larval DNA-EF559311) were a 100% match to *V. litorea fcp* sequence (EF559313) in all cases (Fig. 4). In addition, PCR reactions using *Lhcv 1* primers and the *E. chlorotica* adult genomic DNA, cDNA or veliger larvae genomic DNA as templates each produced a 290 bp product (Figs. 1–3) whose sequences (Accession numbers: adult genomic DNA-EF559305, cDNA-EF559303, larva DNA-EF559304) also produced 100% matches with *V. litorea Lhcp 1* in the database (AF336982) (Fig. 5). The PCR reactions with *Lhcv* 2 primers and both adult and larval genomic DNA produced a 477 bp product (Figs. 1–3) (Accession numbers: adult genomic DNA-EF559308, larval DNA-EF559307) that contained two introns and whose exons matched *V. litorea Lhcp* 2 mRNA coding sequences (AF336985), except for a one bp difference in the larvae sequence (Fig. 6). The introns were identical to those present in *Lhcv 2* that we amplified from *V. litorea* genomic

DNA (EF559309). In addition, the *Lhcv 2* PCR product (363 bp) from the adult cDNA (Accession number-EF559306) was identical to that from *V. litorea Lhcp2* (AF336985) (Figs. 1 and 3).

Since contamination is always a source of concern in these types of experiments, we have done a variety of procedures to insure that the results above are correct. First, all of the results were repeated on different preparations from 3 sets of animals or egg masses done weeks apart with fresh reagents in two different laboratory buildings by two researchers, all with the same results. Second, to test for the presence of contaminating *V. litorea* genomic DNA in the *E. chlorotica* samples, PCR reactions were performed on both the *E. chlorotica* adult and veliger larvae genomic DNA's using primer sequences to *V. litorea* internal transcribed spacer regions (*ITS*), all with negative results (Fig. 7). These results, in addition to earlier ones using similar methodology (Pierce et al., 2003; Green et al., 2000) indicate that algal genomic DNA is not contaminating the slug DNA samples. In addition, we tested genomic DNA from *A. californica*, a non-kleptoplastic sea slug, with the *fcp*, *Lhcv 1-2* and *ITS* primers, again with negative results. Finally, PCR reactions with each primer set were run in the absence of template DNA with negative results demonstrating that reagents were not contaminated with algal DNA.

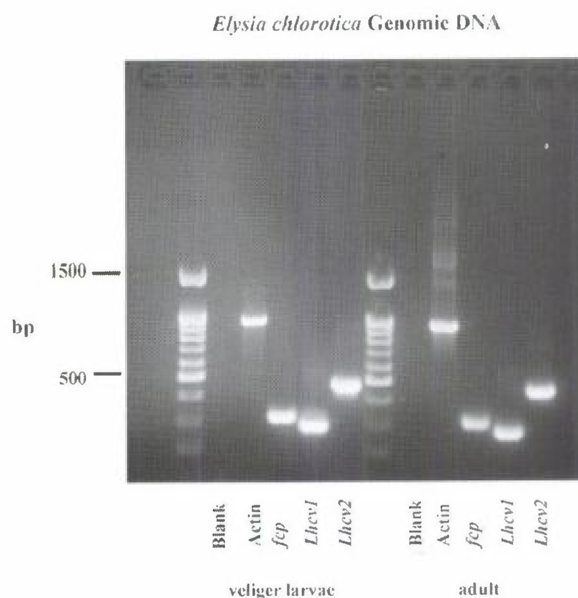

Figure 2. Agarose gel of the PCR products from the reactions of veliger larvae or adult *E. chlorotica* genomic DNA and primer sequences made to *fcp*, *Lhcv 1* and *Lhcv 2*. The lanes labeled Blank were from PCR reactions that lacked template. The lanes labeled Actin were PCR reactions done with primer sequences for the actin gene to verify template and reagents.

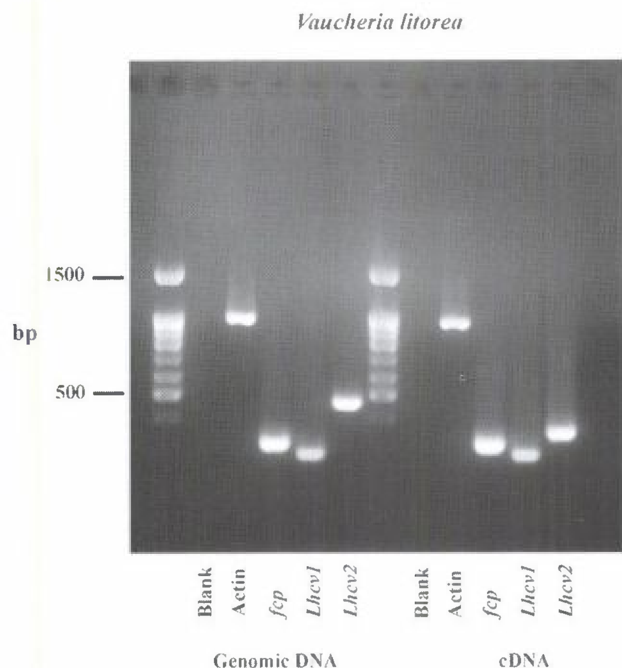

Figure 1. Agarose gel of the PCR products obtained from the reactions of *V. litorea* genomic DNA or cDNA and primer sequences made to *fcp*, *Lhcv 1* and *Lhcv 2*. The lanes labeled Blank were from PCR reactions that lacked template. The lanes labeled Actin were PCR reactions done with primer sequences for the actin gene to verify template and reagents (See methods).

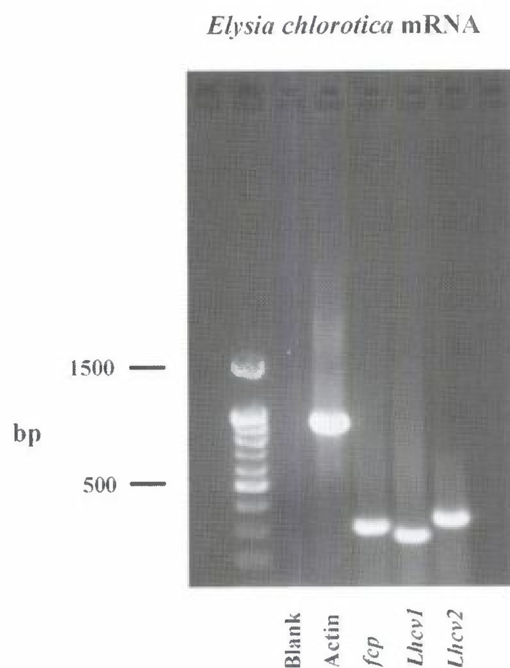

Figure 3. Agarose gel of the PCR products from the reactions of *E. chlorotica* mRNA sequences (as cDNA) and primer sequences made to *fcp*, *Lhcv1* and *Lhcv 2*. The lanes labeled Blank were from PCR reactions that lacked template. The lanes labeled Actin were PCR reactions done with primer sequences for the actin gene to verify template and reagents.

fcp Sequence Comparisons

```
E. chlorotica cDNA            GCTGGATTCACTACTGATGGCGATGAAGAAGCATTCAACCGTAGACGTGCCGTCGAAATC 60
E. chlorotica larvae genomic  GCTGGATTCACTACTGATGGCGATGAAGAAGCATTCAACCGTAGACGTGCCGTCGAAATC
E. chlorotica adult genomic   GCTGGATTCACTACTGATGGCGATGAAGAAGCATTCAACCGTAGACGTGCCGTCGAAATC
V. litorea genomic            GCTGGATTCACTACTGATGGCGATGAAGAAGCATTCAACCGTAGACGTGCCGTCGAAATC
                              ************************************************************

E. chlorotica cDNA            AAGCATGGACGCGTTGCTATGCTTGCCACCATTGGATACATTGTCCCCGATCTTTTCAAA 120
E. chlorotica larvae genomic  AAGCATGGACGCGTTGCTATGCTTGCCACCATTGGATACATTGTCCCCGATCTTTTCAAA
E. chlorotica adult genomic   AAGCATGGACGCGTTGCTATGCTTGCCACCATTGGATACATTGTCCCCGATCTTTTCAAA
V. litorea genomic            AAGCATGGACGCGTTGCTATGCTTGCCACCATTGGATACATTGTCCCCGATCTTTTCAAA
                              ************************************************************

E. chlorotica cDNA            CTTCCAGGAAACATCTCTAACTCAGCCAACCTCAAATTCGCTGACATTCCCAATGGGCTT 180
E. chlorotica larvae genomic  CTTCCAGGAAACATCTCTAACTCAGCCAACCTCAAATTCGCTGACATTCCCAATGGGCTT
E. chlorotica adult genomic   CTTCCAGGAAACATCTCTAACTCAGCCAACCTCAAATTCGCTGACATTCCCAATGGGCTT
V. litorea genomic            CTTCCAGGAAACATCTCTAACTCAGCCAACCTCAAATTCGCTGACATTCCCAATGGGCTT
                              ************************************************************

E. chlorotica cDNA            GGTGCCATCAAGGCAGTCCCTGCTCTCGGATGGGTCCAGATCATTCTCTTCATTGGACTT 240
E. chlorotica larvae genomic  GGTGCCATCAAGGCAGTCCCTGCTCTCGGATGGGTCCAGATCATTCTCTTCATTGGACTT
E. chlorotica adult genomic   GGTGCCATCAAGGCAGTCCCTGCTCTCGGATGGGTCCAGATCATTCTCTTCATTGGACTT
V. litorea genomic            GGTGCCATCAAGGCAGTCCCTGCTCTCGGATGGGTCCAGATCATTCTCTTCATTGGACTT
                              ************************************************************

E. chlorotica cDNA            CTTGAGCTTGTCATCTGGCCTCAGCAAGAAGACAAAGCCCCTGGTGACATTGGAGGCGAC 300
E. chlorotica larvae genomic  CTTGAGCTTGTCATCTGGCCTCAGCAAGAAGACAAAGCCCCTGGTGACATTGGAGGCGAC
E. chlorotica adult genomic   CTTGAGCTTGTCATCTGGCCTCAGCAAGAAGACAAAGCCCCTGGTGACATTGGAGGCGAC
V. litorea genomic            CTTGAGCTTGTCATCTGGCCTCAGCAAGAAGACAAAGCCCCTGGTGACATTGGAGGCGAC
                              ************************************************************

E. chlorotica cDNA            AACTGGGTTAGATACGACGACCC 323
E. chlorotica larvae genomic  AACTGGGTTAGATACGACGACCC
E. chlorotica adult genomic   AACTGGGTTAGATACGACGACCC
V. litorea genomic            AACTGGGTTAGATACGACGACCC
                              ***********************
```

Figure 4. Comparison of consensus sequences of the PCR products produced from the *fcp* primers described in the methods (Table 1) and the template DNA indicated. (*) indicates identical nucleotides.

Lhcv 1 Sequence Comparisons

```
E. chlorotica cDNA            GTGGAAATAAAGCACGGACGCATCGCCATGCTCGCCACCATCGGATACATCGTCCCTTAT 60
E. chlorotica larvae genomic  GTGGAAATAAAGCACGGACGCATCGCCATGCTCGCCACCATCGGATACATCGTCCCTTAT
E. chlorotica adult genomic   GTGGAAATAAAGCACGGACGCATCGCCATGCTCGCCACCATCGGATACATCGTCCCTTAT
V. litorea genomic            GTGGAAATAAAGCACGGACGCATCGCCATGCTCGCCACCATCGGATACATCGTCCCTTAT
                              ************************************************************

E. chlorotica cDNA            TACTTTAAACTCTTCCCAGAAGGCAAACCGGGAACCGAGGCGCTCACAACCATCCCACAG 120
E. chlorotica larvae genomic  TACTTTAAACTCTTCCCAGAAGGCAAACCGGGAACCGAGGCGCTCACAACCATCCCACAG
E. chlorotica adult genomic   TACTTTAAACTCTTCCCAGAAGGCAAACCGGGAACCGAGGCGCTCACAACCATCCCACAG
V. litorea genomic            TACTTTAAACTCTTCCCAGAAGGCAAACCGGGAACCGAGGCGCTCACAACCATCCCACAG
                              ************************************************************

E. chlorotica cDNA            GCCGGCCTGGTGCAGATCTTCGCATTCATCGGCGCACTCGAGCTGTGGGTGTTCTACCAG 180
E. chlorotica larvae genomic  GCCGGCCTGGTGCAGATCTTCGCATTCATCGGCGCACTCGAGCTGTGGGTGTTCTACCAG
E. chlorotica adult genomic   GCCGGCCTGGTGCAGATCTTCGCATTCATCGGCGCACTCGAGCTGTGGGTGTTCTACCAG
V. litorea genomic            GCCGGCCTGGTGCAGATCTTCGCATTCATCGGCGCACTCGAGCTGTGGGTGTTCTACCAG
                              ************************************************************

E. chlorotica cDNA            AGCCCCGAGAAGGAGCCGGGGGACATCGCACCAGAGTATCTGAACTGGAAGCGGTACTCC 240
E. chlorotica larvae genomic  AGCCCCGAGAAGGAGCCGGGGGACATCGCACCAGAGTATCTGAACTGGAAGCGGTACTCC
E. chlorotica adult genomic   AGCCCCGAGAAGGAGCCGGGGGACATCGCACCAGAGTATCTGAACTGGAAGCGGTACTCC
V. litorea genomic            AGCCCCGAGAAGGAGCCGGGGGACATCGCACCAGAGTATCTGAACTGGAAGCGGTACTCC
                              ************************************************************

E. chlorotica cDNA            GACTCCGACGTCCGCACCAAAAAACTCAACATCGAGCTTAACAATGGGCG 290
E. chlorotica larvae genomic  GACTCCGACGTCCGCACCAAAAAACTCAACATCGAGCTTAACAATGGGCG
E. chlorotica adult genomic   GACTCCGACGTCCGCACCAAAAAACTCAACATCGAGCTTAACAATGGGCG
V. litorea genomic            GACTCCGACGTCCGCACCAAAAAACTCAACATCGAGCTTAACAATGGGCG
                              **************************************************
```

Figure 5. Comparison of consensus sequences of the PCR products produced from the *Lhcv 1* primers described in the methods (Table 1) and the template DNA indicated. (*) indicates identical nucleotides.

Lhcv 2 Sequence Comparisons

```
E. chlorotica cDNA               GCCATCAATCCTCACCCGC----------------------------------------- 19
E. chlorotica larvae genomic     GCCATCAATCCTCACCCGCAGGTACGCGCACCTCACCTCAAATCTCATGCAAAAACTCAC 60
E. chlorotica adult genomic      GCCATCAATCCTCACCCGCAGGTACGCGCACCTCACCTCAAATCTCATGCAAAAACTCAC
V. litorea genomic               GCCATCAATCCTCACCCGCAGGTACGCGCACCTCACCTCAAATCTCATGCAAAAACTCAC
                                 *******************

E. chlorotica cDNA               ---------------AGTGGCTCCAAGAGTCTGAAATCAAACACGGAAGAATATGCATGC 64
E. chlorotica larvae genomic     CTTTGTTCAACGACCAGTGGCTCCAAGAGTCTGAAATCAAACACGGAAGAATATGCATGC 120
E. chlorotica adult genomic      CTTTGTTCAACGACCAGTGGCTCCAAGAGTCTGAAATCAAACACGGAAGAATATGCATGC
V. litorea genomic               CTTTGTTCAACGACCAGTGGCTCCAAGAGTCTGAAATCAAACACGGAAGAATATGCATGC
                                                *********************************************

E. chlorotica cDNA               TGGCCTTCGTGGGGACCCTGGTGATCCATGCTGGGATTCATATCCCAAACTTGGGTTACA 124
E. chlorotica larvae genomic     TGGCCTTCGTGGGGACCCTGGTGATCCATGCTGGGATTCATATCCCAAACTTGGGTTACA 180
E. chlorotica adult genomic      TGGCCTTCGTGGGGACCCTGGTGATCCATGCTGGGATTCATATCCCAAACTTGGGTTACA
V. litorea genomic               TGGCCTTCGTGGGGACCCTGGTGATCCATGCTGGGATTCATATCCCAAACTTGGGTTACA
                                 ************************************************************

E. chlorotica cDNA               CTTCGGACTGGTACAACTCATTTCCCGAGTTCGTGGCAAAAAATCCTCTGGGCCTTGCAC 184
E. chlorotica larvae genomic     CTTCGGACTGGTACAACTCATTTCCCGAGTTCGTGGCAAAAAATCCTCTGGGCCTTGCAC 240
E. chlorotica adult genomic      CTTCGGACTGGTACAACTCATTTCCCGAGTTCGTGGCAAAAAATCCTCTGGGCCTTGCAC
V. litorea genomic               CTTCGGACTGGTACAACTCATTTCCCGAGTTCGTGGCAAAAAATCCTCTGGGCCTTGCAC
                                 ************************************************************

E. chlorotica cDNA               AAGTCATCGCAGGGT--------------------------------------------- 199
E. chlorotica larvae genomic     AAGTCATCGCAGGGTATGTGTGACCTATCCATGAAATTTGATTTTATTGCAAGTGACTTT 300
E. chlorotica adult genomic      AAGTCATCGCAGGGTATGTGTGACCTATCCATGAAATTTGATTTTATTGCAAGTGACTTT
V. litorea genomic               AAGTCATCGCAGGGTATGTGTGACCTATCCATGAAATTTGATTTTATTGCAAGTGACTTT
                                 ***************

E. chlorotica cDNA               -------------TGACCATTTGGAGGGGTTCCATGGGACCGAGACTGGGCTGATGTGG 246
E. chlorotica larvae genomic     TTAAATCGAAGGTTGACCATTTGGAGGGGTTCCATGGGACCGAGACTGGGCTGATGTGG 360
E. chlorotica adult genomic      TTAAATCGAAGGTTGACCATTTGGAGGGGTTCCATGGGACCGAGACTGGGCTGATGTGG
V. litorea genomic               TTAAATCGAAGGTTGACCATTTGGAGGGGTTCCATGGGACCGAGACTGGGCTGATGTGG
                                              **********************************************

E. chlorotica cDNA               ACAGGGGAAGCCAACAGGAAGCCCGGGCACCTTAACTTCGATCTTTTGAATCTGATGAAG 306
E. chlorotica larvae genomic     ACAGGGGAAGCCAACAGGAAGCCCGGGCACCTTAACTTAGATCTTTTGAATCTGATGAAG 420
E. chlorotica adult genomic      ACAGGGGAAGCCAACAGGAAGCCCGGGCACCTTAACTTCGATCTTTTGAATCTGATGAAG
V. litorea genomic               ACAGGGGAAGCCAACAGGAAGCCCGGGCACCTTAACTTCGATCTTTTGAATCTGATGAAG
                                 ************************************** *********************

E. chlorotica cDNA               GGGAAGAACGAGAGCCAGTTGAAATCCATCCAACTCAAAGAGATTAAGAACGGAAGG 363
E. chlorotica larvae genomic     GGGAAGAACGAGAGCCAGTTGAAATCCATCCAACTCAAAGAGATTAAGAACGGAAGG 477
E. chlorotica adult genomic      GGGAAGAACGAGAGCCAGTTGAAATCCATCCAACTCAAAGAGATTAAGAACGGAAGG
V. litorea genomic               GGGAAGAACGAGAGCCAGTTGAAATCCATCCAACTCAAAGAGATTAAGAACGGAAGG
                                 *********************************************************
```

Figure 6. Comparison of consensus sequences of the PCR products produced from the *Lhcv 2* primers described in the methods (Table 1) and the DNA template indicated. (*) indicates identical nucleotides. The nucleotides in the introns are labeled in bold and the single nucleotide difference in the larval sequence is labeled in bold and underlined.

4. Discussion

All of these results reveal that at least three nuclear genes from *V. litorea* that encode for chloroplast proteins are present in the *E. chlorotica* genome. These findings in addition to our earlier work demonstrating the synthesis of FCP and LHC 1-6 in the symbiotic plastids (Hanten and Pierce, 2001; Pierce et al., 2003) show that the genes are not only present in the *E. chlorotica* total genomic DNA, but also are transcribed and translated in the slug cell. Thus, they represent an actual example of the horizontal transfer of functional genes between two multicellular species. Furthermore, the presence of the genes in the pre-hatched veliger larvae confirms that they are being passed on vertically now, basically waiting to be expressed in the digestive cells of each new generation of juvenile slugs upon the arrival of the first chloroplasts.

Since the nucleotide sequences present in *E. chlorotica* are identical to those from *V. litorea* DNA, it is likely that the transfer is recent, in an evolutionary sense. The mechanism of intergenome transfer is unknown. However, an interesting possibility is the presence of an endogenous retrovirus in *E. chlorotica*. This virus is expressed towards the end of the annual life cycle at a point when the entire population dies synchronously (Pierce et al., 1999; Mondy and Pierce, 2003). The occurrence of the virus is ubiquitous throughout the population. We have found viruses in every slug tested for more than 15 years. Retroviruses are well

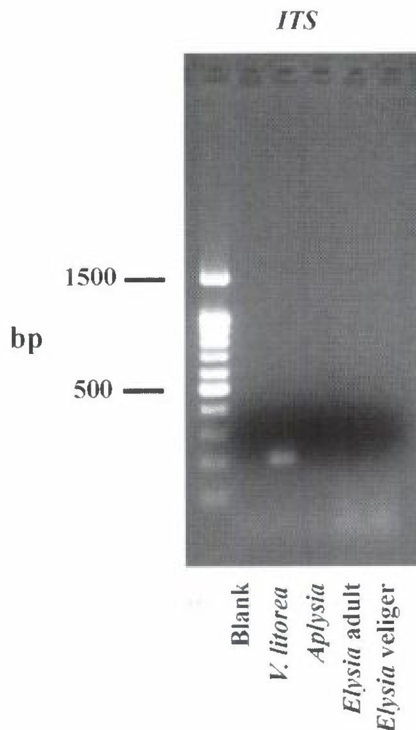

Figure 7. Agarose gel of the PCR products from reactions of primer sequences made to *V. litorea ITS* sequence, which is nuclear encoded, and genomic DNA from *V. litorea*, *A. californica*, *E. chlorotica* adults or veliger larvae. The lane labeled Blank lacked template. The absence of a product in all the lanes, except that containing genomic DNA from the alga, indicates that the slug genomic DNA samples are not contaminated with algal DNA.

known gene movers (Goto et al., 1998; Wolkowicz and Nolan, 2005), so these could be the transfer mechanism, but there are no data at present.

The transferred algal genes we have discovered so far in *E. chlorotica* are all associated with sustenance of photosynthetic function. There are a number of additional, presently unidentified, chloroplast proteins that are synthesized while the plastids reside in the host cytoplasm. The synthesis of several of these proteins is blocked by cycloheximide indicating that their genes should also be located in the host genome and also that other genes have been transferred in addition to those we have found here (Hanten and Pierce, 2001; Pierce et al., 2003). Exactly how much of the algal nuclear genome is necessary to allow plastid survival and function for the year long life cycle of the slug awaits genome sequencing of both alga and slug. However, continued photosynthesis, especially for the 9 month duration of the slug life cycle, requires substantial turnover of several plastid proteins (Stern et al., 1997), including those we have identified here. Although some of these proteins are undoubtedly coded for in the plastid genome, many others are not. Furthermore, at least some of those proteins encoded in the plastid often require nuclear regulation during synthesis (Stern et al., 1997; Merchant and Dreyfuss, 1998). Finally, the size of the *V. litorea* plastid genome is only 120 kb (Green et al., 2000), typical of other chromophyte species (Reith, 1995), but nowhere near large enough to support all plastid function in the absence of a nucleus (Martin and Herrmann, 1998). Thus, all this taken together indicates that much more genetic information has been moved from the alga to the slug then just the few genes we have found so far. It would be extremely interesting to know the amount and composition of the transferred algal DNA, and whether or not it is all together or in several locations in the host genome. Clearly, the best way to determine the genomic context of the transferred material awaits genomic sequencing, but we are currently attempting to compare flanking sequences of *fcp*, *Lhcv 1* and *Lhcv 2* in the slug and alga which may provide some insight.

In conclusion, our results are the first demonstration of the lateral transfer of functional nuclear genes between two multicellular organisms. While chloroplast symbioses are common among sacoglossan species, chloroplast survival ranges from only a few days in some, but lasts the entire annual life cycle in *E. chlorotica* (Clark et al., 1990), making it a species of particular interest with which to examine lateral gene transfer. In addition, not only is the transfer of interest, but understanding the entire process of successful synthesis, targeting and functioning of proteins in a foreign cytoplasm is of high potential significance.

Acknowledgements

This work was supported by NSF grant IBN 0315227. We thank Kathleen Hotchkiss for help with production of the figures.

REFERENCES

Andersson, J.O., Sjögren, Å.M., Davis, L.A.M., Embley, T.M., and Roger, A.J. 2003. Phylogenetic analyses of diplomonad genes reveal frequent lateral gene transfers affecting eukaryotes. *Current Biology* **13**: 94–104.

Clark, K.B., Jensen, K.R., and Stirts, H.M. 1990. Survey for functional kleptoplasty (chloroplast symbiosis) among West Atlantic Ascoglossa (=Sacoglossa) (Mollusca: Opisthobranchia). *Veliger* **33**: 339–345.

Delwiche, C.F. 1999. Tracing the thread of plastid diversity through the tapestry of life. *American Naturalist* **154**: S164–S177.

Goto, T., Nakai, M., and Ikuta, K. 1998. The life-cycle of human immunodeficiency virus type 1. *Micron* **29**: 123–138.

Green, B.J., Li, W-Y., Manhart, J.R., Fox, T.C., Summer, E.J., Kennedy, R.A., Pierce, S.K., and Rumpho, M.E. 2000. Mollusc-algal chloroplast endosymbiosis. photosynthesis, thylakoid protein maintenance, and chloroplast gene expression continue

for many months in the absence of the algal nucleus. *Plant Physiology* **124**: 331–342.

Hanten, J.J. and Pierce, S.K. 2001. Synthesis of several light-harvesting complex I polypeptides is blocked by cycloheximide in symbiotic chloroplasts in the sea slug, *Elysia chlorotica* (Gould): A case for horizontal gene transfer between alga and animal? *Biological Bulletin* **201**: 33–44.

Jain, R., Rivera, M.C., Moore, J.E. and Lak, J.A. 2002. Horizontal gene transfer in microbial genome evolution. *Theoretical Population Biology* **61**: 489–495.

Jansson, S., Green, B., Grossman, A.R., and Hiller, R. 1999. A proposal for extending the nomenclature of light-harvesting proteins of the three transmembrane helix type. *Plant Molecular Reports* **17**: 221–224.

Kennedy, D. and Norman, C. 2005. What don't we know? *Science* **309**: 75–102.

Martin, W. and Herrmann, R.G. 1998. Gene transfer from organelles to the nucleus: how much, what happens and why? *Plant Physiology* **118**: 9–17.

Merchant, S. and Dreyfuss, B.W. 1998. Posttranslational assembly of photosynthetic metalloproteins. *Annual Review of Plant Physiology Plant Molecular Biology* **49**: 25–51.

Mondy, W.L. and Pierce, S.K. 2003. Apoptotic-like morphology is associated with the annual synchronized death of a population of kleptoplastic sea slugs (*Elysia chlorotica*). *Journal of Invertebrate Biology* **122**: 126–137.

Pierce, S.K., Massey, S.E., Hanten, J.J., and Curtis. N.E. 2003. Horizontal transfer of functional nuclear genes between multicellular organisms. *Biological Bulletin* **204**: 237–240.

Pierce, S.K., Maugel, T.K., Rumpho, M.E., Hanten, J.J., and Mondy, W.L. 1999. Annual viral expression in a sea slug population: Life cycle control and symbiotic chloroplast maintenance. *Biological Bulletin* **197**: 1–6.

Pierce, S.K., Biron, R.W., and Rumpho, M.E. 1996. Endosymbiotic chloroplasts in molluscan cells contain proteins synthesized after plastid capture. *Journal of Experimental Biology* **199**: 2323–2330.

Reith, M. 1995. Molecular biology of rhodophyte and chromophyte plastids. *Annual Review of Plant Physiology Plant Molecular Biology* **46**: 549–575.

Stern, D.B., Higgs, D.C., and Yang, J. 1997. Transcription and translation in chloroplasts. *Trends in Plant Science* **2**: 308–315.

West, H.H., Harrigan, J., and Pierce, S.K. 1984. Hybridization of two populations of a marine opisthobranch with different developmental patterns. *Veliger* **26**: 199–206.

Wolkowicz, R. and Nolan, G.P. 2005. Gene therapy progress and prospects: Novel gene therapy approaches for AIDS. *Gene Therapy* **12**: 467–476.