Transfer, integration and expression of functional nuclear genes between multicellular species

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(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 I 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
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 \( I_r \) (NEB, Ipswich, MA), and then precipitated with isopropanol. The precipitated DNA was washed with 75% ethanol, resuspended in nuclese 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 (1:1 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 (Clontech, Mountain View, CA) and random nonamers as per manufacturer’s instructions.

**Primer design**

Oligonucleotide sequence primers (Operon Biotechnologies, 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>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Touchdown temperature range</th>
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| \( fcp \) | Forward: 5' GCTGGATTCACATCTGATGAGC 3'  
Reverse: 5' GGGTCTGCTGATCTAACCAG 3'   | 65-55°C                     |
| \( Lhcp\) 1 | Forward: 5' GTGGAATAAAAGCAGCAAG 3'   
Reverse: 5' CCCCCATGTTAAGCCTC 3'      | 60-50°C                     |
| \( Lhcp\) 2 | Forward: 5' GGCATCAATCTCAGCCCG 3'    
Reverse: 5' CTTTCCGTTATACCTTT 3'      | 60-50°C                     |
| \( V.\ litorea\ ITS\) (Green et al., 2000) | Forward: 5' CCAACGTATTCATCTGCTGC 3'  
Reverse: 5' ATGGCGATTGCTGCTGC 3'      | 52-40°C                     |

Table 1. Primer sequences used in the PCR reactions.
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 mucopolysaccharides, 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 chlorophyceae 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 larval 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 \textit{Lhcv} 2 PCR product (363 bp) from the adult cDNA (Accession number EF559306) was identical to that from \textit{V. litorea} Lhcv2 (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 \textit{V. litorea} genomic DNA in the \textit{E. chlorotica} samples, PCR reactions were performed on both the \textit{E. chlorotica} adult and veliger larvae genomic DNA's using primer sequences to \textit{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 \textit{A. californica}, a non-keleptoplastic sea slug, with the \textit{fcp}, \textit{Lhcv} 1-2 and \textit{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.

\textbf{Figure 1.} Agarose gel of the PCR products obtained from the reactions of \textit{V. litorea} genomic DNA or cDNA and primer sequences made to \textit{fcp}, \textit{Lhcv} 1 and \textit{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).

\textbf{Figure 2.} Agarose gel of the PCR products from the reactions of veliger larvae or adult \textit{E. chlorotica} genomic DNA and primer sequences made to \textit{fcp}, \textit{Lhcv} 1 and \textit{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.

\textbf{Figure 3.} Agarose gel of the PCR products from the reactions of \textit{E. chlorotica} mRNA sequences (as cDNA) and primer sequences made to \textit{fcp}, \textit{Lhcv} 1 and \textit{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**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>E. chlorotica cDNA</td>
<td>GCTGGATTCTACTACTGATGGCGATGAAAGACATCCAAACCCTGACGTGGCCCATGAAAAATC</td>
</tr>
<tr>
<td>E. chlorotica larva genomic</td>
<td>AAGCATTGCGCCCGTGTACGTGGCCGCAATGGAAAGACATTTCAACCCCTGACGTGGCCCATGAAAAATC</td>
</tr>
<tr>
<td>E. chlorotica adult genomic</td>
<td>CTTGGATTCTACTACTGATGGCGATGAAAGACATTTCAACCCCTGACGTGGCCCATGAAAAATC</td>
</tr>
<tr>
<td>V. litorea genomic</td>
<td>AAGCATTGCGCCCGTGTACGTGGCCGCAATGGAAAGACATTTCAACCCCTGACGTGGCCCATGAAAAATC</td>
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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**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. chlorotica cDNA</td>
<td>GTGGGAAATAAACACGGAGCGCATCGCCATGCTCGCCACCATCGGATACATCGTCCCTTAT</td>
</tr>
<tr>
<td>E. chlorotica larva genomic</td>
<td>TCCGGTGTGGCTCACCTGCTGGCCAGCAAGAGACAGGGGACTCAGGTGTTCTACCAG</td>
</tr>
<tr>
<td>E. chlorotica adult genomic</td>
<td>TACTTTAAACTCTTCCCAGAAGGCAAACCGGGAACCGAGGCGCTCACAACCATCCCACAG</td>
</tr>
<tr>
<td>V. litorea genomic</td>
<td>TACTTTAAACTCTTCCCAGAAGGCAAACCGGGAACCGAGGCGCTCACAACCATCCCACAG</td>
</tr>
</tbody>
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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.
E. chlorotica species. Furthermore, the presence of the genes in the pre-hatched veliger larvae confirms that they are being passed through the horizontal transfer of functional genes between two multicellular organisms. Thus, they represent an actual example of the horizontal transfer of genetic material.

Addition to our earlier work demonstrating the synthesis of FCP and LHC I-6 in the symbiotic plastids (Hanten and Pierce, 2001; Pierce et al., 2003) show that the genes are not only present in the V. litorea genome, but also are transcribed and translated in the slug cell.

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 I-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 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

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 I-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 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.

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GENE TRANSFER FROM ALGAE TO SLUGS

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 (Steen 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 than 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 fc1, 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


