Research Article

Algae 2015, 30(4): 303-312
http://dx.doi.org/10.4490/algae.2015.30.4.303
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Transcriptome analysis of the short-term photosynthetic sea slug *Placida dendritica*

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The intimate physical interaction between food algae and sacoglossan sea slug is a pertinent system to test the theory that "you are what you eat." Some sacoglossan mollusks ingest and maintain chloroplasts that they acquire from the algae for photosynthesis. The basis of photosynthesis maintenance in these sea slugs was often explained by extensive horizontal gene transfer (HGT) from the food algae to the animal nucleus. Two large-scale expressed sequence tags databases of the green alga *Bryopsis plumosa* and sea slug *Placida dendritica* were established using 454 pyrosequencing. Comparison of the transcriptomes showed no possible case of putative HGT, except an actin gene from *P. dendritica*, designated as PdActin04, which showed 98.9% identity in DNA sequence with the complementary gene from *B. plumosa*, BpActin03. Highly conserved homologues of this actin gene were found from related green algae, but not in other photosynthetic sea slugs. Phylogenetic analysis showed incongruence between the gene and known organismal phylogenies of the two species. Our data suggest that HGT is not the primary reason underlying the maintenance of short-term kleptoplastidy in *Placida dendritica*.

Key Words: actin; *Bryopsis plumosa*; horizontal gene transfer; kleptoplastidy; *Placida dendritica*; transcriptome

INTRODUCTION

Photosynthesis in some sacoglossan sea slugs offers a unique model to study the possibility of horizontal gene transfer (HGT) between multicellular predator and prey. Sacoglossan mollusks ingest and actively maintain chloroplasts that they acquire from large coenocytic green algae and keep them for up to several months (Wägele et al. 2011). These kleptoplastidic associations vary greatly in terms of specificity of the animal towards its algal prey and in retention time and functionality of the captured plastids (e.g., Rumpho et al. 2011, Klochkova et al. 2013). The basis for long-term maintenance of photosynthesis in these sea slugs has often been explained by extensive HGT from the nucleus of the alga to the animal nucleus, followed by expression of algal genes in the gut to provide essential plastid-destined proteins (Bhattacharya et al. 2013).

Sacoglossan mollusk *Placida dendritica* Alder et Hancock is bonded avidly to its specific algal food *Bryopsis* spp. (Klochkova et al. 2010). When it develops from a veliger larva to a small animal, at final stage of metamorphosis it attaches to and consumes only *Bryopsis* plants for the rest of its life cycle. In north Atlantic waters, *P.*

Received June 18, 2015, **Accepted** October 29, 2015
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Fig. 1. Feeding of *Placida dendritica* on the protoplasm of *Bryopsis plumosa*. (A) Sea slug attached to *B. plumosa* filament and punctured its cell with sharp radula. (B) A fine stream of algal protoplasm is seen through the transparent head of sea slug, entering its digestive system as it feeds.

dendritica is commonly found associated with *Codium fragile* (Suringar) Hariot (Evertsen and Johnsen 2009), but most Korean samples were collected on *Bryopsis* spp. In laboratory culture with mixed diet algae, the animals consumed only *Bryopsis* spp. and less than 10% of them switched to *Codium minus* (Schmidt) Silva or *Derbesia tenuissima* (Moris et De Notaris) P. L. Crouan et H. M. Crouan, even when no *Bryopsis* was available (Klochkova et al. 2010). Chloroplasts were observed in the digestive tract of *P. dendritica* and were found to be functional for a short time (Klochkova et al. 2010).

In this study, we present initial results from the comparative analysis of transcriptomes of *P. dendritica* and its food alga *B. plumosa* that suggest the maintenance of photosynthesis in the sea slug is not directly related with the horizontally transferred genes from algae. However, possible case of putative HGT was found, such as an actin gene from *P. dendritica*.

MATERIALS AND METHODS

Adult animals of *P. dendritica* (Fig. 1) were collected from Wando, southern coast of Korea (34°19′37.32″ N, 120°48′43.55″ E). Collected sea slugs were washed with filtered artificial seawater and kept in marine IMR medium at 15°C with 12 : 12 h L : D cycle and 15 μmol m^{-2} s^{-1} light intensity and without food.

For transcriptome analysis, sea slugs were kept without any food algae (i.e., starved) for 28 days and the Petri dish with culture medium was changed every day. Egg ribbons were harvested from one week after starvation, when no chloroplasts remained in the digestive tract of the sea slugs, because defecation stopped by that time and their body color was turning yellowish by each passing day. The harvested eggs were rinsed with 3% H_2O_2, frozen in liquid nitrogen and kept in deep freezer (-70°C) until use. Other sacoglossan sea slugs, *Elysia atroviridis* Baba and *Elysia nigrocapitata* Baba, were collected from the same locality. The animals were maintained using same method as for *P. dendritica*.

All algae (Appendix 1) were cultured in marine IMR medium at 20°C with 12 : 12 h L : D cycle and 15 μmol m^{-2} s^{-1} light intensity.

Isolation of total RNA and mRNA purification

Total RNA from *B. plumosa* and *P. dendritica* was isolated using Trizol (MRC Inc., Cincinnati, OH, USA) according to manufacturer's protocol. Thirty animals of *P. dendritica*, which had been starved for 28 days, were used. Isolated RNA was quantified spectrophotomertically (260 and 280 nm). mRNA was purified using Oligotex (Qiagen, Valencia, CA, USA) following manufacturer's instructions. Double-strand cDNA was synthesized using Just cDNA Double-stranded cDNA Synthesis Kit (Agilent Technologies, Palo Alto, CA, USA) following manufacturer's instructions. The cDNA was then sent to Macrogen (http://www.macrogen.com/eng/) for 454 pyrosequencing. The library preparation, GS-FLX titanium sequencing, assembly and annotation of sequencing data were carried out by Macrogen (Appendix 2). To analyze the sequence data a web-based pipeline program for expressed sequence tag (EST) data analysis was established (http://genebank2.kongju.ac.kr).

Genomic DNA isolation, polymerase chain reaction (PCR) and sequence determination

DNA was purified from algal samples from different localities (Appendix 1) and the eggs of sea slugs using Intron i-genomic plant DNA extraction mini kit or CTAB DNA extraction mini kit (Intron Biotechnology, Seoul, Korea) following the manufacturer's instructions. Isolated DNA was diluted to 10 ng μL^{-1} of concentration and directly used to PCR reaction. Specific primers were designed based on pyrosequencing database (Appendix 3). PCR was performed as follows: an initial denaturation at 95°C for 4 min, followed by 35 cycles of amplification (denaturation at 94°C for 30 s, annealing at 55°C for 40 s, and extension at 72°C for 1.5 min) with a final extension at 72°C for 10 min.

Bioinformatics

Transcriptome of *B. plumosa* was compared with that of *P. dendritica* by local blast program based on nucleotide sequence (BlastN) from BioEdit ver. 7.0 (Ibis Therapeutics, Carlsbad, CA, USA). All the contigs and singletons of the two species were compared and a table of similar genes was generated. The contigs and singleton sequences smaller than 200 bp were removed from the data set. BlastN parameters were set to expectation value >$1.0e^{-60}$ and identity >85% using BLOSUM62 matrix. Genes were selected by keyword searches from the final spreadsheets obtained from the above annotation process. Functional annotation was used to obtain matches with the following terms: photosynthesis, chlorophyll, light harvesting, intracellular transport, metabolic processes (carbohydrates, lipids, and proteins), organelle organization and biogenesis. Additional homology searches were conducted by comparing our translated EST database directly with the comprehensive chloroplast protein database of *Arabidopsis thaliana* (plastid protein database: http://www.plprot.ethz.ch and AT-Chloro database: http://www.grenoble.prabi.fr/at_chloro) with a cut-off E-value of e^{-50} (Kleffmann et al. 2004). The BioEdit sequence alignment editor program (ver. 7.2.3) was used for sequence homology analysis. An EST database of *Dictyostelium discoideum* Raper obtained from NCBI EST database (dbEST ID: 13952321) was compared with the assembly results of *B. plumosa* using the method described above. As there were no significant matches at the BlastN parameters above, the parameters were lowered to expectation value >$1.0e^{-20}$ and identity >75% using BLOSUM62 matrix.

Phylogenetic analysis

The actin sequences examined in this study were aligned with the actin sequences from GenBank using MacClade 4.08. The nucleotide alignment contained 65 sequences and was trimmed to 778 nucleotide comparable positions without third codon position using Paup*v. b10. Maximum likelihood analysis was performed using RAxML 7.0.4 with rapid bootstrapping option and 1,000 replicates under GTR + I + Γ model. Tree was visualized and graphic versions were exported using FigTree v1.4.0. New actin sequences generated in this study have been deposited in NCBI under the accession numbers listed in Appendix 4. Accession numbers for the sequences from NCBI used to construct phylogenetic tree are listed in Appendix 5.

RESULTS AND DISCUSSION

Functional annotation on *P. dendritica* transcriptome showed no putative gene related to the following terms: light and dark reaction of photosynthesis, chlorophyll assimilation, and light harvesting complex. Additional homology searches comparing *P. dendritica* transcriptome with the comprehensive chloroplast protein database of *A. thaliana* (Kleffmann et al. 2004) showed no significant match, except for some ribosomal genes with a cut-off E-value of e^{-50}.

Comparison of two large-scale ESTs databases of *B. plumosa* and *P. dendritica* showed few candidates of putative HGT except an actin homologue (Table 1). Nine actin homologues were isolated from *P. dendritica* EST database and three from *B. plumosa* (Table 2). One actin homologue from *P. dendritica*, designated as PdActin04, showed 98.9% identity in DNA sequence with the complementary gene from *B. plumosa*, BpActin03, while all the other genes, including other actin homologues, ribosomal proteins, and tubulin genes of the two species showed much lower similarity (≤86%) (Table 1). Full sequence of PdActin04 was obtained from genomic PCR using the egg cells of *P. dendritica*. Highly conserved homologues (93-99% of DNA sequence identity) of this gene were found in eight other ulvophyceaen algae (Appendix 6). However, PdActin04 homologue was not found in the eggs of other related sacoglossan species (*Elysia atroviridis*, *E. nigrocapitata*), which also feed on *Bryopsis* spp. The sequence difference between BpActin03 and PdActin04 was similar to that between species of *Bryopsis*. Most DNA substitution among BpActin03 homologues of ulvophyceaen

algae were synonymous; the translated amino acid sequences were almost identical (>99.7%) to each other. It is noteworthy that three DNA substitutions occurring in PdActin04 were not synonymous and not observed in any other green algae (Fig. 2).

Phylogenetic analysis showed incongruence between the actin gene and known organismal phylogenies of the animals and algae (Fig. 3). Surprisingly, all BpActin03

Table 1. Results of BLAST analysis between the transcriptomes of *Bryopsis plumosa* and *Placida dendritica*

B. plumosa unigene No.	*P. dendritica* unigene No.	Identity (%)	Aligned length (nt)	No. of different nucleotides	Gap	E-value	Candidate gene / Organism
Contig03964	GKI0DVN02FWMUH	98.91	460	2	3	0.0	Actin / *Vannella ebro*
GG4S1ZQ01BMRNG	GKI0DVN02F5TO3	86.36	396	54	0	3.00E-97	Tubulin alpha subunit / *Monocercomonoides* sp.
GG4S1ZQ01DCBGZ	GKI0DVN02IJE3H	84.75	459	67	3	4.00E-90	Beta tubulin / *Bicosoecida* sp.
GG4S1ZQ01A4RL0	GKI0DVN02HT1UO	85.86	382	52	2	4.00E-84	Beta tubulin / *Bicosoecida* sp.
GG4S1ZQ01DRYND	GKI0DVN02HT1UO	85.86	382	52	2	4.00E-84	Beta tubulin / *Bicosoecida* sp.
GG4S1ZQ01ANI4F	GKI0DVN02JFK1H	86.13	346	48	0	2.00E-82	Beta actin / *Lates calcarifer*
Contig02740	GKI0DVN02GALET	86.88	320	42	0	2.00E-80	Ribosomal protein L4
Contig05555	GKI0DVN02HLG12	82.82	483	81	2	2.00E-74	40S ribosomal protein S15 / *Zea mays*
Contig06211	GKI0DVN02ITH6G	81.89	486	88	0	8.00E-70	Tubulin alpha chain / *Micromonas pusilla*
Contig01502	GKI0DVN02INZTJ	82.77	412	70	1	8.00E-64	Alpha tubulin 2 / *Micromonas* sp.

Genes are listed in the order of highest sequence identity.

Table 2. Actin homologues from *Placida dendritica* and *Bryopsis plumosa*

	Unigene No.	E-value	Species (BlastN)	E-value	Species (BlastX)
Placida dendritica					
PdActin01	PdContig08492	0.0	*Aplysia californica* (X52868)	6e-176	*Mizuhopecten yessoensis* (Q26065)
PdActin02	PdContig22473	8e-122	*Urechis unicinctus* (GU592178)	4e-58	*Molgula oculata* (AAC28358)
PdActin03	Pdcontig23501	0.0	*Elysia timida* (HP148203)	3e-145	*Hippoglossus hippoglossus* (ACZ63697)
PdActin04	PdGKI0DVN02FWMUH	5e-174	*Pyrocystis lunula* (AF508263)	1e-71	*Vannella ebro* (AAQ55798)
			Vannella ebro (AY294151)		
PdActin05	PdGKI0DVN02HC94M	0.0	*Aplysia californica* (EZ114794)	3e-48	*Hyriopsis cumingii* (ADG26659)
PdActin06	PdGKI0DVN02F6MNO	1e-75	*Hypsibius klebelsbergi* (HM238268)	4e-87	*Gromia oviformis* (AAT42195)
PdActin07	PdGKI0DVN02H3D9W	e-100	*Rhizamoeba* sp. (EU273459)	3e-43	*Rhizamoeba* sp. (ACA04833)
PdActin08	Pdcontig03862	2e-15	*Elysia timida* (HP141184)	5e-25	*Gallus gallus* (AAA48570)
PdActin09	Pdcontig14953	0.0	*Placobranchus ocellatus* (HP176466)	6e-108	*Biomphalaria glabrata* (AAN31639)
Bryopsis plumosa					
BpActin 01	Bpcontig00392	1e-149	*Oryza sativa* (CT831215)	3e-168	*Chlamydomonas reinhardtii* (XP001699068)
BpActin 02	Bpcontig4636	3e-37	*Candida glabrata* (FN394021)	3e-161	*Nannochloris bacillaris* (BAA25911)
BpActin 03	Bpcontig3964	0.0	*Pyrocysitis lunula* (AF508263)	0.0	*Vannella ebro* (AAQ55798)
			Vannella ebro (AY294151)		

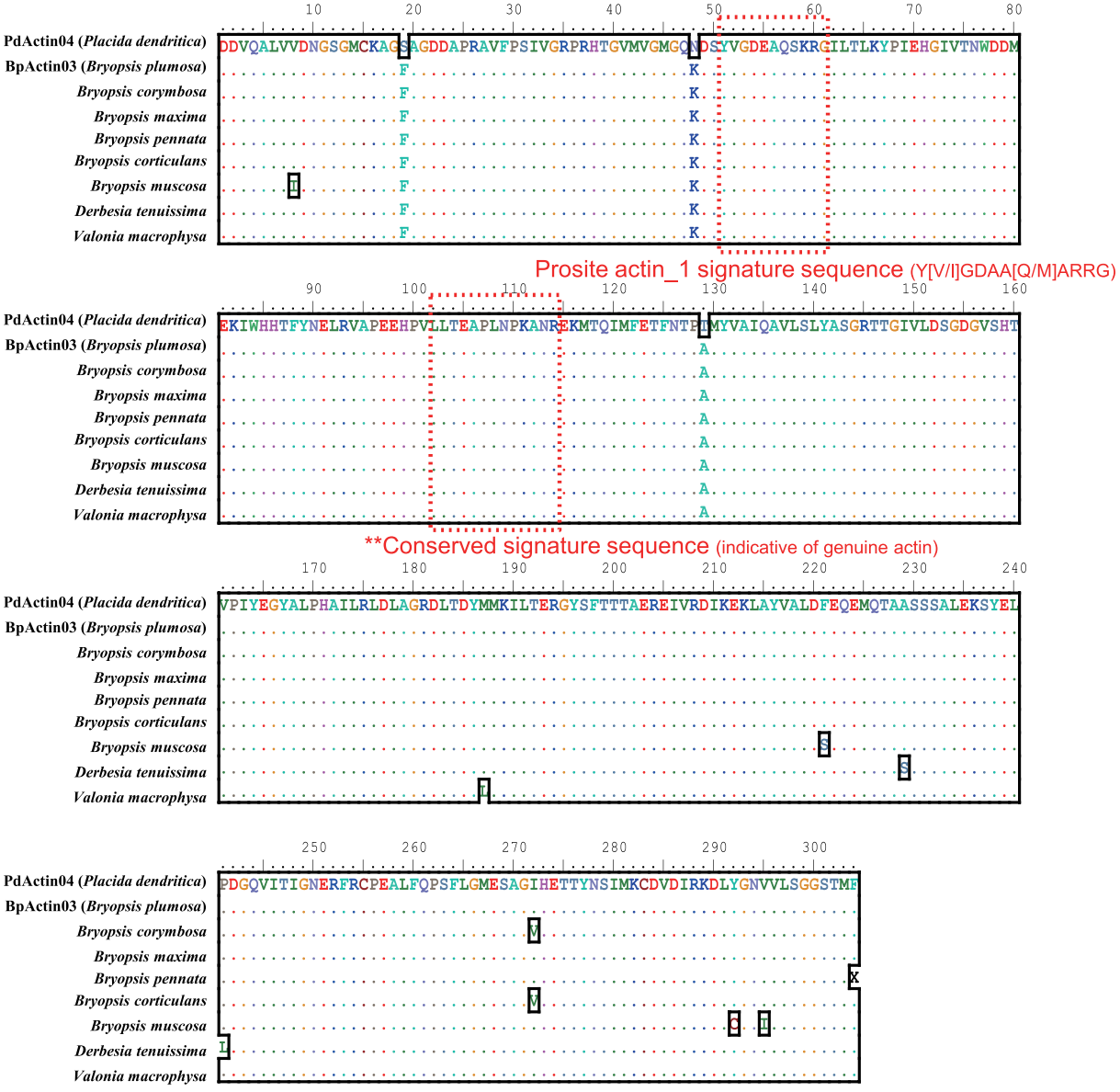

Fig. 2. Aligned amino acid sequence of BpActin03 and homologues isolated from *Placida dendritica* and ulvophyceaen green algae. Signature sequences of actin are marked in the dashed boxes.

Table 3. Results of BLAST analysis between the transcriptomes of *Bryopsis plumosa* and *Dictyostelium discoideum*

B. plumosa	*D. discoideum*	Identity (%)	Aligned length (nt)	No. of different nucleotides	Gap	E-value	Candidate gene
Contig03964	AU284282	87.51	1,121	140	0	0	Actin
Contig00173	AU034477	78.42	329	71	0	5.00E-17	26S proteasome ATPase 2 subunit
Contig07345	AU284686	83.23	310	50	2	2.00E-46	Rho GTPase
Contig00066	C92100	79.7	266	54	0	4.00E-20	Cyclophilin B
Contig06145	C24683	85	260	37	2	2.00E-47	ADP-ribosylation factor
Contig03691	C92100	81.75	252	46	0	7.00E-31	Cyclophilin B
Contig06316	AU034961	87.76	245	28	2	4.00E-60	Ras GTPase

Genes are listed in the order of highest sequence homology.

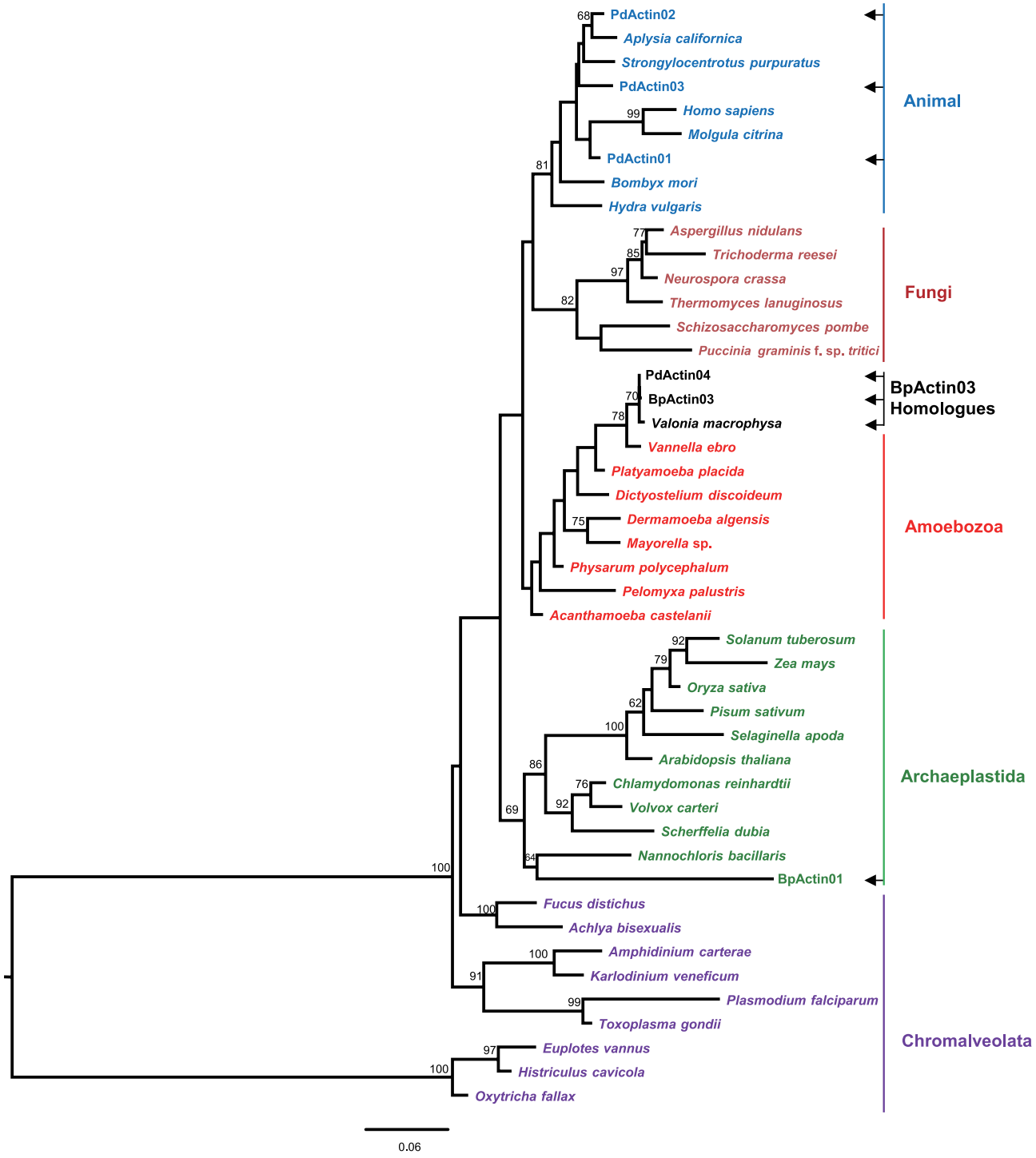

Fig. 3. Phylogenetic analysis of the first and second codon positions (778 nt) of actin genes. This tree was identified with maximum-likelihood method and has been out-rooted with ciliate actin sequences. The bootstrap values (1,000 replicates) are shown above nodes. Only the values over 60% are shown. Arrows show the actin genes detected in this study.

homologues were grouped within a branch of Amoebozoa, while other actin genes from the two species were grouped appropriately in the Archaeplastida and Opisthokonta (Fig. 3). To check if PdActin04 homologues are from simultaneous contamination of some Amoebozoa species, the transcriptomes of *P. dendritica* and *B. plumosa* were compared with that of an amoeba *D. discoideum*. No genes from two EST databases showed significant homology with that of *D. discoideum* (Table 3).

Our results did not show any photosynthesis-related genes in *P. dendritica* transcriptome, which suggest that HGT may not be the primary reason underlying the maintenance of photosynthesis in this mollusk. To become an active nuclear-encoded functional chloroplast protein and return to the plastid, the transferred gene must be assimilated into the host nuclear genome, acquire a transit sequence for targeting the protein to the organelle and be transcribed and processed back into the chloroplast (Bhattacharya et al. 2013). It would be less surprising that a common cytosolic gene like actin which may not require all the steps described above has been successfully transferred and transcribed between two intimately associated organisms. The specialized feeding and use of algal organelles by the sacoglossan mollusks also support the possibility of HGT among them.

The gold standard for identifying HGT with confidence is phylogenetic incongruence and this occurs if there is strong conflict between the phylogenies of the gene and of the organism (Keeling and Palmer 2008). The incongruence between the gene and known organismal phylogenies of the herbivore and algae did not support that PdActin04 has been horizontally transferred from its food algae. PdActin04 and all of its homologues found in green algae were nested in a branch of the Amoebozoa phylogeny (Fig. 3). It is possible that BpActin03 homologues are from a common amoeba contaminating all algal strains as well as the sea slugs simultaneously. If the contamination was from laboratory culture, all BpActin03 homologues should have the same sequence.

However, DNA sequences of nine BpActin03 homologues were clearly different among species. It means there must be nine different amoebas specifically contaminating algal strains, as well as the sea slug. It is hard to believe that each algal strain, collected from different localities of the world in different times, carry a specific amoeba and never mixed during years of laboratory culture. Most of all, the sequence difference among the homologues reflected the phylogenetic distance among ulvophyceaen algae; species closely related showed less sequence difference (Appendix 6). DNA sequence homology among BpActin03 homologues ranged 93-99%.

Direct comparison of large EST databases enabled us to avoid a long-lasting concern with HGT studies, that of contamination of targeted genes. If the materials used for building EST database of *P. dendritica* were contaminated with *B. plumosa* the transcriptomes of the two species would share many genes in common. Simultaneous contamination of the two EST databases by a common amoeba would also reveal more genes in common between *B. plumosa* and *P. dendritica*, not just one actin gene. Although all these evidences indicate that BpActin03 homologues are not from a contaminating amoeba, the questions about how and when this actin gene transferred to green algal lineage still remains.

CONCLUSION

The intimate physical interaction between herbivore and food algae may lead to horizontal transfer of certain genes. The short-term kleptoplastidy occurring in *Placida dendritica* does not seem to be based on any genetic incorporation from the food algae, *Bryopsis* spp. An interesting actin lineage was found and gene was isolated as a candidate of putative HGT between them, but the incongruence between the gene and known organismal phylogenies did not support the possibility of HGT. Highly conserved actin gene lineage found in this study may be useful in interpreting the evolutionary relationship among higher level of taxa.

ACKNOWLEDGEMENTS

This research was supported by Golden Seed Project, Ministry of Agriculture, Food and Rural Affairs (MAFRA), Ministry of Oceans and Fisheries (MOF), Rural Development Administration (RDA) and Korea Forest Service (KFS). This research was also a part of the project titled 'Development of selection techniques of suitable industrial variety in Korean coast', funded by the Ministry of Oceans and Fisheries, Korea. This research was also partially covered by Kongju National University grant to GHK for sabbatical year. Authors express sincere thanks to Dr. G. C. Zuccarello for his critical and very helpful comments regarding phylogenetic results. We also thank Dr. J. A. West for loaning two algal strains used in this study.

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Appendix 1. List of unialgal strains used in this study and information on their collection site or source

Species	Collection locality, date, collector
Bryopsis corticulans	Gangneung, Korea; Nov 7, 2007; Coll.: Klochkova, T. A.
Bryopsis corymbosa	Peter the Great Bay, Japan Sea, Russia; Sep 2003; Coll.: Klochkova, T. A.
Bryopsis maxima	Wando, Chondori, Korea; Feb 2008; Coll.: Klochkova, T. A.
Bryopsis muscosa	Gangneung, Korea; Nov 7, 2007; Coll.: Klochkova, T. A.
Bryopsis pennata	Jeju Island, Korea; Oct 19, 2007; Coll.: Klochkova, T. A.
Bryopsis plumosa	Kacheon, Korea; Apr 2003; Coll.: Klochkova, T. A.
Derbesia tenuissima (No. 4303, sporophyte)[a]	White Beach, Batanes Prov. Philippines; Apr 1987
Valonia macrophysa (No. 1528)[a]	Gran Comoro Islands; Mar 1975

[a]Strains obtained from culture collection of J. A. West.

Appendix 2. Overview of 454 pyrosequencing and assembly results

Characteristics	*Bryopsis plumosa*	*Placida dendritica*
Total number of reads	396,752	567,033
Total number of bases (bp)	158,414,036	247,212,265
Assembled reads	330,192	180,409
Singletons	25,403	98,858
Repeat (reads)	326	164,025
Contigs number (length ≥ 500 bp)	5,710	8,656
Bases (contigs)	5,215,088	6,962,221
Average contig size	913	804

Appendix 3. Primers used for polymerase chain reaction in *Bryopsis plumosa* actin gene study

Actin name (UniGene No.)	Primer name	Direction	Sequences
BpActin01 (Bpcontig 392)	BP_392F_1	Forward	CCTGAACCCAAGAACCATTGCTGCT
	BP_392SF_2	Forward	ACGAGGCCGAGCTCCTCAAGT
	BP_392R_1	Reverse	CCTCATCATCCTTTCCGATGAGAG
	BP_392SR_2	Reverse	GGAGTTGTAAGTGGTGCCAGT
BpActin02 (Bpcontig 4636)	BP_4636F_1	Forward	AGCGTCGACTTCGGAACGGAGG
	BP_4636R_1	Reverse	GGCAGAAACCTGCTTGTGCGA
BpActin03 (Bpcontig 3694)	BP_3694F_12	Forward	ACGATGATGTTCAAGCTCTTGT
	BP_3694SF_1	Forward	ATGAACTTAGAGTTGCTCCAG
	BP_3694R_905	Reverse	GGAACATAGTTGATCCACCGGA

Appendix 4. NCBI accession numbers for the sequences newly generated in this study

Organism	Designated actin strain name	GenBank accession No.
Bryopsis corticulans	BcorActin01	KT950951
Bryopsis corymbosa	BcoryActin01	KT950952
Bryopsis maxima	BmaxActin01	KT950953
Bryopsis muscosa	BmusActin01	KT950954
Bryopsis pennata	BpenActin01	KT950950
Bryopsis plumosa	BpActin01	KT950948
	BpActin03	KT950949
Derbesia tenuissima (No. 4303, sporophyte)[a]	DtActin01	KT950955
Valonia macrophysa (No. 1528)[a]	ValActin01	KT950956
Placida dendritica	PdActin01	KT950957
	PdActin02	KT950958
	PdActin03	KT950959
	PdActin04	KT950960

[a]Strains obtained from culture collection of J. A. West.

Appendix 5. NCBI accession numbers for the sequences used to construct phylogenetic tree in this study

Organism (GenBank accession No.)	
Acanthamoeba castelanii (V00002)	*Oryza sativa* (AC092557)
Achlya bisexualis (X59936)	*Oxytricha fallax* (U63567)
Amphidinium carterae (EU742738)	*Pelomyxa palustris* (AY294156)
Aplysia californica (U01352)	*Physarum polycephalum* (M21501)
Arabidopsis thaliana (U39449)	*Pisum sativum* (U76190)
Aspergillus nidulans (XM_659054)	*Plasmodium falciparum* (M22719)
Bombyx mori (NM_001126253)	*Platyamoeba placida* (AY294153)
Chlamydomonas reinhardtii (D50839)	*Puccinia graminis* f. sp. *tritici* (XP_003323586)
Dermamoeba algensis (AY294159)	*Scherffelia dubia* (AF061018)
Dictyostelium discoideum (M14146)	*Schizosaccharomyces pombe* (D84318)
Euplotes vannus (AF273753)	*Selaginella apoda* (AF090969)
Fucus distichus (U11697)	*Solanum tuberosum* (X55752)
Histriculus cavicola (Y12047)	*Strongylocentrotus purpuratus* (J01202)
Homo sapiens (BC012597)	*Thermomyces lanuginosus* (X07463)
Hydra vulgaris (M32364)	*Toxoplasma gondii* (U10429)
Karlodinium veneficum (GQ152584)	*Trichoderma reesei* (CAA53173)
Mayorella sp. (AY294152)	*Vannella ebro* (AY294151)
Molgula citrina (L21915)	*Volvox carteri* (M33963)
Nannochloris bacillaris (AB013098)	*Zea mays* (J01238)
Neurospora crassa (XM_956040)	

Appendix 6. Aligned DNA sequence of BpActin03 and homologues isolated from *Placida dendritica* and ulvophyceaen green algae