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Comparative genomic analysis of the Hsp70s from five diverse photosynthetic eukaryotes

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Abstract We have identified 24 members of the DnaK subfamily of heat shock 70 proteins (Hsp70s) in the complete genomes of 5 diverse photosynthetic eukaryotes. The Hsp70s are a ubiquitous protein family that is highly conserved across all domains of life. Eukaryotic Hsp70s are found in a number of subcellular compartments in the cell: cytoplasm, mitochondrion (MT), chloroplast (CP), and endoplasmic reticulum (ER). Although the Hsp70s have been the subject of intense study in model organisms, very little is known of the Hsp70s from early diverging photosynthetic lineages. The sequencing of the complete genomes of *Thalassiosira pseudonana* (a diatom), *Cyanidioschyzon merolae* (a red alga), and 3 green algae (*Chlamydomonas reinhardtii*, *Ostreococcus lucimarinus*, *Ostreococcus tauri*) allow us to conduct comparative genomics of the Hsp70s present in these diverse photosynthetic eukaryotes. We have found that the distinct lineages of Hsp70s (MT, CP, ER, and cytoplasmic) each have different evolutionary histories. In general, evolutionary patterns of the mitochondrial and endoplasmic reticulum Hsp70s are relatively stable even among very distantly related organisms. This is not true of the chloroplast Hsp70s and we discuss the distinct evolutionary patterns between ''green'' and ''red'' plastids. Finally, we find that, in contrast to the angiosperms *Arabidopsis thaliana* and *Oryza sativa* that have numerous cytoplasmic Hsp70, the 5 algal species have only 1 cytoplasmic Hsp70 each. The evolutionary and functional implications of these differences are discussed.

INTRODUCTION

The heat shock 70 proteins (Hsp70s) are a ubiquitous protein family that is highly conserved across all domains of life (Gupta and Golding 1993; Karlin and Brocchieri 1998). The Hsp70s are chaperones and are crucial housekeeping proteins. They have roles in the transport of proteins across membranes into organelles, the folding of newly translated proteins, and the repair of misfolded proteins (Bukau and Horwich 1998; Hartl and Hayer-Hartl 2002; Mayer and Bukau 2005). During times of heat stress, certain Hsp70s are upregulated and participate in the refolding of denatured proteins (Bukau and Horowich 1998; Hartl and Hayer-Hartl 2002; Mayer and Bukau 2005). All Hsp70s possess 3 distinct domains: an N-terminal adenosine triphosphatase (ATPase) domain of approximately 400 amino acids, a substrate-binding domain of approximately 200 amino acids, and a highly variable C-terminal domain.

Eukaryotes possess at least 3 types of Hsp70s, each of which localizes to a different cellular compartment: cytoplasm, mitochondrion (MT), and endoplasmic reticulum (ER). In addition, photosynthetic eukaryotes also possess chloroplast (CP) localized Hsp70s. The Hsp70s targeted to specific subcellular compartments share a close evolutionary history (Boorstein et al 1994; Rensing and Maier 1994; Karlin and Brocchieri 1998; Nikolaidis and Nei 2004). Evolutionary analysis of the Hsp70s reveals that they have evolved via 2 different pathways: gene duplication with subsequent divergence (in the case of the ER and cytoplasmic Hsp70s) and endosymbiosis with lateral gene transfer to the nucleus (the MT and CP Hsp70s) (Boorstein et al 1994; Gupta and Golding 1993; Karlin and Brocchieri 1998). Although the evolutionary history of the Hsp70s has been of considerable interest, the taxonomic sampling in previous studies has been un-

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even, primarily due to a lack of complete genome sequence data. For instance, Lin et al (2001) compared the Hsp70s in the complete genome of *Arabidopsis thaliana* (an angiosperm) to those found in yeast. The great evolutionary distance in this comparison was due to the lack of any complete genome datasets for any other photosynthetic eukaryotes. The recent sequencing of the complete genomes of a diatom *Thalassiosira pseudonana*, a red alga *Cyanidioschyzon merolae*, and 3 green algae (*Chlamydomonas reinhardtii*, *Ostreococcus lucimarinus* and *O. tauri*), now allow us to conduct comparative genomics studies of the Hsp70s present in diverse photosynthetic eukaryotic lineages. The purpose of this study was to identify Hsp70 homologs, analyze trends of Hsp70 evolution, and examine hypotheses concerning the diversity of Hsp70s. We hope this work will facilitate future studies of Hsp70s in these and related species.

MATERIALS AND METHODS

Identification of algal Hsp70 homologs

We use the term algae or algal to refer to aquatic photosynthetic eukaryotes. Algae are a diverse group of organisms that all share plastids. Algae are not a monophyletic group and we cannot assume that the organisms themselves have close evolutionary relationships.

The Hsp70 sequences were obtained from the Joint Genome Institute (JGI) genome sites: *Thalassiosira pseudonana* v3.0 (http://genome.jgi-psf.org/thaps3/thaps3.home.html), *Chlamydomonas reinhardtii* v3.0 (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html), *Ostreococcus lucimarinus* v2.0 (http://genome.jgi-psf.org/Ost9901_3/Ost9901_3.home.html), and *Ostreococcus tauri* v.2.0 (http://genome.jgi-psf.org/Ostta4/Ostta4.home.html). The *C. merolae* genome site can be found at (http://merolae.biol.s.u-tokyo.ac.jp/). The databases were queried by both keywords (Hsp70 and heat shock protein 70) and sequence similarity using BLAST (Altschul et al 1997) searches with *A. thaliana* Hsp70 sequences (Lin et al 2001). We used an E-value cut-off of less than 0.001. The genome databases had differing levels of annotation and, in some genomes, multiple gene models for the same chromosomal locations were found during the searches. The most complete gene model for each chromosomal location was chosen for study. These gene models were compared to known expressed sequence tag (EST) sequences (see *EST database searches* section for details). The estimated molecular weights for each protein were determined by using the ProtParam program (Wilkins et al 1999).

Hsp70 protein nomenclature

To easily refer to the proteins discovered in the genome databases examined, we have designated the following naming system: for those HSP70 proteins from *Thalassiosira pseudonana*, Tphsp70-x; *C. merolae*, Cmhsp70-x; *C. reinhardtii*, Crhsp70-x; *O. lucimarinus* Olhsp70-x; *O. tauri;* Othsp70-x. The letter *x* denotes the protein number. This number is given so that the many Hsp70s in each genome can be identified individually. The list of the Hsp70s used in the phylogenetic analysis along with their gene accessions numbers is available in online Supplementary Materials.

Phylogenetic analysis

In order to understand the origins and evolution of the Hsp70s in the diverse species studied here, the Hsp70 protein sequences were imported into the BioEdit Sequence Alignment Editor program (v7.0.5; Hall 1999) and aligned with ClustalW (Thompson et al 1994). Further refinement of the alignment was performed by hand. In this alignment, we included the Hsp70s identified in the 5 genomes mentioned above. In addition, we included Hsp70 homologs from other eukaryotes for which complete genome sequences are available, including *Saccharoymces cerevisiae*, *Plasmodium falciparum*, *Plasmodium yoelii*, *Arabidopsis thaliana*, and *Oryza sativa*. Our choice of Hsp70s included in this alignment was guided by the availability of complete genome data and the desire to include taxa that are closely related to the photosynthetic eukaryotes. For example, the evolutionary jump from green algae to angiosperms (*A. thaliana* and *O. sativa*) is large, but this is due to the lack of available genome datasets. *P. falciparum* and *P. yoelii* were included because they are both apicoplexans, they have relictual plastids that are of red algal origin, and they represent an important early diverging eukaryotic lineage (Baldauf et al 2000; Keeling 2004a, 2004b). Additional *Plasmodium* and other parasitic protist genomes exist; however, addition of these genomes may unnecessarily include additional divergent or long branches in our analysis. The need to clarify the evolutionary relationships of the CP Hsp70s led us to include CP-genome encoded Hsp70s from 5 red algae (*Cyanidium caldarium*, *Gracilaria tenuistipitata*, *Porphyra haitanensis*, *Porphyra purpurea*, and *Porphyra yezoensis*), 1 cyroptophyte (*Guillardia theta*), and 1 diatom (*Odontella sinensis*). The DnaK proteins from the cyanobacteria *Synechocystis* sp strain PCC6803 and *Escherichia coli* also were included. Eighty-one sequences were in the final alignment. The full list of species and accession numbers is available in online Supplementary Materials.

For the phylogenetic analysis, we excluded the variable and difficult to align C-terminal domain. In addition, the variable N-terminal region containing transit or leader sequences also was excluded. The alignment was then of the highly conserved ATPase and peptide-binding domains. However, some amino acid insertions were present in just single or a few proteins; these regions were removed from

the alignment. The final alignment used for phylogenetic analysis is available as online Supplementary Materials.

The phylogenetic relationships of the Hsp70s were analyzed with 2 different phylogenetic tree construction methods: neighbor-joining (NJ) in MEGA v.4.0 (Kumar et al 2004) and Bayesian in MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003). In MEGA, distance matrices were generated using the pairwise deletion option with the Dayhoff amino acid matrix. One thousand bootstrap replicates were created and trees were generated using NJ for each replicate. The bootstrap values reported for each branch reflect the percentage of the 1000 trees that contained that branch.

In MrBayes, we first performed an initial analysis using the mixed amino acid model. This analysis was conducted as described in the program manual (section 4.2.2 in MrBayes v.3.1; Ronquist et al 2005) and determined that the best fixed-rate model of protein evolution for the alignment was the WAG model (Whelan and Goldman 2001). This model (WAG) then was used in our subsequent phylogenetic analysis. Metropolis-coupled Markov chain Monte Carlo (MCMCMC) from a random starting tree was initiated in the Bayesian inference and run 2 000 000 generations with a sample frequency of 1000, print frequency of 100, and 4 chains. Three of the 4 chains run were heated and 1 was cold. All other settings or priors were set to the default used in MrBayes. We determined that the chains converged (the average standard deviation of the split frequencies was below 0.01) after 250 000 generations; this was used as our ''burnin'' and the first 250 trees were discarded. A consensus was created from the remaining trees (1750) and is presented in Figure 2. The topology of the NJ tree was highly congruent with the Bayesian tree and therefore only the bootstrap values are reported for the NJ analysis.

EST database searches

Sequences obtained from the genome databases were used as queries in BLAST searches of available EST databases to determine if the genes are expressed. These EST databases can be found at the *C. merolae* genome site, a diatom site (http://avesthagen.sznbowler.com/), the Chlamy Center website (http://www.Chlamy.org/cgi-bin/webblast.pl), and an *O. tauri* EST site (http://bioinformatics.psb.ugent.be/blast/public/?project=ostreococcus).

The JGI *T. pseudonana* and *C. merolae* and *O. tauri* EST databases do not specify the conditions in which their ESTs were compiled. Therefore, for these species, there is no information on differential expression of Hsp70 EST sequences. However, the Chlamy Center database does list 7 different cDNA libraries from which ESTs were collected: core (normalized), S1D2 (normalized), deflagellation (pH shock and flagellum regrowth), gamete and zygote (nitrogen-deficient medium and collection during gametogenesis), and stress I, stress II, stress III. It is important to note that stress I and II cDNA libraries did not involve heat shock, but instead were grown in varying light conditions and TAP mediums with NO_3, NH_4, H_2O_2, and sorbitol. In addition, the stress III cDNA library was made from *Chlamydomonas reinhardtii* that had been exposed to different levels of copper (Shrager et al 2003). In the EST searches, only sequence matches of 95% sequence identity or higher were considered hits. A higher level of stringency could unnecessarily exclude true matches due to minor sequencing errors. A lower stringency could reflect a match to a closely related but still distinct homolog.

Subcellular predictions

The newly identified Hsp70 sequences were submitted to the prediction programs Psort, Predotar, and TargetP (Nakai and Horton 1999; Emanuelsson et al 2000; Small et al 2004) to determine their possible subcellular localization. Subcellular predictions also were based on the phylogenetic affinity or relationship of the proteins to other proteins with experimentally determined cellular locations (Heazlewood et al 2004).

RESULTS

The green algae

Five full-length Hsp70s from the DnaK subfamily were found in the *Chlamydomonas reinhardtii* nuclear genome (Table 1). Analysis of version 1 of the *C. reinhardtii* genome reported 7 Hsp70s (Schroda 2004); however, an analysis of version 2 and 3 data revealed that these additional 2 Hsp70 sequences are not complete with significant portions of usually conserved regions missing. Of the 5 *Chlamydomonas reinhardtii* Hsp70s, only 1, Crhsp70-3, is clearly a cytosolic protein. It has been established that cytosolic HSP70s have a conserved amino acid motif GP(T/K)(V/I)EEVD at their C-terminus (Boorstein et al 1994; Sung et al 2001). Crhsp70-3 contains the conserved cytosolic C-terminal sequence of GPKIEEVD and lacks any N-terminal signal or transit sequence (Fig 1). Crhsp70-3 is also clearly a member of the cytoplasmic Hsp70 family or lineage (Fig 2).

All of the other *C. reinhardtii* Hsp70 proteins possess some kind of transit sequence. Crhsp70-4 and Crhsp70-5 both possess N-terminal transit sequences and the C-terminal ER retention signal HDEL, suggesting that these are ER proteins. The subcellular prediction programs indicate that these are targeted to the ER (Table 2), and both of these proteins are members of the ER Hsp70 lineage (Fig 2). Crhsp70-1 also possesses an N-terminal transit sequences (Fig 1) and all of the subcellular prediction programs indicate that this is a CP protein (Table

Table 1 Algal HSP70 proteins of the DnaK subfamily

Protein genome location	Mol. wt. (kDa)	Protein ID
Chlamydomonas reinhardtii		
Crhsp70-1 3:3023718-3028013	72.0	126835
Crhsp70-2 22:934666-942280	65.3	137452
Crhsp70-3 64:375804-380164	72.5	185673
Crhsp70-4 7:1048770-1052948	72.6	133650
Crhsp70-5 7:1065515-1069662	71.2	133859
Ostreococcus lucimarinus		
Olhsp70-1 2:668085-672040	74.5	48839
Olhsp70-2 4:702550-704376	64.9	15148
Olhsp70-3 2:580148-582256	73.3	44780
Olhsp70-4 16:360937-363448	71.0	28169
Olhsp70-5 6:131857-133509	60.3	12592
Ostreococcus tauri		
Othsp70-1 02.0001:353442-355202	59.4	15909
Othsp70-2 04.0001:641503-643428	68.3	28024
Othsp70-3 02.0001:258622-260592	72.4	15769
Othsp70-4 17.0001:331347-333715	80.0	22076
Othsp70-5 06.0001:133906-135674	60.0	28374
Cyanidioschyzon merolae		
Cmhsp70-1 8:104737-106557	66.2	CMV163
Cmhsp70-2 12:511588-513660	74.9	CML205C
Cmhsp70-3 16:366191-368173	71.5	CMP145C
Cmhsp70-4 20:255544-257604	76.3	CMT579C
Thalassiosira pseudonana		
Tphsp70-1	65.3	YP_874583
Tpthsp70-2 7:843587-845880	72.2	269240
Tpthsp70-3 5:277722-280084	68.0	28189
Tpthsp70-4 6:1187020-1189401	71.2	269120
Tpthsp70-5 3:2132328-2134446	70.4	27656

Hsp, molecular weight; ID. Genome location refers to the chromosomal location except for *Chlamydomonas reinhardtii*. The genome sequence has not yet been mapped to the *C. reinhardtii* chromosomes. For this species, the location positions refer to scaffold location. Tphsp701 and Cmhsp70-1 are encoded in the chloroplast genome. The gene models for both Crhsp70-2 and Olhsp70-2 missed part of the N-terminal region (see *Results* section for details).

2). The phylogenetic placement of Crhsp70-1 within the lineage containing the CP-localized Hsp70s from *A. thaliana* and *O. sativa* (Fig 2) is consistent with this cellular location. The gene findings programs at the *C. reinhardtii* web site start Cmhsp70-2 at the MEG at positions 77–79 on the alignment (Fig 1). Our examination of the sequences 5′ of this ATG found addition sequence including a region coding for the conserved GIDLGTT region at resides 75–81 in Figure 1. With the addition of these amino acid residues, this protein appears to have a transit sequence but the true start of this protein is not known. EST clones that are an exact match to this gene are present in the EST databases but they are incomplete and do not include the start methionine. However, the phylogenetic placement of this protein clearly indicates that it is an MT-localized protein. Analysis of the EST data for all of the *C. reinhardtii* Hsp70s indicates that all the Hsp70 genes are expressed but that there is differential expression (Table 3). For instance, Crhsp70-2, Crhsp70-3, and Crhsp70-4 are not found in the core library but are found in other libraries.

Each *Ostreococcus* genome (*O. lucimarinus* and *O. tauri*) contains 5 Hsp70s. One is a nuclear-encoded CP Hsp70 (Olhsp70-1, Othsp70-1); another is an MT Hsp70 (Olhsp70-2, Othsp70-2). One ER Hsp70 (Olhsp70-3 and Othsp70-3), 1 cytoplasmic Hsp70 (Olhsp70-4, Othsp70-4), and finally 1 Hsp70 of uncertain location (Olhsp70-5 and Othsp70-5; Tables 1 and 2, Figs 1 and 2) exist. The cytoplasmic Hsp70s contain the conserved consensus motif, and the organelle-localized proteins have the required N-terminal transit sequences (Fig 1). Although all the *O. tauri* Hsp70s were represented in the EST database, the cytoplasmic Othsp70-4 was the most highly represented at 132 matches compared to between 1 and 8 matches for the other Hsp70s. The *O. tauri* CPHsp70, Othsp70-1, is shorter than most other CPHsp70 and Olhsp70-1. It is likely that this gene model is correct. No sequence homologous to the C-terminal region of Olhsp70-1 was found in the *O. tauri* genome. In addition, this short gene model is supported by EST data. The 2 proteins of uncertain location (Olhsp70-5 and Othsp70-5) are closely related to Tphsp70-3. Both Olhsp70-5 and Othsp70-5 are shorter than the other HSP70s and are lacking the variable C-terminal region. These gene models are consistent with EST data, indicating that these proteins are expressed. The phylogenetic placement, outside of the ER+cytoplasmic lineage, of these proteins is not affected by their lack of a C-terminal domain because, due to its high level of variability, this region was excluded from the phylogenetic analysis. We also performed phylogenetic analyses with an even shorter alignment (with less gaps for these proteins) and it had the same topology as the tree in Figure 2. Due to their placement outside of the other Hsp70 lineages, it is not possible to predict where they are found in the cell.

Based on analysis of the EST clone sequences it is clear that both the *O. lucimarinus* (Olhsp70-2) and *O. tauri* (Othsp70-2) MT Hsp70s have N-terminal extensions not reflected in the gene models found at the JGI genome sites. A full-length EST sequence with a 100% match to the DNA sequence of Othsp70-2 (clone ot04g04210) was identified in the *Ostreococcus* EST database. This EST clone contains a clear MT-target sequence. DNA sequence encoding this N-terminal region is present in the genome sequence for OtHsp70-2 and a very similar region was identified in Olhsp70-2; however, the exact start Met residue for Olhsp70-2 is still uncertain. The additional N-terminal sequence protein sequence is presented in Figure 1. Both Othsp70-2 and Olhsp70-2 are clearly members of the MT family of Hsp70s (Fig 2). This is very similar to the situation with the missing N-terminal region of the *C. reinhardtii* MT Hsp70.

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                                                                                                                  100
Crhsp70-1 ---------- ---------- ---------M PVQQMTSMRS QSLAGAPVAP VKAGRAGVSR RG-LAVSVRA EKVVGIDLGT TNSAVAAMEG GKPTIITN-A
Crhsp70-2 ---------- ---------- ---------- ---------- ------TKPS AHLDVFVRQP VCSASAGALS DAVIGIDLGT TNSCVAVMEG KSPRVIEN-A
Crhsp70-3 ---------- ---------- ---------- ---------- ---------- ---------- ------MGKE APAIGIDLGT TYSCVGVWQN DRVEIIAN-D
Crhsp70-4 ---------- ---------- ---------- --------MV GLGLIATLVA ASALASIPQA KAASPTTDKL GTVIGIDLGT TYSCVGVYKN GRVEIIAN-D
Crhsp70-5 ---------- ---------- ---------- ---------- -MAQWKAAVL LLALACASYG FGVWAEEEKL GTVIGIDLGT TYSCVGVYKN GRVEIIAN-D
Olhsp70-1 MYASSPHILT KSTRTFSVQG AAASSRAQRT FGAGRSVDLS TRKLTPGIDV LKSRRYSARV ARKQSLCVRA EKVVGIDLGT TNSAVAAMEG GSPTIVTN-A
Olhsp70-2 ---------- ---------- -------TTR DRVIFLYRQR YAARDAIAPE GGWLNARAAS AAFTRGYAKG GDVIGIDLGT TNSCVAVMEG KNARVIEN-A
Olhsp70-3 ---------- ---------- ---------- -MPIKRASYR NAVVCACASL FLFAVCALPI NAENPT-EIT GTVIGIDLGT TYSCVGAYVN GKVEIIAN-D
Olhsp70-4 ---------- ---------- ---------- ---------- ---------- ---------- -----MSKAE GPAIGIDLGT TYSCVGVWQH DRVEIIAN-D
Olhsp70-5 ---------- ---------- ---------- ---------- ---------- ---------- ---MDNVTLD EHVIGIDLGT TYSCVSVWRN GEAHVLTN-A
Othsp70-1 MYASAHSEFA VSARTNVKHR SAAPNATLRA FSSRPSTQLS SRSLTKGAVG LKCRQPSVRI TGRRSLVVRA EKVVGIDLGT TNSAVAAMEG GSPTIVTN-A
Othsp70-2 ---------- ---------- ----MRRFLT RCGHRAARRA VTRWNAPVSA SVEGRWMHAA PVLSRGYASG GSVIGIDLGT TNSCVAVMEG KNARVIEN-A
Othsp70-3 ---------- ---------- ---------- --------MR RLTLCTLLAV IFAAACAPQG RAESTTSEIT GTVIGIDLGT TYSCVGAYVN GKVEIIAN-D
Othsp70-4 ---------- ---------- ---------- ---------- ---------- ---------- -----MSKAE GPAIGIDLGT TYSCVGVWQH DRVEIIAN-D
Othsp70-5 ---------- ---------- ---------- ---------- ---------- ---------- ---MDNVTLD EYVIGIDLGT TYSCVSVWRN GEAHVLTN-A
Cmhsp70-1 ---------- ---------- ---------- ---------- ---------- ---------- ---------M AKVVGIDLGT TNSVIAVMEG GQPTVVPN-S
Cmhsp70-2 ---------- ---------- -MLRFLLRAG ARALHRAPAL ATLKVPEPVV ELRQPSLLPQ RRLRSTGAVQ GDVVGIDLGT TNSCVAIMEG KAPRVIEN-A
Cmhsp70-3 ---------- ---------- ---------- ---------- ---------- ---------- -------MSK AKAIGIDLGT TYSCVAVMEG NKVEIIAN-E
Cmhsp70-4 ---------- ---------- -MATRRFSYR RVSIWNWALV AVLVHVCCCL FGRAVLVGAK DASSGGGKIE GPVIGIDLGT TYSCVGVFKN GKVEIIAN-E
Tphsp70-1 ---------- ---------- ---------- ---------- ---------- ---------- ---------M NKVVGIDLGT TNSVVAAIEG GQPTVITN-A
Tphsp70-2 ---------- ---------- ---------- --MALQLAAR RGLLASSTSK SAFGGLSSAS SQLRFKSTDA GDVIGIDLGT TNSCVAIMEG RNARVIEN-S
Tphsp70-3 ---------- ---------- ---------- ---------- ---------- ---------- -------MSA EPIIGIDLGT TFSCVACWDD KTNKVEVIES
Tphsp70-4 ---------- ---------- ---------- ---------- ---------- ---------- -----MAQVT GESVGIDLGT TYSCVGVWQN DRVEIIAN-D
Tphsp70-5 ---------- ---------- ---------- ---------- ---------- ----MGMVSP LVSAQEETKV GTTIGIDLGT TYSCVGVFKN GRVEIMAN-D
Consensus ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----GIDLGT T-S------- ----------
                                                                                                                  200
Crhsp70-1 EGGRTTPSVV AFTKTGDRLV GQIAKRQAVV NPENTFFSVK RFIGRRMS-- EVGSESTQVP YRVIEDG-GN VKIKCPNAG- ---KDFAPEE ISAQVLRKLT
Crhsp70-2 EGARTTPSVI AFTDKGERLV GLPAKRQAVT NPTNTVYATK RLIGRGYDDP QTQKEAKMVP YKIVKAK-NG D-AWVEAAG- ---QQYSPSQ MGAFVLTKMK
Crhsp70-3 QGNRTTPSYV AFTDT-ERLI GDAAKNQVAM NPRHTVFDAK RLIGRKFSDP IVQADIKLWP FQVRAG-AHD VPEIVVSYK- NEEKVFKAEE ISSMVLIKMK
Crhsp70-4 QGNRITPSYV AFTDE-ERLI GDAAKNQATV NPKRTIYDVK RLIGRKFSDA DVQRDRKLVS YDIVDR-QG- KPYVAVDVK- GEQKVFSPEE ISAMILQKMK
Crhsp70-5 QGNRITPSYV AFTDE-ERLI GDAAKNQATV NPKRTVYDVK RLIGRKYEDK EVQRDKKLVS YDIVDR-QG- KPYVAVDVK- GEQKVFSPEE ISAMILQKMK
Olhsp70-1 EGGRTTPSVV AYTKTGDRLV GQIAKRQGVV NPENTFFSVK RFIGRKMD-- EVNSESKEIP YSVVNSA-GK VKIECPALG- ---KQFAAEE ISAQVLRKLC
Olhsp70-2 EGARTTPSMV AFTDKGERLV GQPAKRQAVT NPTNTLYATK RLIGRTFEDE HTQKEAKLVP YEIVKAS-NG D-AWVAAGG- ---KQYSPSQ VGAFVLQKMK
Olhsp70-3 QGNRITPSYV AFTDS-ERLI GDSAKNQASA NPTRTVFDAK RLIGRKFTDK EVQRDLKMFP FKVVDK-DS- KPCIEVELK- EGKKVFQAEE ISAMVLTKMK
Olhsp70-4 QGARTTPSYV AFTDS-ERLI GDSAKNQTAM NPMNTVFDAK RLIGRKFSEP QVQADIKDWS FKVEAG-EAD KPMIVVEFH- GEKKKFSAEE ISSMVLVKMK
Olhsp70-5 EGDRTTPSWV AFTEQ-GRLV GDAAKRQAAI NPKNTLFNIK RIIGRQYSE- -CAHELELMP FDVKEG-EGG KPIVSVDVN- GEKKDFAPEQ ISAMVLQKMK
Othsp70-1 EGGRTTPSVV AYTKSGDRLV GQIAKRQAVV NPQNTFASVK RFIGRKMD-- EVKSESKEIP YSVVNAS-GN VKIDCPALG- ---KQFAAEE ISAQVLRKLC
Othsp70-2 EGARTTPSMV AFTDKGERLV GQPAKRQAVT NPTNTLYATK RLIGRTFEDE HTQKEAKLVP YEIIKAP-NG D-AWVSAGG- ---KQYSPSQ VGAFVLQKMK
Othsp70-3 QGNRITPSYV AFTDT-ERLI GDSAKNQASA NPTRTVFDAK RLIGRKFTDK EVQRDLKMFP FKIVDK-DS- KPCIEIELK- DGKKIFQAEE ISAMVLTKMK
Othsp70-4 MGNRTTPSYV AFTDS-ERLI GDSAKNQTAM NPTNTVFDAK RLIGRKFSEP QVQADIKDWS FKVEAG-EHD KPMIAVDFH- GESKKFSAEE ISSMVLVKMK
Othsp70-5 EGDRTTPSWV AFTEQ-GRLI GDTAKRQAAV NPKNTLFNIK RIIGRQYSE- -CADDIALMP FDVKEG-EGG KPVISVEVG- GETKEFAPEQ ISAMVLQKMK
Cmhsp70-1 EGFRTTPSVV AYTKNGDLLV GQIAKRQAVI NPGNTFYSVK RFIGRKFS-- EIEQEAKQVP YPVQADGKGN VRIFCSAKD- ---KFFAPEE ISAQVLRKLV
Cmhsp70-2 EGQRTTPSVV AFTSSGERLV GIAAKRQAVT NPENTIFAAK RLIGRRYEDP EVQRDVKIMP YKIVRAD-NG D-AWVEAQG- ---NRYSPAQ IGAFVLQKMK
Cmhsp70-3 QGNRTTPSYV AFTET-ERLI GDAAKNQVAL NPENTVFDAK RLIGRKFSDP TVQEDMKHWP FKVVQG-PGD KPLIQVVAH- GDVKRFSPEE ISAMVLTKMK
Cmhsp70-4 QGNRITPSYV AFTDK-ERLI GDAAKNQLAL NPERTIFDVK RLIGRRFDEE TVQKDIKLLP YKVVNK-DG- KPYIRVEVRD GEVKTFSPEE ISAMILGKMK
Tphsp70-1 EGFRTTPSIV AYTKKQELLV GQLAKRQSVV NAENTFFSVK RFIGCKAD-- EISEESKELP YKVIKDSNGN IKIKCSSLN- ---KDFSPEE ISAQVIRKLI
Tphsp70-2 EGARTTPSVV AITDDSTRLV GMAAKRQAVT NPENTFYAVK RLIGRSFSDK EVKDIQGLVP YNIVKSD-NN DDAWVEARG- ---KKFSPSQ IGSMVLGKMK
Tphsp70-3 PSGRTCPSWV SFTKE-GKLV GTAAKSQVAS NPRNTVYDIK RIIGRSFTDP VTAEECKNFP FEVSEGGAHG EPKIVVEWR- GEKKELRPEE ISAMVLAELK
Tphsp70-4 QGNRTTPSYV AFTET-ERLI GDAAKSQAAM NASNTVFDAK RLIGRKFSDP GVQSDMKHWP FKVIPG-TGG TPIIEVEYK- GETKQFKAEE ISSMVLTKMK
Tphsp70-5 QGNRITPSYV AFMDNGERLV GDAAKNQATI NPENTVFDVK RLIGRNFSDK SVQADKKLVP YKIVSN-EN- KPMVEVDIE- GKPQRFAPEE VSAMILQKMK
Consensus ---R------ ---------- G--AK-Q--- N--------K RLIG------ ---------- ---------- ---------- ---------- ----------
```

Fig 1. Alignment of heat shock 70 proteins (HSP70s) amino acid sequences from *Chlamydomonas reinhardtii* (Cr), *Ostreococcus lucimarinus* (Ol), *Ostreococcus tauri* (Ot), *Cyandioschyzon merolae* (Cm), and *Thalassiosira pseudonana* (Tp). Amino acid residues 1–70 in the alignment include the variable N-terminal region. This region is absent in cytoplasmically localized HSP70s and contains the transit sequences for mitochondrion (MT), chloroplast (CP), and endoplasmic reticulum (ER) HSP70s. The much more highly conserved adenosine triphosphatase (ATPase) domain includes residues 70–475. This region displays considerable sequence conservation but also has regions of insertion or deletion of 1 to a few residues. The peptide-binding domain (residues 490–645) is extremely well conserved. The variable C-terminal region (645–760) is absent in some proteins and highly variable in others, and its function is not well established. It also contains ER and cytoplasmic consensus sequences. The cytoplasmic consensus sequence GP(T/K)(V/I)EEVD at residues 762–769 is in bold. The ER consensus sequence HDEL at residues 765–769 is underlined.

C. merolae: a red alga

Three Hsp70s from the DnaK subfamily have been found in the *C. merolae* nuclear genome (Table 1). Also an Hsp70 is in the *C. merolae* CP genome (Table 1). All of these Hsp70s are represented in the *C. merolae* EST database. Of the 4 *C. merolae* Hsp70s, only 1, Cmhsp70-3 (71.5 kDa), has a cytoplasmic Hsp70 sequence motif: GPTVEEVD (Fig 1). Cmhsp70-4 has a N-terminal transit sequence and ends in HDEL, suggesting that this could be an ER protein. This is consistent with its phylogenetic placement (Fig 2) and with the subcellular predictions (Table 2). Cmhsp70-2 also possesses an N-terminal transit sequence (Fig 1). The results of the subcellular predictions (Table 2) and the phylogenetic analysis indicate that Cmhsp70-2 is targeted to the mitochondria. The CP Hsp70, CmHsp70-1, is found within the larger plastid Hsp70s but is within the subfamily of CP-encoded Hsp70s, including Tphsp70-1 and other red algal and diatom CP Hsp70s (Fig 1).

Thalassiosira pseudonana: a diatom

Thalassiosira pseudonana has 5 Hsp70s. One Hsp70 (Tphsp70-1) is encoded in the CP genome (Table 1; Fig

```
                                                                                                                           300
Crhsp70-1 EDAAKFLN-- DKVEKAVITV PAYFNDSQRQ ATKDAGKIAG LEVLRIINEP TAASLAYGFD KK-------- --ANETILVF DLGGGTFDVS VLEVGDGVFE
Crhsp70-2 ETAEAYLG-- HPVSKAVITV PAYFNDSQRQ ATKDAGKIAG LEVLRIINEP TAAALAYGTD KK-------- --EG-LIAVY DLGGGTFDIS ILEIMGGVFE
Crhsp70-3 ETAQAFLGAD REVKKAVVTV PAYFNDSQRQ ATKDAGMIAG LEVLRIINEP TAAAIAYGLD KKDSG----- -LGERNVLIF DLGGGTFDVS LLTIEEGIFE
Crhsp70-4 DTAEAYLG-- KTVKHAVVTV PAYFNDAQRQ ATKDAGTISG LNVVRIINEP TAAAIAYGLD KKGG------ ---EKNILVF DLGGGTFDVS ILTIDNGVFE
Crhsp70-5 DTAEAYLG-- KTVKHAVVTV PAYFNDAQRQ ATKDAGTISG LNVVRIINEP TAAAIAYGLD KKGG------ ---EKNILVF DLGGGTFDVS ILTIDNGVFE
Olhsp70-1 DDAASFLG-- DAVTKAVVTV PAYFNDSQRQ ATKDAGQIAG IEVLRIINEP TAASLAYGFD RK-------- --SNETILIF DLGGGTFDVS VLEVGDGVFE
Olhsp70-2 ETAEAYLG-- HGVSQAVVTV PAYFNDAQRQ ATKDAGKIAG LDVLRIINEP TAAALSYGVD KK-------- --EG-LVAVY DLGGGTFDVS ILEISGGVFE
Olhsp70-3 ETAEAYLG-- KDIKHAVVTV PAYFNDAQRQ ATKDAGVIAG LNVARIINEP TAAAIAYGLD KKG------- ---EKNILVF DLGGGTFDVS ILTIDNGVFE
Olhsp70-4 EVAEAYLG-- KDIKNAVVTV PAYFNDSQRQ ATKDAAVISG LNCLRIINEP TAAAIAYGLD KRHEA----- NGAEKNVLIF DLGGGTFDVS LLTIEEGIFE
Olhsp70-5 ATAEAQLG-- VPITKAVVTV PAYFNDAQRR QTKDAGAIAG LDVLRIINEP TAAALAYGLD RREG--ENGE VIKNQCILVF DLGGGTFDVS LLNLQDGVFE
Othsp70-1 DDAATFLG-- DTVTKAVVTV PAYFNDSQRQ ATKDAGQIAG IEVLRIINEP TAASLAYGFD RK-------- --SNETILIF DLGGGTFDVS ILEVGDGVFE
Othsp70-2 ETAEAYLG-- SNVSQAVITV PAYFNDAQRQ ATKDAGKIAG LEVLRIINEP TAAALSYGVD KK-------- --EG-LVAVY DLGGGTFDVS ILEISGGVFE
Othsp70-3 ETAEAYLG-- KDIKNAVVTV PAYFNDAQRQ ATKDAGIIAG LNVARIINEP TAAAIAYGLD KKG------- ---EKNILVF DLGGGTFDVS ILTIDNGVFE
Othsp70-4 ETAEAYLG-- KEIKNAVVTV PAYFNDSQRQ ATKDAAVIAG LNCLRIINEP TAAAIAYGLD KRNEN----- NGAEKNVLIF DLGGGTFDVS LLTIEEGIFE
Othsp70-5 ATAEAQLG-- VPITKAVVTV PAYFNDAQRR QTKDAGAIAG LDVLRIINEP TAAALAYGLD RREG--ADGE VIKSQCILVF DLGGGTFDVS LLALQDGVFE
Cmhsp70-1 DSASQYLG-- EKVTQAVITV PAYFNDSQRQ ATKDAGKIAG LDVLRIINEP TAASLAYGLD KK-------- --SNEKILVF DLGGGTFDVS ILEIGDGVFE
Cmhsp70-2 ETAESFLG-- RTVNNAVITV PAYFNDAQRQ ATKDAGRIAG LNVLRIINEP TAAALAYGLD KAD------- --EGRVVAVY DLGGGTFDVS ILEISGGVFE
Cmhsp70-3 DIAESYLG-- TKVTDAVITV PAYFNDSQRQ ATKDAGTIAG LNVLRIINEP TAAAIAYGID KKTSD----- -NKERNVLIY DLGGGTFDVS LLSVDSGIFE
Cmhsp70-4 KIAEDYLG-- KPVKNAVVTV PAYFNDAQRQ ATKDAGTIAG LTVQRIINEP TAAAIAYGLD KGGS------ ---EKNILVF DLGGGTFDVT LLTLDDGVFE
Tphsp70-1 ADAKEYLG-- QDVTKAVITV PAYFNDSQRQ ATVDAGKIAG IEVLRIINEP TAASLAYGLD KK-------- --QNETILVF DLGGGTFDVS ILEVGDGIFE
Tphsp70-2 ETAEGFLG-- RDVTKAVVTV PAYFNDSQRQ ATKDAGKIAG LDVLRIINEP TAAALAYGMD KA-------- --DGKTIAVF DLGGGTFDVS ILEISGGVFE
Tphsp70-3 LAAERHLG-- REVKGAVITV PAHFNNQQRQ ATKDAGRIAG LDVKRIINEP TAAALSYGLH AKKEREESGA EQKKANVVIF DLGGGTFDVS VLAMDSGVFE
Tphsp70-4 EIAEAYLG-- KEVKNAVVTV PAYFNDSQRQ ATKDAGAISG LNVLRIINEP TAAAIAYGLD QK-------- -GEEKNVLIF DLGGGTFDVS LLTIEEGIFE
Tphsp70-5 STAETFLG-- KEIKNAVVTV PAYFNDAQRQ ATKDAGTISG MKVERIINEP TAAAIAYGMD KTGG------ ---ESNVLVF DLGGGTFDVT LLTIDNGVFE
Consensus ---------- -----AV-TV PA-FN-SQR- AT-DA-K--G ----RIINEP TAA---YG-- ---------- ---------- DLGGGTFD-- --------FE
                                                                                                                           400
Crhsp70-1 VLSTSGDTHL GGDDFDKRIV DFLADDFKKS E-GIDLR--- KDRQALQRLT EAAEKAKIEL SGMAQTSINL PFITATADGP KHIDTQLTRA KFEEMCNDLL
Crhsp70-2 VKATNGDTFL GGEDFDNTIL NYLVGEFKKE S-GIDLS--- KDRLAVQRLR EASEKAKCEL SSTTSTDINL PFITADASGP KHLNMQLTRA KLELLVKELL
Crhsp70-3 VKATAGDTHL GGEDFDERLV NHFANEFQRK Y-KKDLK--- TSPRALRRLR TACERAKRTL SSAAQTTIEL DSLFEGVD-- --FATSITRA RFEELCMDLF
Crhsp70-4 VISTNGDTHL GGEDFDQRVM EYFIKLIKKK Y-KKDIS--- GDARALQKLR REAERAKRAL SSQHQVRVEI EALYEGID-- --LSEPLTRA RFEELNMDLF
Crhsp70-5 VISTNGDTHL GGEDFDQRVM EYFIKLIKKK Y-KKDIS--- GDARALQKLR REAERAKRAL SSQHQVRVEI EALYEGID-- --LSEPLTRA RFEELNMDLF
Olhsp70-1 VLSTSGDTHL GGDDFDKRIV EWLAGDFEKS E-GIDLM--- SDKQALQRLT EAAEKAKMEL STTSSTSISL PFITATADGP KHIDTSLTRP KFEQLCDDLI
Olhsp70-2 VKATNGDTFL GGEDFDTVLL DHFVDNFKKD Q-GIDLK--- QDKLAVQRLR EAAEKAKIEL SSAQSTDINL PFITADASGP KHMAMTLSRA KLEELVGSLL
Olhsp70-3 VISTNGDTHL GGEDFDQRIM EYFMKLIKRK H-GKDVS--- GDVKAVQKLR REAERAKRTL SNQHQVRVEI EALFDGID-- --FSEPLTRA RFEELNNDLF
Olhsp70-4 VKATAGDTHL GGEDFDARLL QHFIAEFKRK N-KKDIT--- GNPKALRRLR SACERAKRTL SSTAQTSIEI DSLFEGVD-- --FYTSITRA RFEELCMDLF
Olhsp70-5 VLSTAGDTHL GGEDFDTSLA AFAQKEIEKE RGADIFT--- GDEKALRKLR TACEKAKREL S--VANHANI ECFIGEIE-- --INMKITRE QFEKVCEPTF
Othsp70-1 VLSTSGDTHL GGDDFDKRVV EWLADDFKKA E-GIDLK--- NDKQALQRLT EASEKAKMEL SSTSSTSISL PFITATADGP KHIDTSLTRT KFEQLCDDLI
Othsp70-2 VKATNGDTFL GGEDFDTVLL DYFVDNFKKD Q-GIDLK--- QDKLAVQRLR EAAEKAKIEL SSAASTDINL PFITADATGP KHMALQLTRA KLEELVGGLL
Othsp70-3 VISTNGDTHL GGEDFDQRIM EYFIKLIKRK H-GKDIS--- SDSKAVQKLR REAERAKRTL SNQHQVRVEI EALFDGMD-- --FSEPLTRA RFEELNNDLF
Othsp70-4 VKATAGDTHL GGEDFDNRLL QHFIQEFKRK H-KKDIT--- GNPKALRRLR TACERAKRTL SSTAQTSVEI DSLFEGVD-- --FYTSITRA RFEELCMDLF
Othsp70-5 VLSTAGDTHL GGEDFDTALA AYAQKEIEKQ KGSDIFA--- GDAKSLRKLR TACEKAKREL S--VANHANI ECFIGEHE-- --INLKVSRE EFNKVNEPTF
Cmhsp70-1 VLATSGDTHL GGDDFDKKIV DWLIDNWKRI E-GIDLS--- KDKQALQRLT EAAEKAKIEL SNVTQTDINL PFITATADGP KHLDQTLTRA QFEQLTSDLI
Cmhsp70-2 VKATNGDTHL GGEDFDNVLL NHLVSEFKKD Q-GIDLS--- RDRLALQRLR EAAEKAKVEL SSTMQTEINL PFITADASGP KHMNMKLTRA KFESLVDHLV
Cmhsp70-3 VRAVSGNSHL GGEDFDSRMV DYFVKEFKRK Y-KEDIT--- GNNRAMRRLR TACERAKRTL SSATQATIEI DSLYNGID-- --FYSSITRA KFEELCGDLF
Cmhsp70-4 VLATNGDTHL GGEDFDQRVM DYLVKQFKKK NDGLDLR--- TDKRAMAKLR REVEKAKRAL SYQTQVRIEI ESIMDGKD-- --LIEELTRA RFENLNMDLF
Tphsp70-1 VLSTAGDTNL GGDDFDKALV RWLVEDFEAK E-GTNLT--- KDIQALQRLT EAAEKAKMEL SNVEKTTINL PFITADKNGP KHIQQELTRE KFESLCQDLI
Tphsp70-2 VKATNGNTML GGEDFDEELL EYLMKTFKKD S-GIDLS--- GDNLAMQRLR EAAEKAKREL DGLAQTDVSL PFITADATGP KHLNIKITKA QFENMVSELV
Tphsp70-3 VKATGGDTHL GGEDFDNSVM KWCLEQI-KA KNADVWAKLQ GNPRAQSRLR RAVENAKRTL SSSMSTEIEV DSLVGDFD-- --FTVTLDRA KFEELNAVLF
Tphsp70-4 VKATAGDTHL GGEDFDNRMV DYFLQDFKRR T-KKDMS--- QNQRSLRRLR TACERAKRTL SSSTQAHIEI DSLFDGVD-- --FNSTITRA RFEDLCMDYF
Tphsp70-5 VLATNGDTHL GGEDFDQRVM QYFIKMMKKK S-NVDIS--- GDKRALQKLR KEVERVKRAL SSQQQARLEI EDLAEGFD-- --FSETLTRA RFEELNNDLF
Consensus -----G---L GGEDFD---- ---------- ---------- ---------- ---E-AK--- ---------- ---------- --------R- ----------
```

Fig 1. Continued.

2). An additional 4 genes for Hsp70s were identified in the JGI *Thalassiosira pseudonana* v3.0 nuclear genome (Table 1). All of these Hsp70s are represented in the Diatom EST database. Analysis of the alignment in Figure 2 reveals that Tphsp70-2 possesses an N-terminal leader sequence, suggesting either CP or MT localization (see also Table 2). Based on phylogenetic relationship (Fig 2) and subcellular prediction (Table 2) it is clear that Tphsp70-2 is MT protein. Tphsp70-4 contains the cytoplasmic amino acid motif (GPTIEEID). This evidence plus the placement of this protein within the cytoplasmic lineage suggests that it is cytoplasmically localized. Tphsp70-5 possesses a short N-terminal signal sequence and ends in DDEL (Fig 1), which indicates that this is ER localized. The ER location is consistent with Tphsp70-5 placement in the ER family in the phylogenetic tree (Fig 2). Tphsp70-3 lacks any N-terminal signal or transit sequence, suggesting a cytoplasmic location (Table 2; Fig 1). However, the C-terminal region is shorter than the cytoplasmic protein, and Tphsp70-3 lacks the cytoplasmic consensus region. Further, its placement outside of both the ER and cytoplasmic lineages (Fig 2) makes a prediction based on phylogenetic relationships problematic. At this time, the cellular location of this protein is unknown.

Gene family evolution

Analysis of the phylogenetic tree of Hsp70s in Figure 2 indicates that there are 5 well-supported lineages of Hsp70s. The first lineage (the branch leading to this lineage is labeled 1) includes all the MT-located Hsp70s (Fig 2), which are all nuclear encoded. The second major lineage includes the plastid Hsp70s. This lineage is closely related to *Synechocystis* DnaK1 and DnaK3, and it includes *Synechocystis* DnaK2. Within this CP lineage there are 2 distinct subfamilies. One subfamily includes the

```
                                                                                                                             500
Crhsp70-1 ERCKVPVQQA LRDAKLSISD IQEVILVGGS TRIPAVQEIV RKLSGG-KDP NVTVNPDEVV ALGAAVQAGV LAG------- ------EVSD IVLLDVTPLS
Crhsp70-2 ERTKQPCLQA MKDAGVQPKD IQEVLLVGGM TRMPKVNEIV KEVFQ--RDP SKGVNPDEVV AMGAAIQGGV LRG------- ------DVKD ILLLDVTPLS
Crhsp70-3 RKCMDPVEKC LRDAKMDKMT VHDVVLVGGS TRIPKVQQLL QDFFNG-KEL NKSINPDEAV AYGAAVQAAI LTGEG----- ----GEKVQD LLLLDVTPLS
Crhsp70-4 KKTMGPVKKA MDDANLKKTE IDEIVLVGGS TRIPKVQDLL REWFGG-KEP NKGVNPDEAV AYGAAVQGAI LSGEE----- ----EESTEG LIVIDRTPLS
Crhsp70-5 KKTMGPVKKA MDDANLKKTE IDEIVLVGGS TRIPKVQDLL REWFDG-KEP NKGVNPDEAV AYGAAVQGGI LGGEG----- ----GDEVKD ILLLDVAPLS
Olhsp70-1 NRCKVPVQQA LKDAKLSLAE VDEVILVGGS TRIPAIRELV KSLTK--KEP NMSVNPDEVV ALGAAVQAGV LAG------- ------EVSD IVLLDVTPLS
Olhsp70-2 ERTKQPCKNC LKDAGVSTGE ISEVLLVGGM SRMPKVQGIV KDLFG--RDP SKGVNPDEVV AMGAAIQGGV LRG------- ------DVKD ILLLDVTPLS
Olhsp70-3 RKTMGPVKKA MDDAGMKKSE IDEIVLVGGS TRIPKVQDLL RDFFDG-KEP NRGVNPDEAV AYGAAVQGGI LSGEG----- ----GDETKD ILLLDVAPLT
Olhsp70-4 RKCMDPVEKT LRDAKMDKSQ VHEVVLVGGS TRIPKVQQLL SDFFNG-KDL CKSINPDEAV AYGAAVQAAI LSGEG----- ----NEKVQD LLLLDVSPLS
Olhsp70-5 QRCLDSVKRV LSDAGKKKEE VDEIVLVGGS TRVPRVQGIL TEYFDG-KTL NKSVHPDEAV AYGAAVQGAI LAG------- --VRDKQTSR VLLMDVVPLS
Othsp70-1 NRCKVPVEQA LKDAKLSLSD VDEVILVGGS TRIPAVRELV KKLTS--KDP NMSVNPDEVV ALGAAVQAGV LAG------- ------EVSD IVLLDVTPLS
Othsp70-2 ERTKQPCKNC LKDAGVSTSE ISEVLLVGGM SRMPKVQQIV KDLFG--REP SKGVNPDEVV AMGAAIQGGV LRG------- ------DVKD ILLLDVTPLS
Othsp70-3 RKTMGPVKKA MDDAGMKKSE IDEIVLVGGS TRIPKVQDLL RDFFDG-KEP NKGVNPDEAV AYGAAVQGGI LSGEG----- ----GDETKD ILLLDVAPLT
Othsp70-4 RKCMDPVEKC LRDSKMDKSS VHEVVLVGGS TRIPKVQQLL SDFFNG-KEL CKSINPDEAV AYGAAVQAAI LSGEG----- ----NEKVQD LLLLDVSPLS
Othsp70-5 QRCMDAVKRV LSDASRKKEE VNEIVLVGGS TRVPRVQEIL TEYFDG-KPL NKSVHPDEAV AYGAAVQGAI LAG------- --VRDKQTSR VLLMDVVPLS
Cmhsp70-1 ERCRKPVEQA LTDAKLSKQD IDEVVLVGGS TRIPAVQQLV KDLLG--KQP NQSVNPDEVV AIGAAIQAGV LAG------- ------EVKN ILLLDVCPLS
Cmhsp70-2 QRTLEPMKLC LKDAGMSAKD ISDVLLVGGM TRVPAVQRLV QDFFG--RAP NKSVNPDEVV AMGAAIQGGV LRG------- ------DVKD ILLLDVTPLS
Cmhsp70-3 RSTLDPVERV LKDANLSKSQ VDDVVLVGGS TRIPKIQQLL SQFFNG-KEL CKSINPDEAV AYGAAVQAAI LSGHE----- ----SETTKD ILLLDVTPLS
Cmhsp70-4 RKTLKPVEIV LKDAKKEKKD IDEIVLVGGS TRIPKIQELI TEFFDG-KQP SKGINPDEAV AYGAAVQGAI LSGEG----- ----GETTKD ILLLDVTPLS
Tphsp70-1 NRCRIPVEKA LKDAKLDQSG INEVVLVGGS TRIPAIQQLV ESLTG--KKP NKSVNPDEVV AIGAAIQAGI LAG------- ------EITD ILLLDVTPLS
Tphsp70-2 QKTVDPCQKC MKDADVSKAE IHEVILVGGM TRMPKVQETV ENFFG--KKP SRGVNPDEVV AMGAAIQGGV LKG------- ------DVKD ILLLDVTPLS
Tphsp70-3 KRCIDTVNEV LNDAGCSQDE VTDLVLVGGS TRIPSLQTSL YDMFGGRIEL CKSVHPDEAV AHGAAVQGHI LATGGSGGGQ DLAGAEMTTD LLLLDVTPLS
Tphsp70-4 KKCMDPCEKV LRDAKIAKNQ VDEVVLVGGS TRIPKIQSML AEFFNG-KEP NKGINPDEAV AYGATVQAAI LSGADK---- ----SEKLSE LLLLDVTPLS
Tphsp70-5 KKTLGPVGRV LEDADVSKSE VDEIVLVGGS TRIPKVQSLI SEFFGG-KEP SKGINPDEAV AYGAAVQGGI LSGEG----- ----GDATSE ILLLDVTPLS
Consensus ---------- --D------- ------VGG- -R-P------ ---------- -----PDE-- --GA--Q--- L--------- ---------- ----D--PL-
                                                                                                                             600
Crhsp70-1 LGLETLGGVM TKLIPRNTTL PTSKSEVFST AADGQTSVEI NVLQGEREFA RDNKSLGTFR LDGIPPAPRG VPQIEVKFDI DANGILSVTA TDKGTSKKQD
Crhsp70-2 LGIETLGGVF TRMINRNTTI PTKKSQVFST AADNQTQVGI KVFQGEREMA ADNKLLGQFD LVGIPPAPRG VPQVEVTFDI DANGIVHVSA KDKATGKEQS
Crhsp70-3 LGLETAGGVM TVLIPRNTTI PTKKEQVFST YSDNQPGVLI QVYEGERART KDNNLLGKFE LTGIPPAPRG VPQINVIFDI DANGILNVSA EDKTTGNKNK
Crhsp70-4 LGIETTGGVM TNLIPRNSVI PTKKSQTFST AADNQPTVSI QVYEGERALT KDNHKLGQFD LNGIPPAPRG TPQIEVTFEV DANGILTVSA QDKGTGKKEK
Crhsp70-5 LGIETVGGVM TKLIPRNTVI PTKKSQTFTT YQDQQTTVSI QVYEGERAMT KDNHKLGQFD LNGIPPAPRG TPQIEVTFEV DANGILNVAA EDKGTGKKEK
Olhsp70-1 LGLETLGGVM TKLIPRNTTL PTSKSEVFST AADGQTSVEI NVLQGEREFV KDNKSLGNFR LDGIPSAPRG VPQIEVKFDI DANGILSVEA CDKGTGKKQD
Olhsp70-2 LGIETLGGVF TRLISRNTTI PTKKSQTFST AADNQTQVGI KVLQGEREMA ADNKTLGQFD LVGIPPAPRG VPQIEVTFDI DANGIVNVSA KDKATNKEQK
Olhsp70-3 QGIETVGGVM TKLIPRNTVI PTKKSQTFTT YQDNQQTVMI QVFEGERAMT KDNHLLGKFE LTSIPPAPRG VPQIEVTFEI DANGILNVGA EDKGTGKSEK
Olhsp70-4 MGLETVGGVM TVLIPRNTTI PTKKEQVFST YSDNQPGVLI QVFEGERSRT RDNHLLGKFE LSGIPPAPRG VPQINVCFDI DANGILNVSA EDKGSGQKNK
Olhsp70-5 LGVECEGRQF AKVVQRNTAI PCKKKSEFTT VYDNQDEIDV RIFEGERSNT DGNHLLGEFQ ISGIERASAG EPKIDVTFEV NTNGLLTVTA KDRVTGVEAN
Othsp70-1 LGLETLGGVM TKLIPRNTTL PTSKSEVFST AADGQTSVEI NVLQGEREFV KDNKSLGTFR LDGIPSAPLN ARTCSSTVAH LDVGSAFEPT SASHMLVQ--
Othsp70-2 LGIETLGGVF TRLISRNTTI PTKKSQTFST AADNQTQVGI KVLQGEREMA ADNKTLGQFD LVGIPPAPRG VPQIEVTFDI DANGIVNVSA KDKATGKEQK
Othsp70-3 QGIETVGGVM TKLIPRNTVI PTKKSQTFTT YQDNQQTVMI QVFEGERAMT KDNHLLGKFE LTSIPPAPRG VPQIEVTFEI DANGILNVGA EDKGTGKSEK
Othsp70-4 MGLETVGGVM TVLIPRNTTI PTKKEQVFST YSDNQPGVLI QVFEGERSRT RDNHLLGKFE LSGIPPAPRG VPQINVCFDI DANGILNVSA EDKASGQKNK
Othsp70-5 LGVECEGRQF AKVVPRNTSI PCKKKSEFTT VYDNQDEIDV RIFEGERQNT DGNHLLGEFQ ISGIERASAG EPKIDVTFEV NTNGLLTVTA KDRVTGVEAN
Cmhsp70-1 LGVETLGGIM TKMIPRNTTI PTRKTEIYST AVDNQTNVEI HVLQGERELA KDNKSLGTFR LDGIPPAPRG VPQIEVTFDI DANGILSVTA KERSTGKQQS
Cmhsp70-2 LGIETLGGVF TKLIPRNSTI PTKKSQVFST AADNQTQVGI KVLQGERPMA AENKLLGQFD LVGIPPAPRG VPQIEVIFDI DANGILHVSA VDKGTGKRNE
Cmhsp70-3 LGIETAGGVM TVLIPRNSTI PTKKSQIFTT YADNQPAVTI QVYEGERAMT KDNHLLGQFT LTGIPPAPRG VPQIEVTFDL DANGILNVTA VDKTTGKSER
Cmhsp70-4 LGIETVGGVM TKIINRGTTI PTKKSQIFTT HVDRQSSVLI QVYEGERVAT KDNHLLGKFE LTGIPPAPRG VPQIEVTFEI DANGILQVSA EEKTAGKREK
Tphsp70-1 LGVETVGGIM TKLISRNTTI PVKKSELFST AADNQTNVEI HVLQGEREVV SGNKSLGNFK LEGIPQAPKG KPQIEVTFDI NVDGILSVTA KENESGKEQN
Tphsp70-2 LGIETLGGVM TKLIPRNTTI PTKKQQTFST AVDNQPQVQI KVMQGEREMA ADNKNLGEFD LVGIPSAPRG VPQIEVSFDI DADGILNVSA KDKGTGKEQN
Tphsp70-3 LGIELEGKQM STLIKRNTAI PCKKTRTYTT VDDWQTEIDV VVFEGERPHV DANNKLGSFV ISGVQRARAG EPKVDVTFAL DANGILNVSA RDQVTGAEAR
Tphsp70-4 LGLETAGGVM TTLIKRNTTV PAKKTQTFST YADNQPGVLI QVFEGERSMT RDNNLLGKFN LDGIPPMPRG QPQIDVIFDM DANGILNVSA LEKSTGKENK
Tphsp70-5 QGIETVGGVM TKLINRGTTI PTKKSQTFST HQDNQPAVLI QVFEGERSMT KDNHLLGKFE LTGIPPAPRG VPQIEVSFEV DANGILQVSA EDKGTGKAEK
Consensus -G-E--G--- -----R---- P--------- --D------- ----GER--- --N--LG--- ---------- ---------- ---G------ ----------
```

Fig 1. Continued.

''green'' plastid Hsp70s (the angiosperm and green algal CP Hsp70s). These proteins are all nuclear encoded. The other CP Hsp70 subfamily or the ''red'' plastid lineage includes the *C. merolae* and *T. pseudonana* CP-encoded Hsp70s, along with other CP-encoded Hsp70s. Another major branch in the Hsp70 tree (branch 3) includes both the ER and cytoplasmic Hsp70s. Within this lineage there are the well-supported ER (branch 4) and cytoplasmic (branch 5) Hsp70 lineages. Three Hsp70s fall outside of the ER+cytoplasmic lineage: Tphsp70-3, Olhsp70-5, and Othsp70-5.

DISCUSSION

In this study, we identified Hsp70 homologs in 5 complete genomes: *C. reinhardtii*, *O. lucimarinus*, *O. tauri*, *T. pseudonana*, and *C. merolae*. We found that each species had between 4 and 5 Hsp70s, with at least 1 each belonging to CP, MT, ER, and cytoplasmic lineages. This is considerably less than the 14 Hsp70s present in the *A. thaliana* genome and the 18 Hsp70s in *O. sativa*. As we describe the relationships and evolutionary history of the Hsp70s, it is useful to review the features and evolutionary relationships of the species examined in this study.

C. merolae, a red alga, is a single-celled organism that lives in acidic hot springs (Matsuzaki et al 2004). Therefore, it is well adapted to high temperatures. *C. merolae* has a small and compact genome (Matsuzaki et al 2004). The marine diatom *T. pseudonana* is also single-celled, has a worldwide distribution, and like other diatoms has silicified cell walls (Armbrust et al 2004). It also has a relatively small genome. *C. reinhardtii* is a chlorophyte green alga and, as such, is more closely related to land plants than are the diatoms and red algae (Baldauf 2000; Yoon et al 2004). *Chlamydomonas* is also single-celled and is not known to be adapted to extreme temperatures or other

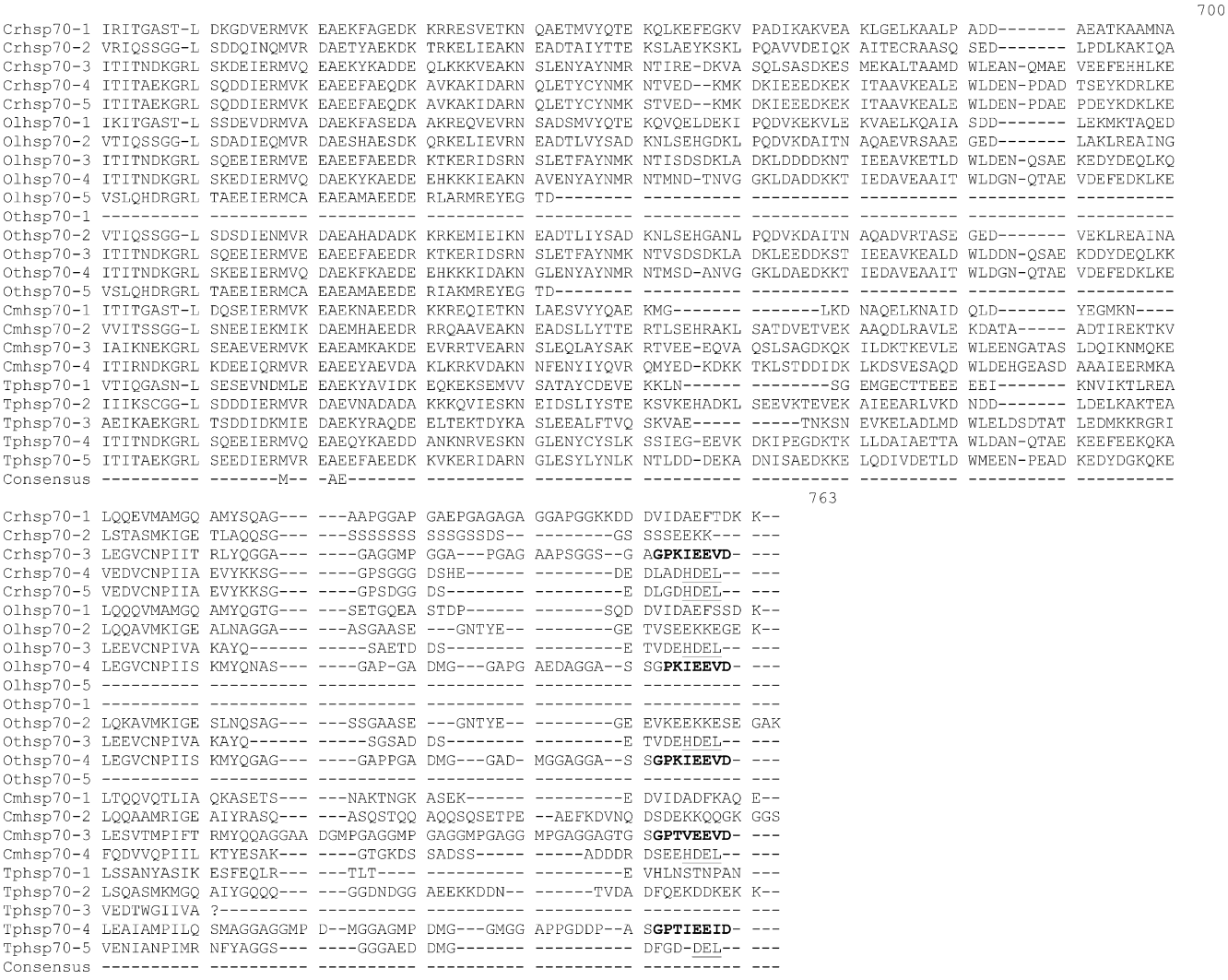
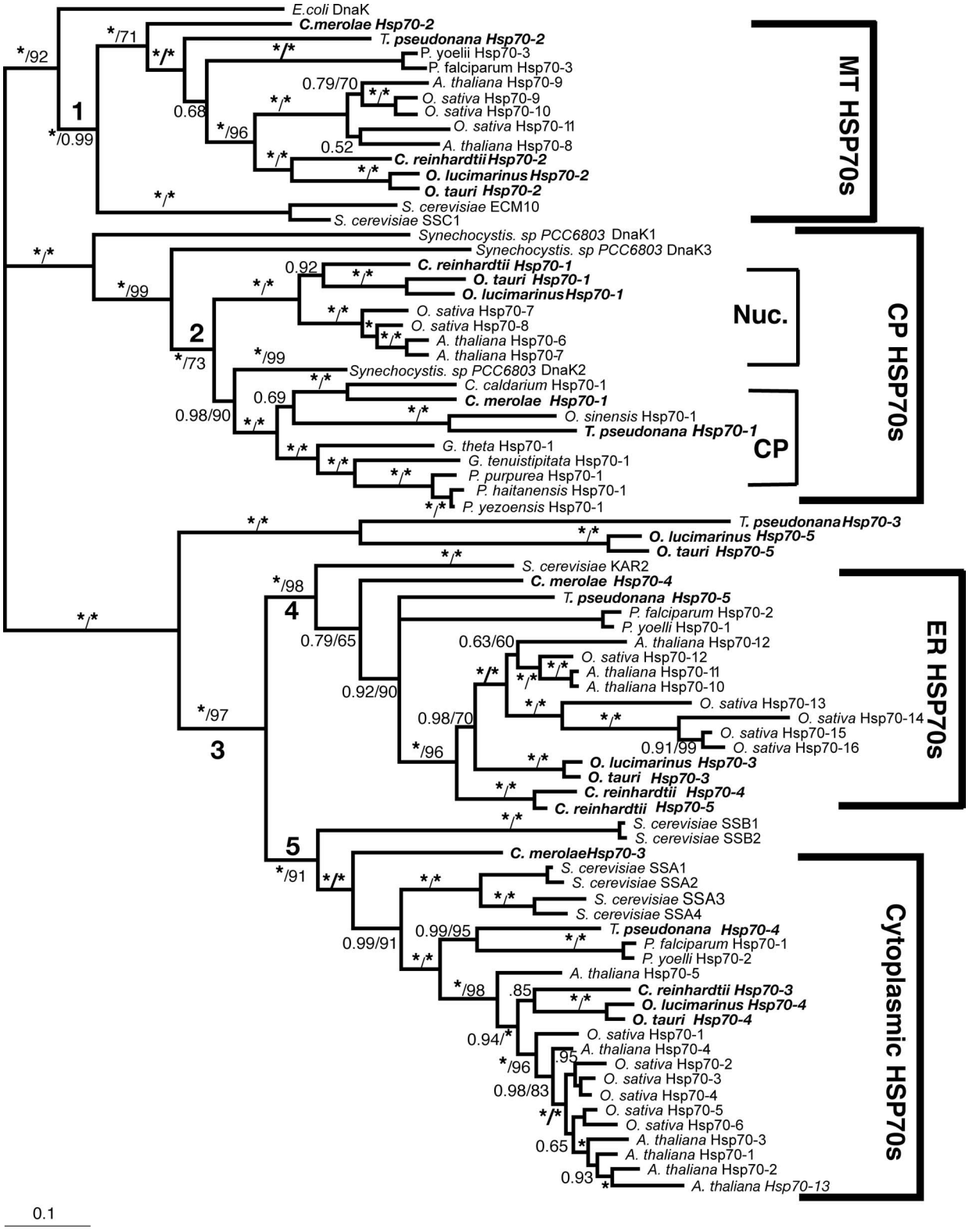


Fig 1. Continued.

extreme conditions. It does not possess a streamlined genome. The *Ostreococcus* isolates are very interesting. They are Prasinophytes and members of the green algal lineage. *O. tauri* and *lucimarinus* are extremely small single-celled organisms; in fact, it has been reported that they are the smallest known free-living eukaryotes. They, like *C. merolae*, have very small and highly dense genomes (Derelle et al 2006). Both are found in marine environments. *O. lucimarinus*, usually isolated from surface waters, is adapted to high light intensities. *O. tauri* most often is found deeper in the water column.

C. merolae, *C. reinhardtii*, *O. lucimarinus*, *O. tauri*, and *T. pseudonana* all have 1 nuclear-encoded MT Hsp70 protein. This is comparable to the number of MT Hsp70s found in other organisms. Each *Plasmodium* genome has 1, *S. cerevisiae* and *A. thaliana* have 2, and *O. sativa* has 3 MT Hsp70s. The mitochondrion evolved once, very early in eukaryote evolution, prior to the divergence of the major eukaryotic lineages (Embley 2006). The transfer to the nucleus of many MT-endosymbiont genes occurred soon after the establishment of this endosymbiont (Embley and Martin 2006). In fact, the presence of Hsp70 (and Hsp60) genes in the nucleus of eukaryotes that now lack MT has provided the key evidence that MT were gained once in evolution, with multiple subsequent losses (Embley 2006; Embley and Martin 2006). The relationships of the early diverging eukaryote lineages still are uncertain and a single protein phylogeny is not expected to resolve these relationships (Embley and Martin 2006). However, the relationships of the MT Hsp70s (Fig 2, branch 1) in this study mostly follow organismal relationships. The green plant lineage (green algae plus plants or Chlorobiota) forms a well-supported lineage. The red alga and diatom fall outside of this lineage with the *Plasmodium* species. The relative lack of resolution among the red alga, diatom, and *Plasmodium* species is not unexpected because


E.coli DnaK
C.merolae Hsp70-2
T. pseudonana Hsp70-2
P. yoelii Hsp70-3
P. falciparum Hsp70-3
A. thaliana Hsp70-9
O. sativa Hsp70-9
O. sativa Hsp70-10
O. sativa Hsp70-11
A. thaliana Hsp70-8
C. reinhardtii Hsp70-2
O. lucimarinus Hsp70-2
O. tauri Hsp70-2
S. cerevisiae ECM10
S. cerevisiae SSC1
Synechocystis. sp PCC6803 DnaK1
Synechocystis. sp PCC6803 DnaK3
C. reinhardtii Hsp70-1
O. tauri Hsp70-1
O. lucimarinus Hsp70-1
O. sativa Hsp70-7
O. sativa Hsp70-8
A. thaliana Hsp70-6
A. thaliana Hsp70-7
Synechocystis. sp PCC6803 DnaK2
C. caldarium Hsp70-1
C. merolae Hsp70-1
O. sinensis Hsp70-1
T. pseudonana Hsp70-1
G. theta Hsp70-1
G. tenuistipitata Hsp70-1
P. purpurea Hsp70-1
P. haitanensis Hsp70-1
P. yezoensis Hsp70-1
T. pseudonana Hsp70-3
O. lucimarinus Hsp70-5
O. tauri Hsp70-5
S. cerevisiae KAR2
C. merolae Hsp70-4
T. pseudonana Hsp70-5
P. falciparum Hsp70-2
P. yoelli Hsp70-1
A. thaliana Hsp70-12
O. sativa Hsp70-12
A. thaliana Hsp70-11
A. thaliana Hsp70-10
O. sativa Hsp70-13
O. sativa Hsp70-14
O. sativa Hsp70-15
O. sativa Hsp70-16
O. lucimarinus Hsp70-3
O. tauri Hsp70-3
C. reinhardtii Hsp70-4
C. reinhardtii Hsp70-5
S. cerevisiae SSB1
S. cerevisiae SSB2
C. merolae Hsp70-3
S. cerevisiae SSA1
S. cerevisiae SSA2
S. cerevisiae SSA3
S. cerevisiae SSA4
T. pseudonana Hsp70-4
P. falciparum Hsp70-1
P. yoelli Hsp70-2
A. thaliana Hsp70-5
C. reinhardtii Hsp70-3
O. lucimarinus Hsp70-4
O. tauri Hsp70-4
O. sativa Hsp70-1
A. thaliana Hsp70-4
O. sativa Hsp70-2
O. sativa Hsp70-3
O. sativa Hsp70-4
O. sativa Hsp70-5
O. sativa Hsp70-6
A. thaliana Hsp70-3
A. thaliana Hsp70-1
A. thaliana Hsp70-2
A. thaliana Hsp70-13
MT HSP70s
CP HSP70s
Nuc.
CP
ER HSP70s
Cytoplasmic HSP70s
0.1

they represent early diverging lineages. The phylogenetic patterns among the MT Hsp70s indicate a fairly consistent evolutionary pattern for this protein across organismal lineages. However, it is clear that 1 duplication of the MT Hsp70s occurred prior to the monocot-dicot divergence and an additional duplication occurred within the lineage leading to rice.

The chloroplasts also are derived from bacterial endosymbionts, but the CP Hsp70s (Fig 2, branch 2) have a very different evolutionary history compared to the MT Hsp70s. All of the algal species examined here have 1 CP Hsp70. Again we see evidence of gene duplication within the angiosperms because *A. thaliana* and *O. sativa* each have 2 CP Hsp70s. What is most notable concerning the CP Hsp70s is that the green algae and plants all have nuclear-encoded CP Hsp70s, and the red algae and diatoms have CP-encoded Hsp70s. Reith and Munholland (1991) were the first to report that a red alga, *Porphyra umbilicalis*, had a CP-encoded Hsp70. Now, with the complete genome of *C. merolae*, we know that red algae do not have also a nuclear-encoded CP Hsp70.

A short discussion of plastid evolution is useful here in our evaluation of the CP Hsp70s. It is now clear that there was a single origin of primary plastids. However, the primary green, red, and glaucocystophyte plastid lineages diverged very early in plastid evolution (Keeling 2004a). The primary plastids are the product of a single endosymbiotic event in which a nonphotosynthetic eukaryote engulfed a cyanobacterium (Keeling 2004a). The primary plastids in turn have been involved in numerous secondary endosymbiotic events (Keeling 2004a, 2004b). In secondary endosymbiosis, nonphotosynthetic eukaryotes engulf a photosynthetic eukaryote (usually either a green or red alga) with a plastid (Armbrust et al 2004; Keeling 2004b). Much of the red or green alga then disappears after becoming an endosymbiont, leaving a plastid with multiple membranes. The plastids in diatoms are a product of a secondary endosymbiosis of a red alga. Therefore, the CP genomes of red algae and diatoms are much more closely related than the red algal and diatom nuclear genomes. From this we might expect more similarity between the *C. merolae* and *T. pseudonana* CP Hsp70s than we see in the other nuclear-encoded Hsp70 homologs in these 2 species.

From our phylogenetic analysis (Fig 2) it is clear that, although the CP Hsp70s in all the species studied are derived from the cyanobacterial endosymbiont, the green and red algal Hsp70s form 2 distinct lineages. Although it has been reported that much of the endosymbiont genome was transferred to the nucleus prior to the split of the plastid lineages (green and red) (Martin et al 1998; Martin 2003; Keeling 2004a, 2004b), it is apparent that the Hsp70 gene was not in 1 of these early transfer events. Rather, there was a transfer to the nucleus from the green plastid after the green and red plastid lineages diverged. No transfer to the nucleus of the CP Hsp70 gene occurred in the red plastid lineage.

We know that extant cyanobacteria have multiple DnaK proteins (Nimura et al 2001). This suggests that the CP endosymbiont also had multiple DnaKs. From our phylogeny, it appears that the red CP Hsp70s are more closely related to *Synechocystis* DnaK2 than are the green CP Hsp70s. It is then possible that the green and red algal CP Hsp70s are derived from different cyanobacterial DnaKs. We do not yet have a complete understanding of the functional differences among the DnaK homologs in cyanobacteria, but there is evidence that differences do exist (Kovacs et al 2001; Varvasovszki et al 2003). Many functional and biochemical studies have been done of green algal CP Hsp70s (mostly studies of the *C. reinhardtii* CP Hsp70s). In contrast, very little is known of the red algal CP Hsp70s. The different evolutionary histories of the green and red algal CP Hsp70s suggest functional differences between these CP Hsp70s. Comparative studies of the functional differences among the *Synechocystis* DnaKs, and red and green CP Hsp70s clearly are needed to address this interesting question.

The other major lineage of Hsp70s includes the ER and cytoplasmic Hsp70s (Fig 2, branch 3). This branching pattern is consistent with an early gene duplication generating the ER and cytoplasmic lineages. It is interesting that the TpHsp70-3, OlHsp70-5, and Othsp70-5 proteins fall outside this lineage. It is unlikely that they represent a family of Hsp70s that have been lost in other eukaryotic lineages because these species are not closely related. The branch uniting these proteins was well supported in both the NJ and Bayesian analysis. However, it is possible that they are divergent cytoplasmic Hsp70s whose placement

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Fig 2. Phylogenetic tree of HSP70s based on Bayesian analysis. The alignment used in this analysis excluded variable N- and C-terminal regions. This alignment and all the accession numbers for the proteins in the alignment are available as online Supplementary Materials. The relative support for each branch is indicated by both the posterior probability from the Bayesian analysis (highest value is 1.00) and the bootstrap value percentage (based on 1000 bootstrap replicates) from a neighbor-joining analysis of the same alignment (provided in Supplementary Materials available online). In order to save space, an asterisk (*) is used to denote a posterior probability of 1.00 or a bootstrap value of 100. The symbol */* would denote both 1.0 posterior probability and a 100 bootstrap value. Whenever possible, support values are above each branch. When branches are short, these values are below the branch. The numbered branches refer to the well-supported major heat shock protein (Hsp) 70 lineages: 1 = mitochondrion (MT) Hsp70s; 2 = chloroplast (CP) Hsp70s; 3 = endoplasmic reticulum (ER) + cytoplasmic Hsp70s; 4 = ER Hsp70s; 5 = cytoplasmic Hsp70s. The scale bar indicates the expected number of substitutions per site.

Table 2 Algal HSP70 predicted subcellular locations

Protein	Subcellular prediction			Phylogenetic affinity
	Psort	Predotar	TargetP	
Chlamydomonas reinhardtii				
Crhsp70-1	CP (0.400)	Plastid (0.59)	CP (0.532)	CP
Crhsp70-2				MT
Crhsp70-3	Cytoplasm (0.65)	NA	NA	Cytoplasm
Crhsp70-4	Out (0.724)	ER (0.98)	SP (0.858)	ER
Crhsp70-5	Out (0.757)	ER (0.99)	SP (0.972)	ER
Ostreococcus lucimarinus				
Olhsp70-1	CP (.86)	CP (.59)	CP (.91)	CP
Olhsp70-2				MT
Olhsp70-3	Outside (.82) NA	NA	SP (.97)	ER
Olhsp70-4	Nucleus (.88)	NA	NA	Cytoplasm
Olhsp70-5	Cytoplasm (.65)	NA	NA	
Ostreococcus tauri				
Othsp70-1	CP (.56)	CP (.89)	CP (.97)	CP
Othsp70-2	MT (0.92)	MT (.80)	MT (.94)	MT
Othsp70-3	Outside (.82)	ER (.99)	SP (.81)	ER
Othsp70-4	Nucleus (.96)	NA	NA	Cytoplasm
Othsp70-5	Cytoplasm (.65)	NA	NA	
Cyanidioschyzon merolae				
Cmhsp70-1				CP
Cmhsp70-2	MT (0.80)	MT (0.91)	MT (0.932)	MT
Cmhsp70-3	Nucleus (0.76)	NA	NA	Cytoplasm
Cmhsp70-4	Out (0.82)	ER (0.55)	MT (0.881) SP (0.813)	ER
Thalassiosira pseudonana				
Tpthsp70-1				CP
Tpthsp70-2	CP (0.88) MT (0.80)	NA	MT (0.683)	MT
Tpthsp70-3	Cytoplasm (0.45)	NA	NA	
Tpthsp70-4	Nucleus (0.76)	NA	NA	Cytoplasm
Tpthsp70-5	Nucleus (0.76)	NA	NA	ER

Hsp, heat shock protein; CP, chloroplast; MT, mitochondrion; NA; ER, endoplasmic reticulum; SP, subcellular prediction. Predictions were not made for Cmhsp70-1 and Tphsp70-1; both are encoded in the CP genome. Predictions were not made for Crhsp70-2 and Olhsp70-2 due to uncertainty about sequence at the N-terminal region for these genes. For all other Hsp70 proteins, subcellular location predictions were obtained. Each program uses different terminology and predictions: Psort predicts all cellular locations including CP, MT, ER, PR (peroxisome), and cytoplasm. Predotar predicts CP, MT, ER, or elsewhere. TargetP predicts CP, MT, ER, SP (secretory pathway), or other. NA indicates a prediction of elsewhere or other. Phylogenetic affinity is based on placement within the phylogenetic tree in Fig. 2.

outside of the cytoplasmic lineage is due to long-branch effects. Examination of additional homologs from other species will be needed to fully understand the evolution and function of these 3 Hsp70s. Like the MT Hsp70s, the ER Hsp70s (Fig 2, branch 4) display a stable or consistent evolutionary history across organismal lineages. Within the ER Hsp70s, phylogenetic relationships generally reflect organismal relationships. Of the algal species examined here, only *C. reinhardtii* has more than 1 ER Hsp70; by comparison *A. thaliana* has 3 and *O. sativa* has 5.

The evolution of the cytoplasmic Hsp70s deserves considerable attention. Though there is strong support for a cytoplasmic Hsp70 lineage (Fig 2, branch 5), within this lineage the relationships of the cytoplasmic Hsp70s do not reflect organismal relationships. This indicates a complex history of gene duplication, possible gene loss, and gene conversion. One *A. thaliana* cytoplasmic Hsp70, Athsp70-4, is found in a more basal position than the green algal cytoplasmic Hsp70s. However, most of the cytoplasmic *A. thaliana* and *O. sativa* Hsp70s form species-specific groups. These groupings suggest either recent gene duplication or gene conversion. It is also possible that both of these forces are acting on the cytoplasmic Hsp70s. Gene conversion among the angiosperm cytoplasmic Hsp70s quite likely is as rapid gene conversion among cytoplasmic Hsp70s as has been reported in *Drosophila* (Bettencourt et al 2002). In addition, in a study of *Caenorhabditis elegans* and *C. briggsae* Hsp70s, Nikolaidis and Nei (2004) also reported gene conversion among cytoplasmic Hsp70 genes. If gene conversion is active among the angiosperm cytoplasmic Hsp70s, then these

Table 3 Number of matches to *Chlamydomonas reinhardtii* Hsp70s in *C. reinhardtii* EST libraries

Protein	Core	Stress I	Stress II	Stress III	S1D2	Deflagellation	Gamete and zygote
Crhsp70-1	4	2	5	0	0	6	6
Crhsp70-2	0	1	0	0	0	0	0
Crhsp70-3	0	1	2	1	0	5	0
Crhsp70-4	0	1	0	0	1	1	2
Crhsp70-5	1	1	0	0	1	1	2

Hsp, heat shock protein; EST, expressed sequence tag. The numbers in the table indicate the number of times each sequence was present in each *C. reinhardtii* library. The libraries are described in detail in Shrager et al 2003. The core and SID2 libraries are normalized, nonstressed libraries. The stress I and II libraries were made from tissues under nutrient and light stress. The stress III library is based on copper- and iron-stressed tissues. The deflagellation library is from tissue that was under pH shock. The gamete and zygote library is from tissue grown under nitrogen deficiency and collected during gamete and zygote development.

duplicates could be quite old, and gene conversion, not recent duplication, is responsible for the high sequence similarity within species.

The complex evolutionary history of the cytoplasmic Hsp70s is also evident when the number of proteins across species is examined. Although some gene duplication has occurred within the organelle-localized Hsp70 lineages, it is evident from Figure 2 that these protein lineages have remained relatively stable over long periods of evolutionary time. In contrast, it appears that there has been the evolution of considerable diversity in the cytoplasmic Hsp70s since the last common ancestor between *C. reinhardtii* and the angiosperms. The angiosperms *A. thaliana* and *O. sativa* have 7 and 8 cytoplasmic Hsp70s, respectively, and the algal species studied have 1 each. In their study of the *A. thaliana* Hsp70s, Lin et al 2001 suggest that the reason that *A. thaliana* has so many more Hsp70s than other eukaryotes is the presence of the plastid. However, all 5 of our study species are photosynthetic and have plastids. Therefore, there must be another explanation for the differences in diversity of Hsp70s between the angiosperms (represented by *A. thaliana* and *O. sativa*) and these photosynthetic eukaryotes. An important distinction between the algae studied here and the flowering plants is that all the algae examined are single-celled. Is it possible that multiple cytoplasmic Hsp70s are associated with multicellularity? However, it has been noted that considerable differences in the numbers of cytoplasmic Hsp70s exist among other lineages. For example, the ascidian *Ciona intestinalis* (a multicellular animal) has only 2 cytoplasmic Hsp70s, although humans have 8 (Wada et al 2006). Other lineages that have multiple cytoplasmic Hsp70s include single-celled yeast and multicellular *Drosophila*. From these comparisons, it appears that there is no clear relationship between multicellularity and the number of cytoplasmic Hsp70s.

However, it has been well established that the cytoplasmic Hsp70s are an important part of the heat shock response and that these proteins can confer thermal tolerance. The importance of multiple copies of cytoplasmic Hsp70s in the ability of *Drosophila* to withstand high temperature stress has been shown in a number of studies (Feder and Krebs 1998; Krebs and Feder 1998; Garbuz et al 2003; Lerman and Feder 2004). These findings suggest that the multiple cytoplasmic Hsp70s in angiosperms may be related to an increased thermal tolerance in angiosperms compared to algae that have only 1 cytoplasmic Hsp70. However, the red alga *C. merolae* is adapted to extreme conditions, and the lack of additional Hsp70 homologs in this species indicates this adaptation was not gained by the evolution of diverse Hsp70s, suggesting that not all thermotolerant organisms have multiple cytoplasmic Hsp70s. The plant cytoplasmic Hsp70s have not been studied to the extent that their animal homologs have (Sung et al 2001) and further functional analysis of these proteins clearly is needed.

It is known that there have been numerous polyploidy events within the land plant lineages and that these events have played an important role in gene family diversification within plants. It is possible that the additional cytoplasmic Hsp70s in angiosperms are a product of multiple polyploid events. A fascinating study showed that duplications of cytoplasmically localized proteins are more likely to be retained than duplications of organelle-localized proteins (Blanc and Wolfe 2004). Other studies have found gene family expansions when angiosperms are compared to algae. For example, in a study of kinesins, it was found that *C. merolae* has 5, *T. pseudonana* has 22, *C. reinhardtii* has 23, and *A. thaliana* has 61 kinesins. The large increase in angiosperm kinesins compared to the *C. reinhardtii* is due to expansion in only 2 families of kinesins (Richardson et al 2006). It is also noteworthy that within the plant lineage there also has been a lineage-specific amplification of the small heat shock proteins or Hsp20s (Waters 2003). However, early diverging land plants also have a diversity of small heat shock proteins and so this expansion was not directly related to polyploidy in vascular plants. It has been suggested that the stresses of moving onto land, which included increased desiccation, increased ultraviolet exposure, and increased temperature extremes may have been a selective pressure that favored or drove an increase in the types and numbers of molecular chaperones (Waters 2003). Distinguishing between duplication due to the selective pressures of life on land and the effects of polyploidy for the Hsp70s and other gene families will require considerable additional information including the complete genome sequences of a number of plants representing early diverg-

ing land plant lineages. When this data is available, it will be possible to determine if multiple cytoplasmic Hsp70s are found in all land plants, suggesting that selection pressure for thermal tolerance early in land plant evolution drove the duplication of plant cytoplasmic Hsp70s or if only those land plant lineages that have undergone multiple polyploidy events have a diversity of cytoplasmic Hsp70s.

SUMMARY

From the complete genome sequences of 5 distantly related photosynthetic eukaryotes or algae we identified 24 Hsp70s of the DnaK subfamily. Analysis of these 24 proteins indicates that all are expressed and that they all possess the highly conserved ATPase and substrate-binding domains. Some, but not all, also possess the transit sequences for targeting to particular organelles and a few lack the highly variable C-terminal domain. The MT and ER Hsp70s have relatively stable evolutionary histories and the protein phylogenies approximate the organismal relationships. The CP Hsp70s have a very interesting evolutionary history that suggests the possibility of functional differences between red and green CP Hsp70. The green CP Hsp70s are all nuclear encoded. The red CP Hsp70s are all encoded in the CP genome. Our analysis also indicates that the red and green CP Hsp70 may be derived from distinct cyanobacterial DnaK homologs. Finally, the cytoplasmic Hsp70s have a much more complex evolutionary history than the MT and ER Hsp70s. We describe the absence of diverse cytoplasmic Hsp70s in the algal species. Each species appears to have only 1 Hsp70 that is clearly within the cytoplasmic Hsp70 lineage. This is quite distinct from the diverse number of cytoplasmic Hsp70s found in angiosperms or flowering plants.

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