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Kirsten Haussuehl, Pitter F Huesgen, Marc Meier, Patrick Dessi, Elzbieta Glaser, et al.. The eukaryotic GCP1 is a conserved mitochondrial protein required for progression of embryo development beyond the globular stage in *Arabidopsis thaliana*. *Biochemical Journal*, 2009, 423 (3), pp.333-341. 10.1042/BJ20091023 . hal-00479220

HAL Id: hal-00479220

<https://hal.science/hal-00479220>

Submitted on 30 Apr 2010

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The eukaryotic GCP1 is a conserved mitochondrial protein required for progression of embryo development beyond the globular stage in *Arabidopsis thaliana*

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Running Head: Essential mitochondrial GCP1

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ABSTRACT

Glycoproteases (GCPs) are members of the HSP70-actin ATPase superfamily that are highly conserved in taxonomically diverse species from bacteria to man, suggesting an essential physiological role. Although originally identified and annotated as putative endopeptidases, a proteolytic activity could not be confirmed for these proteins. Our survey of genome database revealed that all eukaryotic organisms contain two *GCP* genes (called *GCP1* and *GCP2/Kae1*), while prokaryotes have only one either of the *GCP1*- (Bacteria) or the *GCP2/Kae1* (Archaea)-type. *GCP2/Kae1* is essential for telomere elongation and transcription of essential genes, whereas little is known about localization, expression and physiological role of *GCP1*. In this report on *GCP1*-type proteins from eukaryotic organisms we demonstrate that *GCP1* is a mitochondrial protein in *Homo sapiens* (called here *GCP1/OSGEPL1*) and *Arabidopsis thaliana* that is located/anchored to the inner mitochondrial membrane. Analysis of mRNA and protein levels revealed that the expression of *GCP1/OSGEPL1* in *A. thaliana* and *H. sapiens* is tissue- and organ-specific and depends on developmental stage, suggesting a more specialized function for this protein. We show that homozygous *A. thaliana GCP1* T-DNA insertion lines are embryo-lethal. Embryos in homozygous seeds are arrested at the globular stage and fail to undergo the transition to heart stage. Based on this data we propose that the mitochondrial *GCP1* is essential for embryo development in plants.

Key words: *Arabidopsis thaliana*, embryo development, human, mitochondrial localization, phylogeny, tissue specific expression

Abbreviations used: COXII, the cytochrome c oxidase; EKC, endopeptidase-like and kinase associated to transcribed chromatin; F1 β , the F1 β subunit of the ATP-synthase; GCP, glycoprotease; GDC-H, the subunit H of the glycine decarboxylase; Kae1, kinase-associated endopeptidase 1; KEOPS, kinase, endopeptidase and other proteins of small size; LHCB2, the light-harvesting chlorophyll *a/b*-binding protein of photosystem II; OSGEPL, O-sialoglycoprotein endopeptidase; T-DNA, transfer DNA; WT, wild type.

INTRODUCTION

Proteins called GCPs (for glycoproteases) belong of the ASKHA superfamily (containing acetate and sugar kinases, HSP70 and actin) of phosphotransferases but differ from other members of this superfamily by the presence of an insertion within the HSP70-actin fold that is a protein structural motif binding ATP in the presence of Ca^{2+} or Mg^{2+} (<http://merops.sanger.ac.uk>). It was proposed that during evolution GCPs might have acquired the protease function by grafting the metal-binding motif HXEXH onto the structural framework of the HSP70 domain, thus creating a protease active site [1]. A proteolytic activity against O-sialoglycosylated proteins was reported for a GCP orthologue (EC 3.4.24.57) in the Gram-negative pathogenic bacterium *Mannheimia (Pasteurella) haemolytica* [2]. However, such a protease activity was never confirmed for other GCP orthologues.

Here we show that two GCP types, called GCP1 (found in Bacteria and Eukarya) and GCP2 (found in Archaea and Eukarya), are present in prokaryotic and eukaryotic organisms. It was reported that GCP2 in yeast, called Kae1 (for kinase-associated endopeptidase 1), is a component of the EKC (endopeptidase-like and kinase associated to transcribed chromatin)/KEOPS (for kinase, endopeptidase and other proteins of small size) complex that is essential for telomere elongation and transcription of essential genes [3, 4]. GCP2/Kae1 is also found in a similar complex in Archaea [5, 6]. Archaeal GCP2/Kae1 was further reported to bind DNA and exhibit class I apurinic-endonuclease activity [7, 8]. However, the precise biochemical function and biological role of GCP2/Kae1 still remains to be determined.

It was shown that GCP1 is an essential protein and its knockout is lethal in *Escherichia coli* [9, 10], *Staphylococcus aureus* [11] and *Arabidopsis thaliana* (this work). Originally, it was described that GCP1 from *M. haemolytica* is secreted outside the cell and might be involved in the induction of an immune response [2]. *E. coli* contains two GCP1 orthologues, called YgjD and YeaZ [10]. While the amino acid sequence of YgjD resembles those of plant GCP1, yeast (*Saccharomyces cerevisiae*) Qri7 or worm (*Caenorhabditis elegans*) and human (*Homo sapiens*) OSGEPL (for O-sialoglycoprotein endopeptidase), the YeaZ sequence is truncated and restricted to Bacteria. It was reported that the repression of the YeaZ expression results in cells with highly condensed nucleoids, while the repression of YgjD results in a large cells with unusual peripheral distribution of nucleoids [10]. Recently, the involvement of yeast Qri7 and worm OSGEPL in mitochondrial genome maintenance was proposed [12].

We selected GCP1 orthologues from *A. thaliana* and human (called here GCP1/OSGEPL1) for detailed analysis in order to gain insights into the function of these conserved proteins in higher eukaryotes. We demonstrate that GCP1/OSGEPL1 is located in mitochondria of both organisms. In plants, GCP1 is located/anchored to the inner mitochondrial membrane and is expressed in young developing organs. Also human GCP1/OSGEPL1 is expressed in certain organs and tissue types with the maximal expression measured in pituitary gland, prostate, rectum and uterus. Furthermore, homozygous *gcp1* knockout mutants of *A. thaliana* are lethal at the early stages of embryo development indicating that GCP1 is essential for plant viability. Our data suggest an unexpected connection between mitochondrial functions and progression of embryo development beyond the globular stage in *A. thaliana*.

EXPERIMENTAL

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia (*Col-0*) were grown in a growth chamber on soil at 20°C at a photon flux density of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ under the light regime of 8 h dark/16 h light. The T-DNA insertion mutants GABI-Kat 322F06 (*gcp1-1*) and GABI-Kat 158E07 (*gcp1-2*) were generated in the context of the GABI-Kat program and provided by Bernd Weisshaar (MPI for Plant Breeding Research; Cologne, Germany; <http://www.gabi-kat.de>)[13]. T-DNA insertion sites were verified by PCR using gene specific primers annealing upstream and downstream of the insertion sites of 322F06 (5'-CTT TCA TGG CAT TGC TTA TAT CAG G-3' and 5'-GTT CTA ATG CCC AGT CTA TTG CTC-3'), 158E07 (5'-GTG AGA GGT AAT GGT GAA ATT CTC-3' and 5'-AAA CCT AAT CCC TAC GGT GAA ACT-3') and a T-DNA specific primer (5'-CCC ATT TGG ACG TGA ATG TAG ACA C-3'). Obtained PCR products were purified with a kit (Qiagen GmbH) and sequenced (GATC, Konstanz, Germany). T3 generation mutant plants were selected by resistance against sulfadiazine on agar with MS medium [14]. T4 generation mutant plants for analysis of embryonic development were grown on soil and selected by PCR screening.

Gene cloning and sequencing

Based on the *A. thaliana GCP1* genomic sequence (GenBank accession: AAB82636; Locus: At2g45270) a pair of primers (5'-ATG GTT CGT CTG TTT CTT ACA CTT TC-3' and 5'-GTT GAA GAG AAT CTG CTC TAA -3') were designed and used for amplification of the *GCP1* ORF from the l-ZAP (Stratagene) cDNA library (1×10^3 p.f.u.) prepared from *A. thaliana* seedlings. The PCR cycling profile (30 cycles) was denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 2 min. The amplified 1443 bp cDNA fragment was purified (QiaEx, Qiagen GmbH) and cloned into the pCR2.1 cloning vector (Invitrogen) prior to sequencing of both DNA strands (CyberGene, Stockholm, Sweden).

RNA isolation and analysis

Total plant RNA was extracted from material frozen in liquid nitrogen using an RNeasy mini kit (Qiagen GmbH) according to the manufacturer's protocol. After separation of 10 μg of RNA in 1.2% agarose gel, RNA was transferred to a Hybond-N+ membrane (Amersham Biosciences) prior to the hybridization. The full-length *GCP1* cDNA probe was labelled with [α - ^{32}P]dCTP using a megaprime DNA labelling kit (Amersham Biosciences). The hybridization was performed as described elsewhere [15].

For measurement of human tissue expression a cDNA-panel representing 48 different human tissues (TissueScan Human Major Tissue qPCR Array HMRT102, Origene, USA) was used. The cDNA content of a single sample in the panel was normalized to equal levels of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by the manufacturer. PCR was performed in a real-time 7900HT PCR-system (Applied Biosystems) using Power SybrGreen Master Mix with ROX (Applied Biosystems) in 96 well system. Melting curves were recorded for single wells for verification of amplification specificity. Results of the qPCR (ct values) were analyzed via SDS 2.2 software (Applied Biosystems). The following primers were used for the amplification of human *GCP1/OSGEPL1* (GenBank accession: AAH11904): forward 5'-CTGCCACAGTACAGCACACA-3' and reverse 5'-GGAGGAGGACACAACAAAGTG-3'. Their specificity was verified with dilution series of HeLa cell line cDNA. The resulting signals of each tissue were normalized against *GAPDH*-signals. The qPCR results (ct values) are given as $1/\exp(\Delta \text{ct})$ for the difference between *GCP1/OSGEPL1* and *GAPDH* ct. The values represent the ct *GCP1/OSGEPL1* - ct *GAPDH* recalculated as reciprocal values $1/(\text{ct } GCP1/OSGEPL1 - \text{ct } GAPDH)$. We used further

exponent function to facilitate presentation of differences. Sequencing of the resulting PCR-products further proved specificity of amplification.

Recombinant protein overexpression, purification and polyclonal antibodies sources

The *A. thaliana* *GCP1* cDNA was amplified using a pair of PCR primers (5'- GTT CGT CTG TTT CTT ACA CTT TC-3' and 5'-TTG AAG AGA ATC TGC TCT AAT G-3') and a full-length *GCP1* cDNA clone (see above) as template. The *GCP1* was expressed in *E. coli* as a fusion protein with the N-terminal-attached thioredoxin and the C-terminal-attached His₆-tag using a kit (pBAD/Topo[®] ThioFusion[™] Expression System, Invitrogen) according to the manufacturer's protocol. Recombinant *A. thaliana* *GCP1* was purified under denaturing conditions by affinity chromatography on Ni-NTA-agarose (Qiaexpress, Qiagen) and eluted from the column with 250 mM imidazole. The *GCP1*-containing fractions were separated by SDS-PAGE, proteins transferred to a nitrocellulose membrane and the *GCP1* band excised from the blot prior to raising polyclonal antibodies (BioGenex, Berlin, Germany).

The polyclonal antibodies against plant proteins, such as the subunit II of the cytochrome *c* oxidase (COXII), the subunit *H* of the glycine decarboxylase (GDC-H) and the light-harvesting chlorophyll *a/b*-binding protein of photosystem II (LHCB2) were purchased from Agrisera AB (Vännäs, Sweden).

Protein analysis

Total plant proteins were isolated as described [16]. Total or mitochondrial proteins (see below) were separated by SDS-PAGE [17] generally using 12% polyacrylamide minigels (Hoefer mini gel system). The gels were loaded on equal 5 µg protein basis. Immunoblotting was carried out according to [18] using a nitrocellulose membrane with 45-µm pores (Hybond-P, Amersham Biosciences) and an enhanced chemiluminescence assay (ECL, Amersham Biosciences) as the detection system.

Isolation and fractionation of organelles

Intact chloroplasts of *A. thaliana* were purified on Percoll gradients according to Cline [19]. Mitochondria from rat liver [20] and HeLa cells [21] were isolated as described. Intact mitochondria from *A. thaliana* were isolated either from 2-3-week-old seedlings (developing leaves) or from 4-6-week-old (mature) leaves of *A. thaliana* using a density step gradient centrifugation as described [22]. For fractionation into soluble and membrane fractions mitochondria were osmotically lysed for 10 min on ice in 10 mM Tris, pH 7.5 and centrifuged for 1 h at 4°C at 130,000 g. The pellet (total mitochondrial membranes) and supernatant (total soluble fractions) were collected, proteins in the supernatant precipitated with 5% (v/v, final concentration) TCA on ice for 30 min and collected by centrifugation at 14,000 g at 4°C for 10 min. Both subfractions were solubilised in equal volumes of Laemmli sample buffer [17]. For isolation of peripheral and integral membrane proteins, isolated mitochondrial membranes were resuspended in 25 mM MES, pH 6.5 at a protein concentration of 0.1 mg mL⁻¹ and subjected to washes with salt and chaotropic agents as described [23]. For protease protection assay, intact or osmotically lysed mitochondria were resuspended in 50 mM Hepes, pH 8.8 and 330 mM sorbitol at a protein concentration of 1 mg mL⁻¹ and incubated for 30 min at 4°C in the absence or the presence of 50 µg mL⁻¹ trypsin. After TCA precipitation (5% v/v, final concentration) for 30 min on ice, proteins were pelleted by centrifugation at 14,000 g at 4°C for 10 min and used for immunoblotting as described above.

Immunohistochemistry

Human HeLa cells were grown under humidified standard conditions (37°C, 5% CO₂) in

High Glucose Dulbecco's Modified Eagle Medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% (v/v) fetal bovine serum (FBS, Biochrom AG, Berlin, Germany), 2 mM L-GlutamaxI (Invitrogen), and 100 U mL⁻¹ penicillin/100 µg mL⁻¹ streptomycin (Invitrogen). The cells were incubated for 30 min with a fresh staining medium containing minimum essential medium with 10% (v/v) FBS and 300 nM MitoTracker Orange. After removal of medium, cells were washed twice with PBS and fixed with 3.7 % (v/v) formaldehyde for 10 min at 37 °C. To enable binding of specific antibodies, fixed cells were permeabilised with 0.5 % (v/v) Triton X-100 for 5 min at 25°C. To prevent unspecific binding of proteins, blocking with 3 % (w/v) bovine serum albumin (BSA) in PBS was done for 30 min at 25°C. Incubation of primary antibody α-GCP1/OSGEPL1 in 200 µL solution (1:1,000 in 3 %, w/v, BSA in PBS) for 1 h at 25°C was followed by two washing steps and incubation with a secondary antibodies conjugated to fluorescent marker Cy3 (Dianova) (1:2,000 in 3 %, w/v, BSA in PBS). In order to stain the nuclei, the cells after washing were incubated with a solution of Hoechst 33342 (Invitrogen) diluted 1:5,000 in PBS for 2 min at 25°C. After washing twice with PBS, the cover slips with the cells were fixed on glass slides on a drop of Vectashield (Vectalabs). Subcellular localization was analyzed by fluorescence microscopy on a Zeiss Axiophot (Carl Zeiss, Oberkochen, Germany) microscope using a 40x oil immersion objective and the ISIS software (MetaSystems, Altlussheim, Germany).

Phenotypic analysis of *A. thaliana* mutants

Developing siliques of heterozygous mutants and wild type (WT) plants were sliced longitudinal and analyzed under a stereomicroscope (Carl Zeiss, Oberkochen, Germany). Seeds were counted and classified as normal (green seeds) or abnormal (white seeds). For microscopical analysis, developing siliques of heterozygous mutants were fixed and cleared essentially as described [24], with three additional washing steps with 50% and 25% (v/v) ethanol and distilled H₂O before the addition of the clearing solution. The seeds were dissected before viewing transmitted light images with Nomarski's differential interference contrast illumination using an Olympus BX51 epifluorescence microscope equipped with a Nikon DXM1200 digital camera system (Olympus Europe). Digital pictures were acquired with the ACT-1 software (Nikon GmbH).

Bioinformatics

Similarity searches were done using the Advanced BLAST program. The transmembrane regions were predicted using the dense alignment surface method and the hydropathy plot was drawn according to [25]. Prediction of subcellular location and determination of processing site were analyzed by TargetP v.1.1 and MitoProt II 1.0a4 programs. Prediction of protein pattern and motifs were performed using sequence motif search and protein motif fingerprint databases. All software programs used are accessible on the Internet (<http://www.expasy.org/tools> and <http://www.genome.ad.jp>). The phylogenetic tree was prepared using full-length amino acid sequences of GCP1 and GCP2 orthologues from selected organisms. Sequences were aligned with T-Coffee software [26] and the neighbour-joining tree was reconstructed from this alignment with the MEGA software package version 3.1 [27].

Accession numbers

The GenBank accession numbers for the nucleotide sequence (*A. thaliana* Col-0) described in this article is AY024338 (*GCP1* cDNA). The accession numbers for the sequence data shown in Figure 1A are listed in Supplemental Table S1.

RESULTS AND DISCUSSION

Conservation of GCP1 in Bacteria and Eukarya

Two highly conserved GCP paralogues, called here GCP1 and GCP2/Kae1, are present in all Archaea, Bacteria and Eukarya investigated so far (Figure 1A) [12]. The only two exceptions are the highly reduced genomes of the endosymbiotic bacteria *Carsonella ruddii* and *Sulcia muelleri* [7]. Phylogenetic analysis revealed that GCP1 and GCP2/Kae1 form two distinct clades (Figure 1A). The GCP1 clade gathers orthologues present in Bacteria and Eukarya, while the GCP2/Kae1 clade clusters orthologues present in Archaea and Eukarya (Figure 1A). We focused our present study on the eukaryotic GCP1 orthologues from *A. thaliana* (NCBI Gene ID: 819135) and *H. sapiens* (NCBI Gene ID: 64172).

Recently, the atomic structures of the KEOPS complex [6] and GCP2/Kae1 protein from Archaea [5, 8] were solved. Based on these structures GCP2/Kae1 is a metalloprotein containing an iron ion directly linked to ATP via the γ -phosphate. As predicted by Aravind and Koonin [1] the archaeal GCP2/Kae1 contains the ASKHA fold that consists of two subdomains with the active site HXEXH residing in the cleft region between these subdomains [5, 6, 8]. The ferric ion in GCP2/Kae1 from the archaeon *Pyrococcus abyssi* is tetrahedrally liganded by His-107, His-111, Tyr-127 and Asp-285 [8]. A comparison of the conserved domains of GCP1 orthologues from various organisms (alternative names are given on the left) revealed the conservation of two histidines (Figure 1B and 1C). While both His (His-194 and His-198 in *A. thaliana* and His-147 and His-151 in *H. sapiens*) and Asp (Asp-402 in *A. thaliana* and Asp-358 in *H. sapiens*) are conserved in GCP1 orthologues the Tyr ligand is missing (Supplemental Fig. S1). A highly conserved His present +23 to +25 amino acid downstream from the His dyad might provide a fourth metal ligand in GCP1 orthologues (Supplemental Fig. S1). Furthermore, eukaryotic GCP1 orthologues possess an N-terminal transit peptide present in proteins targeted to mitochondria (predicted for human) and to chloroplasts (predicted for plants) (Supplemental Fig. S1).

Orthologous GCP1 from *M. haemolytica* has been previously reported to exhibit protease activity [2]. Later the authors of the original annotation suggested that the identification of GCP1 as a glycoprotease might have been misleading [28]. Similarly, we could not detect proteolytic activity when we tested recombinant refolded *A. thaliana* GCP1 *in vitro* against several universal protease substrates, such as β -casein, gelatine, haemoglobin, azocoll, albumin or specific glycosylated substrates, like glycophorin A or bovine milk κ -casein (data not shown). Also the GCP2/Kae1 from Archaea did not exhibit endopeptidase activity *in vitro* [8]. This suggests that purified GCP1 (and possibly also GCP2/Kae1) might not be correctly folded, has a narrow substrate specificity, requires specific interacting partners, cofactors or posttranslational modification for its activity, or finally and most probably, has a function other than proteolysis. It was further reported that mutation of the two conserved His residues in yeast GCP2/Kae1 completely inactivated its function and led to several defects in cell cycle progression and polarized growth that were ascribed to a defective transcriptional response [3]. This is in agreement with a study showing that the ATPase catalytic function of GCP2/Kae1 in Archaea depends on bound metal ions [6]. This further implies a similar importance of the conserved His residues for GCP1 function.

The crystal structure of YeaZ from *Salmonella typhimurium* revealed the lack of conserved His dyad involved in metal binding in GCP2/Kae1, although it was suggested that both paralogous, YeaZ and YgjD, might bind the same ligands due to their conserved pocket [29]. It was further suggested that YeaZ requires interaction with a partner protein to adopt an active conformation in order to bind its ligand. Interestingly, an interaction between YeaZ and YgjD in *E. coli* has recently been reported [10].

Localization and expression of GCP1 in *A. thaliana*

In order to perform localization and expression studies at the protein level we expressed *A. thaliana* GCP1 in *E. coli*. The recombinant protein accumulated in inclusion bodies and was purified under denaturing conditions prior to raising polyclonal antibodies. The obtained α -GCP1 antibody crossreacted with two distinct bands in *A. thaliana* membrane protein extracts (Figure 2). We assume that the lower band might result either from posttranslational modifications or limited proteolysis of GCP1 since it appeared in varying ratio depending on sample storage and treatment.

To prove the cellular location of GCP1 we performed Western blot analysis using total cell extracts, isolated intact mitochondria and chloroplasts. GCP1 was detected in the total cell extract and mitochondria, but not in chloroplasts (Figure 2A). Interestingly, a strong GCP1 signal was obtained only in mitochondria isolated from very young seedlings and not in those isolated from mature leaves (Figure 2A). Immunoblots with two marker proteins, the mitochondria-located F1 β subunit of the ATP-synthase (F1 β)[30] and the chloroplast-located light-harvesting chlorophyll *a/b*-binding protein of photosystem II (LHCB2)[31], confirmed the purity of preparations. Also the pattern of proteins, as visualized by Coomassie staining, differed for each fraction (Figure 2A). These data are in agreement with a mitochondrial location predicted for the GCP1 orthologue from *S. cerevisiae* and experimentally proven for *C. elegans* [12].

To test whether GCP1 is a membrane or a soluble protein we fractionated purified mitochondria into the membrane fraction, containing inner and outer mitochondrial membranes, and the soluble fraction, containing mitochondrial matrix and intramembrane space. Immunoblot analysis with the α -GCP1 antibody found GCP1 exclusively in the membrane fraction (Figure 2B). Immunoblots using antibodies against the inner membrane-located subunit II of the cytochrome *c* oxidase (COXII)[32] and the matrix-located subunit *H* of the glycine decarboxylase (GDC-H)[33] confirmed the purity of the obtained fractions.

According to the hydropathy plot GCP1 contains two hydrophobic stretches that might theoretically provide membrane attachment sites (not shown). However, based on the GCP2/Kae1 structure these alpha-helices form a hydrophobic core of the protein [5, 6, 8]. To determine whether GCP1 is a peripheral protein or a membrane anchored protein we washed isolated mitochondrial membranes with various salt and chaotropic agents to release extrinsic membrane proteins [23]. The pellet fraction containing integral and membrane anchored proteins and the supernatant fraction containing peripheral membrane proteins were used for immunoblotting. The presence of GCP1 in the pellet fraction indicated its membrane location (Figure 2C). In contrast, F1 β known to be a peripheral membrane protein [30] was partially or completely released from the membrane, depending of the stringency of washes.

To investigate whether GCP1 is located in the outer or the inner mitochondrial membrane we performed protease protection assays with intact or osmotically lysed mitochondria. The protection of GCP1 in intact mitochondria and its susceptibility to trypsin digestion in osmotically broken organelles suggested that GCP1 is located/anchored in the inner mitochondrial membrane (Figure 2D).

Expression studies revealed that GCP1 transcript and protein accumulated transiently at the early stages of seedling development (Figure 3A). The maximal transcript level of GCP1 in total seedlings was reached during week 3 after seed germination as shown by Northern blotting (Figure 3A). During weeks 1, 2 and 4 only traces of GCP1 transcripts were detected. In contrast, a significant amount of GCP1 protein accumulated already during week 1 after seed germination and this amount increased 3- and 5-fold, during week 2 and 3, respectively. During week 4 the amount of GCP1 decreased again reaching the level present after week 1 (Figure 3A).

We investigated the GCP1 expression during development of various organs. Immunoblotting detected GCP1 in young developing leaves, roots, flowers and siliques

(Figure 3B). Much lower amounts of GCP1 were found in mature roots, flowers and siliques and no immunoblot signals were visible in mature leaves or mature dried or imbibed seeds.

Localization and expression of GCP1/OSGEPL1 in *H. sapiens*

The high conservation of GCP1 among various taxa prompted us to test *A. thaliana* α -GCP1 antibody for its crossreactivity with mitochondria isolated from yeast, human and rat. While no positive reaction was obtained using yeast samples (not shown), a strong specific GCP1/OSGEPL1 signal was observed in human and rat mitochondrial extracts (Figure 4A). Three protein bands were recognized by α -GCP1/OSGEPL1 antibody in human mitochondria samples that might correspond to different GCP1/OSGEPL1 isoforms. At least nine isoforms are reported for human GCP1/OSGEPL [34].

Taking advantage of the crossreactivity of *A. thaliana* α -GCP1 antibody with its human orthologue, we investigated the localization of GCP1/OSGEPL1 in HeLa cells by immunocytochemistry. Colocalisation of a fluorescent mitochondrial marker MitoTracker and signals from labelled GCP1/OSGEPL1 antibodies demonstrated the presence of GCP1/OSGEPL1 in mitochondria of HeLa cells (Figure 4B).

A cDNA-panel representing 48 human tissues was used to investigate GCP1/OSGEPL1 expression. Similar to *A. thaliana* the expression of human *GCP1/OSGEPL1* transcripts was tissue-specific (Supplemental Fig. S2). The maximal expression level was measured in prostate, pituitary glands, uterus and rectum. No or very low expression of *GCP1/OSGEPL1* transcripts was assayed in bone marrow, liver, mammary gland, muscle, nasal mucosa and oviduct (Supplemental Fig. S2). This suggests that in contrast to GCP2/Kae1 the physiological function of GCP1/OSGEPL1 is less general and connected to certain organs and tissues.

Disruption of the *GCP1* gene in *A. thaliana*

The conservation of GCP1 orthologues across taxonomically diverse species implies an essential physiological function. To elucidate this function we analyzed two *A. thaliana* mutant lines of the GABI-Kat collection carrying T-DNA insertions within the *GCP1* gene [13]. PCR analysis showed that the insertion in the *gcp1-1* (GABI-Kat line 322F06) mutant is located in the third intron and that the *gcp1-2* (GABI-Kat line 158E07) mutant carries an inverted tandem repeat T-DNA insertion in the seventh intron (Figure 5A). The PCR screening of T3 and T4 seedling generations grown on selective media detected only heterozygous plants. This suggested that the disruption of the *GCP1* gene might be lethal at the early stages of seed formation and embryo development. Therefore, we investigated individual immature siliques of heterozygous plants for abnormal seeds [35]. While WT siliques showed consistently maturing seeds, abnormal seeds were present beside normally formed and developed seeds in young siliques of heterozygous plants. Out of 794 and 736 seeds analyzed for heterozygous *gcp1-1* and *gcp1-2* mutant plants, 183 (23%) and 188 (26%), respectively, were abnormally formed (Table 1). According to statistical analysis with the χ^2 test these numbers are in agreement with a recessive lethal segregation of seeds homozygous for the insertion [24, 35]. We further followed embryo development in immature siliques of heterozygous plants by interference contrast light microscopy. We noticed that while embryos in WT and heterozygous seeds underwent the well-defined developmental stages, such as globular, heart, torpedo, walking-stick or curled, embryos in presumably homozygous seeds were arrested at the globular stage and did not undergo transition to heart stage (Figure 5B). Both *GCP1* alleles conferred the same embryo phenotypes.

It was reported that a number of critical developmental events occur during the globular/heart transition period, including the differentiation of primary embryonic tissue layers and the initiation of embryonic organs, which make this period particularly sensitive to

mutations [36]. Thus, we expect that *A. thaliana* GCP1 is essential for one of these events. This is supported by a very high expression level of *GCP1* transcripts in globular to early heart embryos as revealed by microarray analysis (NASCArray set and AtGenExpress Plus-Extended Tissues set available through http://bbc.botany.utoronto.ca/ntods/cgi-bin/ntods_expression_angler.cgi).

There are some reports on other mitochondrial proteins essential for embryonic development. The *A. thaliana* *short integuments 2-1* (*sin2-1*) mutant produces ovules with short integuments due to early cessation of cell division in this structure. *Sin2-1* was isolated and encodes a putative GTPase, related to mitochondrial GTPases essential for biogenesis of large ribosomal subunit [37]. The *A. thaliana* *HUELLENLOS* gene, encoding a mitochondrial ribosomal protein was reported to be essential for normal ovule development [38]. Recent studies showed that the disruption of translation in mitochondria by the deletion of various aminoacyl-tRNA synthetases lead to the typical knockout phenotype, where embryo was arrested at the globular state before transition to heart state [39].

Recent studies demonstrated that Qri7 in *S. cerevisiae* and OSGEPL in *C. elegans* are involved in mitochondrial genome maintenance [12]. The *gri7*Δ mutant of yeast contained highly fragmented mitochondria that lost their mtDNA [12]. The *osgl-1*Δ mutant of *C. elegans* has enhanced longevity, showed increased resistance to oxidative stress and the mitochondrial network in such a mutant was disorganized [12]. Although all these reports implied the importance of GCP1 for mitochondrial physiology the exact role of this protein still remains to be elucidated.

CONCLUSIONS

Based on their conservation throughout the genomes GCPs were included into a set of approximately 60 universal proteins that were probably present in the last universal cellular ancestor of all extant life [40]. Therefore, understanding the biological role of the universal GCPs remains a major challenge.

Localization and expression studies suggest that the physiological function of GCP1 in Eukarya and Bacteria might differ from that reported for GCP2/Kae1 in yeast and Archaea [3, 7, 8]. First, the GCP2/Kae1 is part of the EKC/KEOPS transcription complex and is associated with serine-threonine Bud32/PRPK (p53-related protein kinase) kinase in Archaea [6], yeast [41, 42] and human [43]. No subunits of EKC/KEOPS complex have been identified in Bacteria [6, 8] or in mitochondria that are of bacterial origin [44]. Second, the expression of GCP1/OSGEPL1 in plants and human is restricted to certain developmental stages, tissues and organs, which suggests a more specific function of GCP1/OSGEPL1. The putative protease activity of GCP proteins can still not be completely dismissed, although neither the crystal structure of GCP2/Kae1 [5, 6] nor biochemical activity tests with recombinant GCP1 (this work) and GCP2/Kae1 [8] provided evidence for an endopeptidase activity.

ACKNOWLEDGEMENTS

We thank Dr. Dietmar Funck for advice on microscopy and genetic analysis and express our gratitude to Dr. Christian Spielhaupter for his helpful discussions on real-time PCR. This work was supported by research grants from the Deutsche Forschungsgemeinschaft (SFB-TR11 grant 650/04, TPC11), the Konstanz University grant (to I.A.), the Helmholtz Zentrum München grant (to J.A.), and the Swedish Research Council (to E.G.).

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FIGURE LEGENDS

Figure 1 Conservation of GCPs in prokaryotic and eukaryotic organisms

(A) Phylogenetic analysis of GCP1 and GCP2/Kae1 paralogues from various prokaryotic and eukaryotic organisms. A non-rooted phylogenetic tree was generated as described in Materials and Methods. Bootstrap percentage supports are indicated. Abbreviations used: Ath, *Arabidopsis thaliana*; Bme, *Brucella melitensis*; Bsu, *Bacillus subtilis*; Dme, *Drosophila melanogaster*; Dre, *Danio rerio*; Eco, *Escherichia coli*; Hin, *Haemophilus influenzae*; Hsa, *Homo sapiens*; Mmu, *Mus musculus*; Mth, *Methanobacterium thermoautotrophicum*; Osa, *Oryza sativa*; Pfu, *Pyrococcus furiosus*; Rco, *Rickettsia conorii*; Spo, *Schizosaccharomyces pombe*; Tac, *Thermoplasma acidophilum*. The accession numbers of sequences are listed in Supplemental Table S1. (B) Schematic representation of the predicted structure of GCP1 from *A. thaliana*. Abbreviations used: aa, amino acid; stars mark conserved His residues; mGCP1, mature processed form; TP, predicted transit peptide (grey). (C) Sequence alignment of potential catalytic domains of GCP1 orthologues from various organisms. The common gene names are given on the left. Asterisks mark the predicted catalytic His dyad. For sequence accession numbers see Materials and Methods.

Figure 2 Localization of GCP1 in *A. thaliana*

(A) Localization of GCP1 in a total cell extract (Total), isolated intact chloroplasts (Chloro) and mitochondria from young developing seedlings (Mito-D) and mature leaves (Mito-M) assayed by immunoblotting. The light-harvesting chlorophyll *a/b*-binding protein of photosystem II (LHCB2) and the F1 β subunit of the ATP-synthase (F1 β) were assayed as markers for chloroplast and mitochondria, respectively. The protein pattern of isolated fractions was analyzed on SDS-gels stained with Coomassie. (B) Isolated intact mitochondria were lysed osmotically and separated into soluble and membrane fractions followed by immunoblotting with the α -GCP1 antibody. As references, the distribution of the inner membrane-located subunit II of the cytochrome *c* oxidase (COXII) and the matrix-located subunit *H* of the glycine decarboxylase (GDC-H) was analyzed by immunoblotting. (C) Isolated mitochondrial membranes were incubated in the absence (Control) or in the presence of various salt and chaotropic agents to remove peripheral membrane proteins. The membrane pellets (M) containing integral/anchored membrane proteins and the supernatants (S) containing extracted peripheral membrane proteins were used for immunoblotting with the α -GCP1 antibody. As a reference for the distribution of the peripheral membrane proteins, the F1 β is shown by immunoblotting. (D) A protease protection assay carried out in the absence (-) or the presence (+) of trypsin added to intact (Intact) or osmotically lysed (Lysed) mitochondria prior to immunoblotting with the α -GCP1 antibody.

Figure 3 Expression of GCP1 in *A. thaliana*

(A) Expression of *GCP1* transcript (Top) and protein (Middle) during 1-4 weeks of seedling development assayed by Northern and Western blotting, respectively. All Northern blots contained 10 μ g total RNA and were hybridized to a ³²P-labeled *GCP1* cDNA probe. As a reference, the rRNA pattern in the gel stained by ethidium bromide, is shown. An equal loading of proteins (5 μ g) is shown by Coomassie staining of a constantly expressed protein of unknown identity (loading control). (B) Accumulation of GCP1 in various organs. Abbreviations: D, developing; M, mature; Md, mature dried; Mi, mature imbibed. An equal loading of proteins (5 μ g) is shown by Coomassie staining of a constantly expressed protein of unknown identity (Loading control).

Figure 4 Localization of GCP1/OSGEPL1 in *H. sapiens*

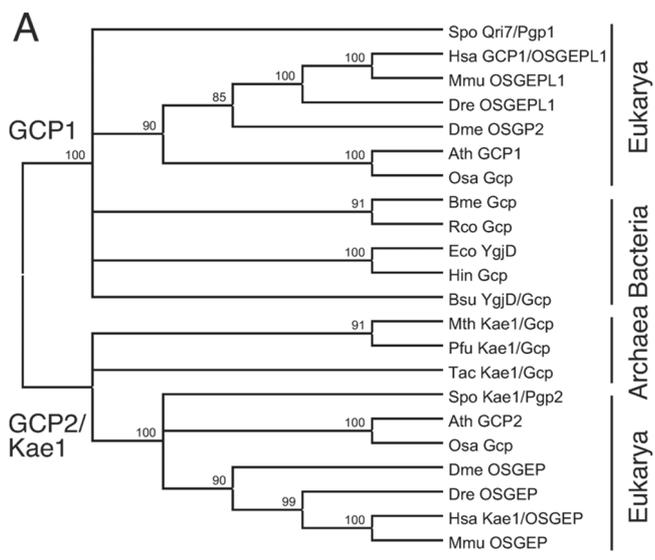
(A) Crossreactivity of *A. thaliana* α -GCP1 antibody with mitochondria isolated from rat liver and human cell cultures tested by immunoblotting. (B) Fluorescent histochemical staining. Mitochondria of HeLa cells were labelled with MitoTracker (green), GCP1/OSGEPL1 was detected with a rabbit α -GCP1/OSGEPL1 antibody and with a secondary antibody labelled with Cy3 fluorochrome (red) and co-localization of GCP1/OSGEPL1 and mitochondria is confirmed by overlapping both signals (yellow). The nuclei were stained with Hoechst 33342 dye (blue).

Figure 5 GCP1 knockout in *A. thaliana* is embryo lethal

(A) Schematic gene structure of *GCP1*. Black squares represent exons, lines introns. Arrows indicate T-DNA insertion sites and direction of inserted sequence in two GABI-Kat mutant lines *gcp1-1* and *gcp1-2*. (B) Differential interference contrast light microscopy photographs of fixed and cleared seeds from developing siliques of heterozygous *gcp1-1* and *gcp1-2* mutants. Arrowheads mark position of globular embryos.

Table 1. Quantitative analysis of abnormal developed seeds in heterozygous *gcp1-1* and *gcp1-2* mutants of *A. thaliana*.

	<i>gcp1-1</i>	<i>gcp1-2</i>
abnormal	183	188
wild type	611	548
total	794	736
% abnormal	23%	26%



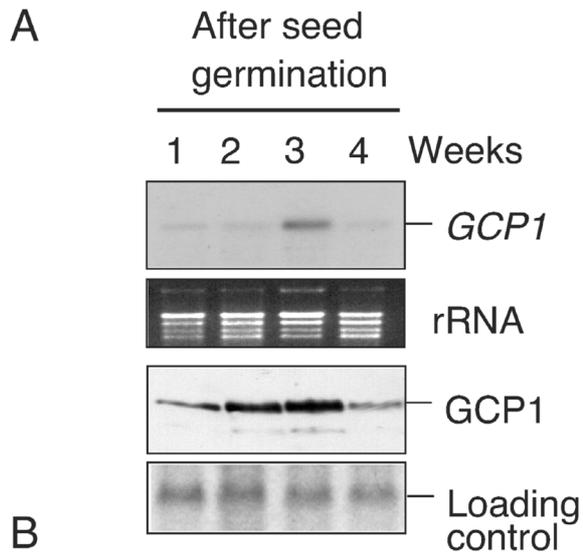
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GCP1	Arabidopsis	★ ★	VGVHHMEAHALV
YgjD	Escherichia		IPVHHMEGHLA
YdiE/Gcp	Bacillus		VGVHHIAGHIYA
Qri7/Pgp1	Yeast		LAVHHMQAHALA
OSGP2	Fruitfly		LPVHHMEAHALQ
OSGEPL1	Zebrafish		IPIHHMEAHALT
OSGEPL1	Mouse		IPIHHMEAHALT
GCP1/OSGEPL1	Human		IPIHHMEAHALT

Haussuehl et al_Fig. 1

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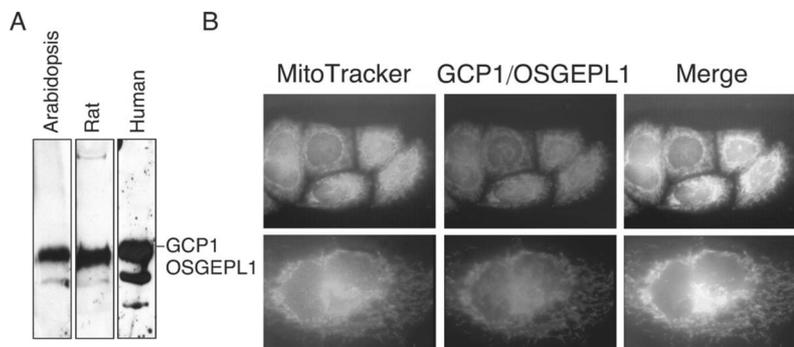
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Haussuehl et al_Fig. 3

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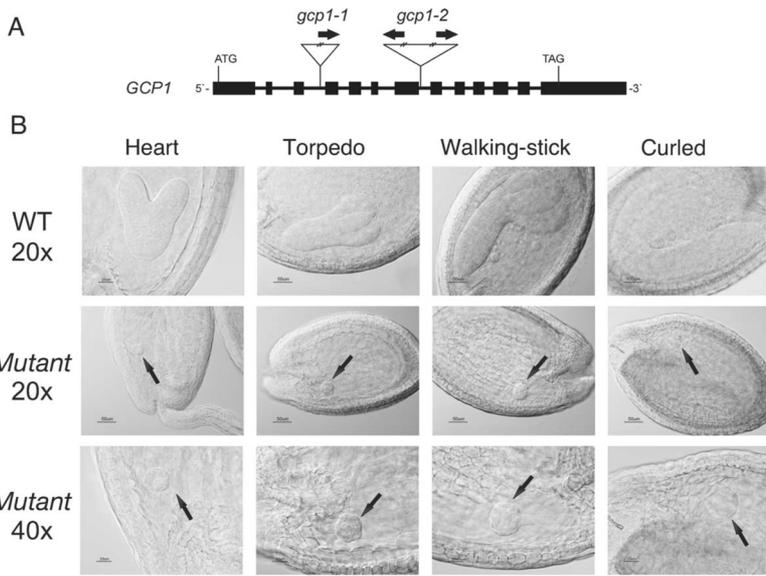
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Haussuehl et al_Fig. 4

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Haussuehl et al_Fig. 5

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