Supporting Information

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SI Text

Methods

Purification of Mitochondria from Potato Tubers (Protocol Derived from ref. 9). Tubers were homogenized in a juice extractor and the suspension mixed with one-third volume of grinding buffer. After filtration on a 45-μm nylon meshes, cell debris were removed by centrifugation at 1,600 $\times$ *g* for 10 min. The supernatant was then centrifuged at 13,000 $\times$ *g* for 15 min, and the crude mitochondria were resuspended in washing buffer with a Dounce homogenizer. After a second round of differential centrifugation, the resuspended mitochondrial pellet was loaded on a 28% (vol/vol) Percoll solution in washing buffer and centrifuged at 40,000 $\times$ *g* for 20 min (*TLA100.4 rotor*, *Optima TL Ultracentrifuge*) or 1 h (JA25.5 rotor). Mitochondria were collected from the bottom third of the Percoll gradient, diluted five to six times with the washing buffer and centrifuged at 12,000 $\times$ *g* for 10 min. The mitochondrial pellet was washed twice with five to six volumes of the washing buffer without BSA and centrifuged for 10 min at 10,000 $\times$ *g*.

Purification of Mitochondria from *A. thaliana* cells. *Buffers.* Grinding buffer (pH 7.5): 900 mM sucrose, 90 mM sodium pyrophosphate, 6 mM EDTA, 12 mM cysteine, 15 mM glycine, 2.4% (wt/vol), polyvinylpyrrolidone 25000, 0.9% (wt/vol) BSA, 6 mM 2-mercaptoethanol, pH is adjusted with 37% HCl.
Washing buffer (pH 7.2). 300 mM sucrose, 10 mM potassium phosphate pH 7.2, 1 mM EDTA, 5 mM glycine, 0.1% (wt/vol) BSA (derived from ref. 10).

Protoplasts were prepared from 5-day-old *A. thaliana* cell culture growing in the dark. Typically, 70 g of cells were collected from 1 liter of culture and incubated 3 h at 25°C in 130 ml of A buffer (0.4 M mannitol, 3.5 mM Mes-NaOH, pH 5.7) containing 1% (wt/vol) cellulase RS (Yakult) and 0.1% (wt/vol) pectolyase Y-23 (Seishin Pharmaceutical). Protoplasts were harvested by centrifugation at 800 $\times$ *g* for 10 min and washed three times with an equal volume of A buffer without the enzymes. Protoplasts were then resuspended in 50 ml of B buffer [0.4 M mannitol, 50 mM Tris-HCl, pH 7.5, 3 mM EDTA, 2 mM DTT and 0.1% (wt/vol) BSA] and ruptured by forcing them successively through 45-μm, 30-μm and 10-μm nylon meshes. Cell debris and nuclei were sedimented by centrifugation at 1,000 $\times$ *g* for 15 min. The supernatant was centrifuged at 12,000 $\times$ *g* for 15 min to pellet mitochondria, which were washed in C buffer (300 mM mannitol, 50 mM Tris-HCl, pH 7.5, 3 mM EDTA). The mitochondria-enriched fraction was subjected to a second round of differential centrifugations, then loaded onto a 18%/23%/40% (vol/vol) Percoll step gradient in C buffer and centrifuged at 40,000 $\times$ *g* for 1 h. Intact mitochondria were collected at the 23%/40% interface, diluted in buffer C and pelleted by three centrifugations at 17,000 $\times$ *g* for 15 min.

Isolation of Chloroplasts from Pea or Potato Leaves (from ref. 11). Chloroplasts were isolated from 8- to 10-day-old dwarf pea (variety Douce Provence) seedlings or 4-week-old potato plants. Leaves (30 g) were homogenized with a blender in 90 ml of grinding buffer (50 mM Hepes pH 7.3, 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.1% BSA, 0.1% reduced glutathione) by three bursts of 1 s each. The homogenate was filtered through six layers of gauze, and the filtrate was centrifuged at 2,000 $\times$ *g* for 5 min. Each pellet was carefully resuspended in 1 ml of grinding buffer and overlaid on top of four 25-ml Percoll gradients (50% Percoll solution in grinding buffer) preformed by centrifugation for 35 min at 40,000 $\times$ *g*. The gradients were centrifuged at 6,000 $\times$ *g* for 30 min. Intact chloroplasts in the lower band were gently collected with pipette, washed with 4–5 volumes of import buffer (50 mM Hepes, pH 7.3, 0.33 M sorbitol) and then pelleted at 2,000 $\times$ *g* for 4 min. The chlorophyll concentration was determined by: C (μg/ml) = 8.02 A_{663} + 20.2 A_{645}.

Isolation of Chloroplasts from *A. thaliana* cells. *Arabidopsis* chloroplasts were prepared by using a slightly modified protocol of ref. 12. Briefly, 30 g of 8-week-old *Arabidopsis* leaves were homogenized in buffer HB (0.45 mM sorbitol, 20 mM tricine, pH 8.4, 10 mM EDTA, 10 mM $NaHCO_3$). A crude plastid pellet was obtained by centrifugation at 5,000 $\times$ *g* for 3 min. Plastids were suspended in RB buffer (0.3 mM sorbitol, 20 mM tricine, pH 7.6, 5 mM $MgCl_2$, 2.5 mM EDTA) and further purified by centrifugation through a 40% Percoll cushion at 5,000 $\times$ *g* for 5 min. Intact chloroplasts were recovered as a pellet, washed twice with RB buffer.

***In Vitro* Import Assays into Isolated Chloroplasts and Mitochondria.** *In vitro* transcription/translation was carried out with a TNT Coupled Reticulocyte Lysate System (Promega) in the presence of [^{35}S]-methionine according to the manufacturer's instructions.

In vitro import into isolated pea chloroplasts was carried out essentially according to ref. 11. Four reaction mixtures were prepared. Each contains purified chloroplasts (equivalent to 100 μg of chlorophyll) in 1$\times$ import buffer (see isolation of pea chloroplasts) and 22.7 mM methionine (Table S2).

After import, the chloroplasts were isolated by centrifugation through a Percoll cushion (40% (vol/vol) Percoll solution in import buffer) at 5,000 $\times$ *g* for 5 min. The pellet was further washed with import buffer and centrifuged at 5,000 $\times$ *g* for 5 min. The final pellet was resuspended in SDS/PAGE sample buffer.

In vitro import into isolated mitochondria was performed essentially as described in ref. 13. Four reaction mixtures were prepared containing potato purified mitochondria (equivalent to 50 μg of mitochondrial protein), 0.3 M mannitol, 20 mM KCl, 1 mM potassium phosphate, 1 mM malate, 1 mM NADH, 1 mM DTT, 10 mM Hepes pH 7.5, 40 μM ADP, 2 mM ATP in a total volume of 45 μl (Table S3).

After import, intact mitochondria were isolated by centrifugation through a 27% sucrose cushion (27% (vol/vol) sucrose, 10 mM potassium phosphate pH 7.5, 1 mM EDTA pH 8.0, 0.1% BSA) at 10.000 $\times$ *g* for 10 min. The pellet was resuspended in SDS/PAGE sample buffer.

In both import assays, proteins are separated in a polyacrylamide–SDS gel. Radiolabeled proteins are visualized by using a BAS2500 image plate scanner.

In both assays, the radioactive preprotein is incubated with isolated mitochondria or chloroplasts (Tubes 1) and a smaller mature peptide should appear showing that the preprotein is imported into the organelles and processed into the mature form by cleavage of the targeting sequence.

The addition of proteinase K to the import mixture (Tubes 2) should reduce the signal corresponding to the preprotein but should not affect the signal corresponding to the mature protein, that is protected by mitochondrial or chloroplastic membranes.

When the mitochondria or the chloroplasts are preincubated with valinomycine or in the dark respectively, protein import is inhibited (Tubes 3 and 4). In these conditions the formation of

the mature protein should be prevented. Comparison of proteinase K treatment in Tubes 2 and 4 should confirm that the signal observed in tube 2 represents genuine imported proteins.

Western Blot Analysis. Mitochondrial and chloroplastic extracts were obtained by resuspending mitochondria or chloroplasts in SDS/PAGE sample buffer. The *A. thaliana* cytosolic fraction corresponds to the supernatant obtained after disruption of the cells (see above, purification of *A. thaliana* mitochondria) and centrifugation (10,000 × *g*). The potato total extract was prepared from leaves that were frozen in liquid nitrogen and ground in Extraction Buffer (20 mM Tris pH 7.5, 2 mM EDTA, 0.5% 2-mercaptoethanol, 0.3% SDS). The homogenate was centrifuged at 12000 g for 10 min. The supernatant was mixed to SDS/PAGE sample buffer.

Proteins were separated by polyacrylamide–SDS gel electrophoresis, electrotransferred onto Immobilon-P membranes (Millipore) and submitted to immunological detection following classical protocols (14). Binding of the primary antibodies was revealed by chemiluminescence by using peroxidase-conjugated secondary antibodies and ECL reagents (Amersham Pharmacia Biotech).

Preparation of [^{14}C]Glu-tRNAs and [^{14}C]Asp-tRNAs. Plant mitochondrial GluRS was isolated from a potato mitochondrial enzymatic extract by gel filtration chromatography (Superdex 200, Amersham) (15). [^{14}C]Glu-tRNA was prepared in a 50-μl mixture containing 100 mM Na-Hepes pH 7.2, 30 mM KCl, 10 mM ATP, 15 mM $MgCl_2$, 20 μM [^{14}C]Glu (330 cpm/pmol), 1 nmol total mitochondrial tRNA and 1.5 μg partially purified mitochondrial GluRS (quantities for one amidation test). The incubation was 15 min at 37°C.

For [^{14}C]Asp-tRNA, the standard aminoacylation mixture (100 μl) containing 100 mM Na-Hepes pH 7.2, 30 mM KCl, 2 mM ATP, 12 mM $MgCl_2$, 50 μM l-[^{14}C]Asp (300 cpm/pmol), 5 μM of purified *T. thermophilus* $tRNA^{Asn}$ overexpressed in *E. coli*, and 1 μM *T. thermophilus* nondiscriminating AspRS was incubated 10 min at 50°C.

For both [^{14}C]Asp-tRNAs and [^{14}C]Glu-tRNAs, reactions were stopped by sequential acid-buffered phenol and chloroform extractions. The [^{14}C]aa-tRNAs were precipitated with ethanol, pelleted, dissolved in water and the concentration was determined by scintillation counting of an aliquots after TCA precipitation.

tRNA-Dependent Amidation Assays. The standard reaction mixture of 50 μl containing 100 mM Na–Hepes buffer pH 7.2, 12 mM $MgCl_2$, 2 mM Gln, 10 mM ATP, 100 pmol [^{14}C]Asp-tRNA or 10 pmol [^{14}C]Glu-tRNA and 1 μM of *A. thaliana* pure enzyme or 15 μg of *A. thaliana* mitochondrial or chloroplastic crude extract was incubated during 15 min at 37°C. After phenol/chloroform extractions, the [^{14}C]aa-tRNA was precipitated with ethanol, sedimented by centrifugation, dried and dissolved in 50 μl of water. Deacylation was performed during 30 min at 37°C in presence of 30 mM KOH. The hydrolysate was neutralized by addition of 30 mM HCl and dried in a Speed-Vac, dissolved in 3 μl of water and fractionated by TLC on cellulose plates (20 × 20 cm) extended by a 3MM Whatman paper sheet (5 × 20 cm), with a solvent containing 2-propanol/formic acid/water (80:4:20, by vol). The [^{14}C] amino acids were revealed by scanning the exposed image plate with a Fuji Bioimager.

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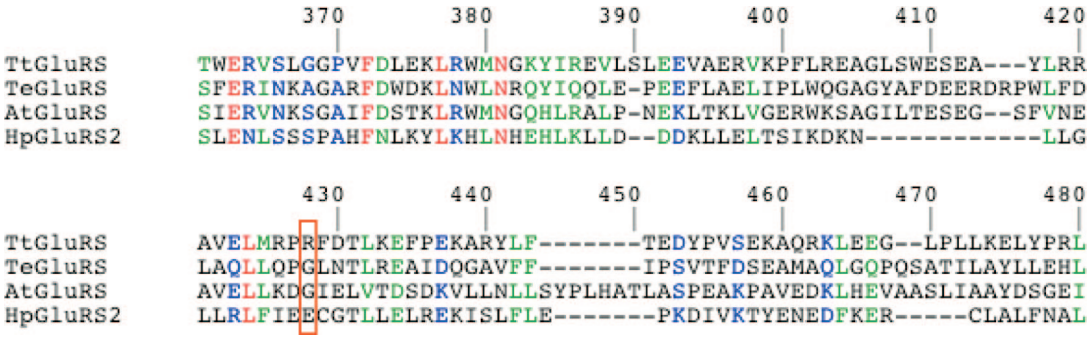

Fig. S1. Alignment of GluRS Sequences. GluRS sequences from *Thermus thermophilus* (Tt), *Thermosynechococcus elongatus* (Te), *Arabidopsis thaliana* (At; organellar GluRS), and *Helicobacter pylori* (Hp, GluRS2) were aligned by using Clustal W (http://pbil.univ-lyon1.fr). Te, At and Hp GluRS are nondiscriminating (ND) GluRS, as Tt GluRS is discriminating (D). The crystal structure of the Tt GluRS.tRNA^{Glu} complex was obtained and showed the interaction of the third nucleotide of the anticodon with an arginine residue (Arg-358; boxed in red). Mutation of this Arg transformed the D-GluRS into a ND-GluRS able to aminoacylate both tRNA^{Gln} and tRNA^{Glu} (1). The crystal structure of Te ND-GluRS was also obtained and the Arg-358 is replaced by a Gly (Gly-366) in this structure (2). In Hp ND-GluRS2 the Arg-358 is replaced by a Glu (Glu-334). Mutation of this Glu into Arg reduced the catalytic efficiency of the enzyme toward tRNA^{Gln} but has no effect toward tRNA^{Glu} (3). In At GluRS, a Gly (Gly-428) is found at this discriminating position, in accordance with its nondiscriminating activity. Only the sequences around the discriminating position are shown. The numbers refer to At sequence.

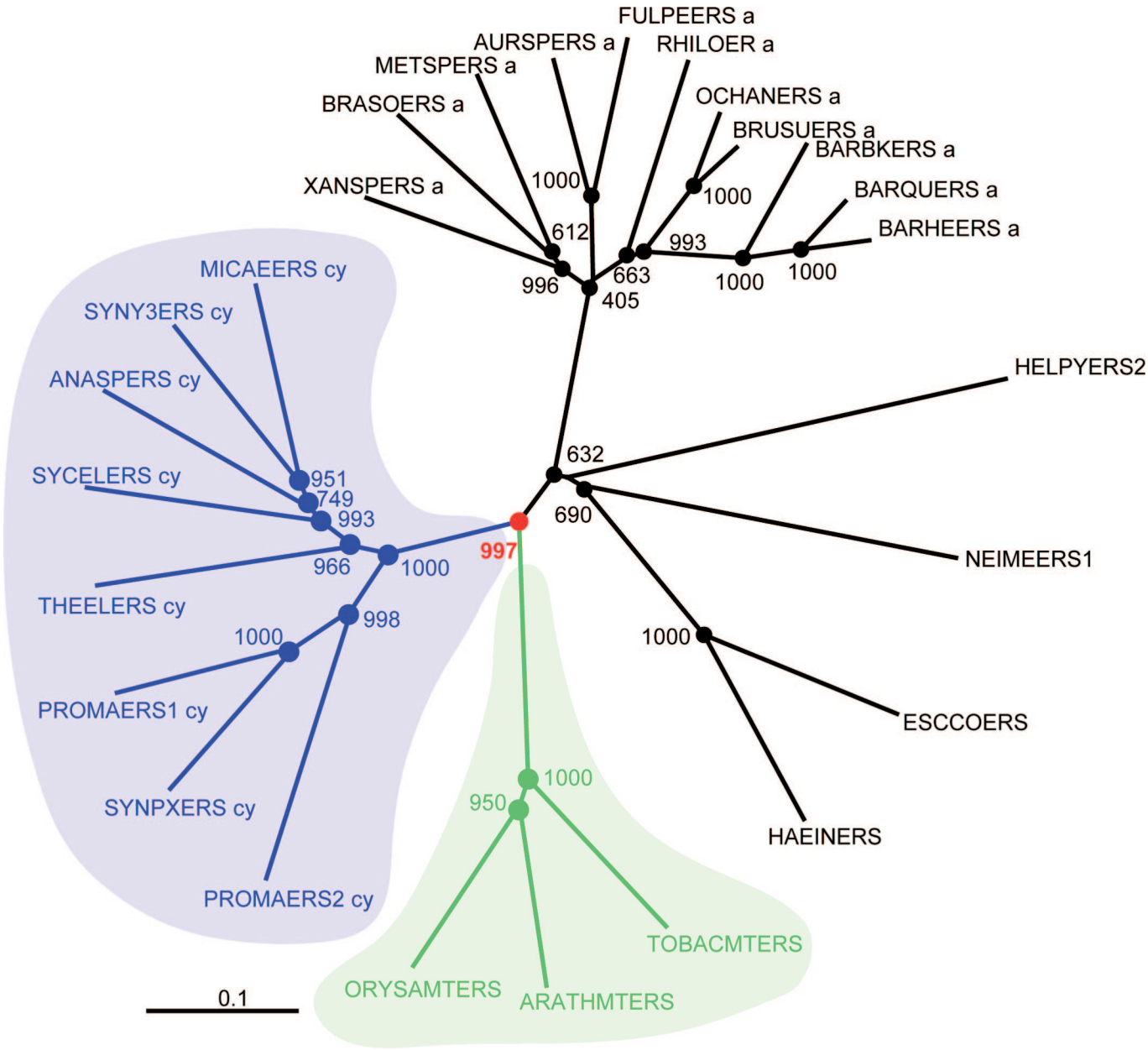

Fig. S2. Analysis of the Phylogenetic Origin of Organellar Plant GluRS. The sequences of three plant organellar GluRSs (in green) were aligned with those of a sampling of eight cyanobacteria (cy, in purple) and 11 alpha-proteobacteria (a). Alignments were performed by using the CLUSTALX program, Version 1.81 (4). The trees were constructed from a bootstrap of 1000 replicates inferred by the neighbor-joining method by using the PHYLIP package, Version 3.57c (J. Felsenstein, University of Washington, Seattle), with the Dayhoff PAM matrix. Trees were edited with the TREEVIEW program, Version 1.5. A tree with archaea and eukaryotes GluRS sequences can be seen in the supplementary information of ref. 5.

Fig. S3. Phylogenetic analysis of GatA, GatB, and GatC sequences. The archaea are represented in pink, bacteria in red, eukaryotes in blue and plants in green. α-proteobacteria are marked by # and cyanobacteria are marked by *. The Gat sequences were downloaded through SwissProt databases, except for the following sequences, which were identified by BLAST: GATA_YEAST, NP_014021; GATA_MOUSE, BAE41646; GATA_HUMAN, NP_060762; GATA_Orysa, EAZ32175; GATB_Orysa, NP_001068056 and GATC_Orysa, NP_001050789. The entire proteins sequences were aligned by using MUSCLE v3.52 (6). The phylogeny was performed with the PhyML program (7). The unrooted trees were built by using the neighbor joining method and were drawn with Treedyn (8). Bootstrap values above 50 (using 100 replications) are indicated along branches. The scale bar indicates the evolution distance (amino acid substitutions per site). The complete list of species used in this phylogenetic analysis is shown below in Table S1. Plant GatA and GatB are poorly related to other eukaryotic GatA and GatB. Plant GatA and GatB are related to cyanobacteria. By contrast mammals and yeast GatA form a distinct branch related to α-proteobacteria. Mammals GatB are also close to α-proteobacteria, and yeast GatB appears distinct. Last GatC sequences are poorly conserved. So the origin of plant Gat proteins seems different from the other eukaryotic Gat orthologs.

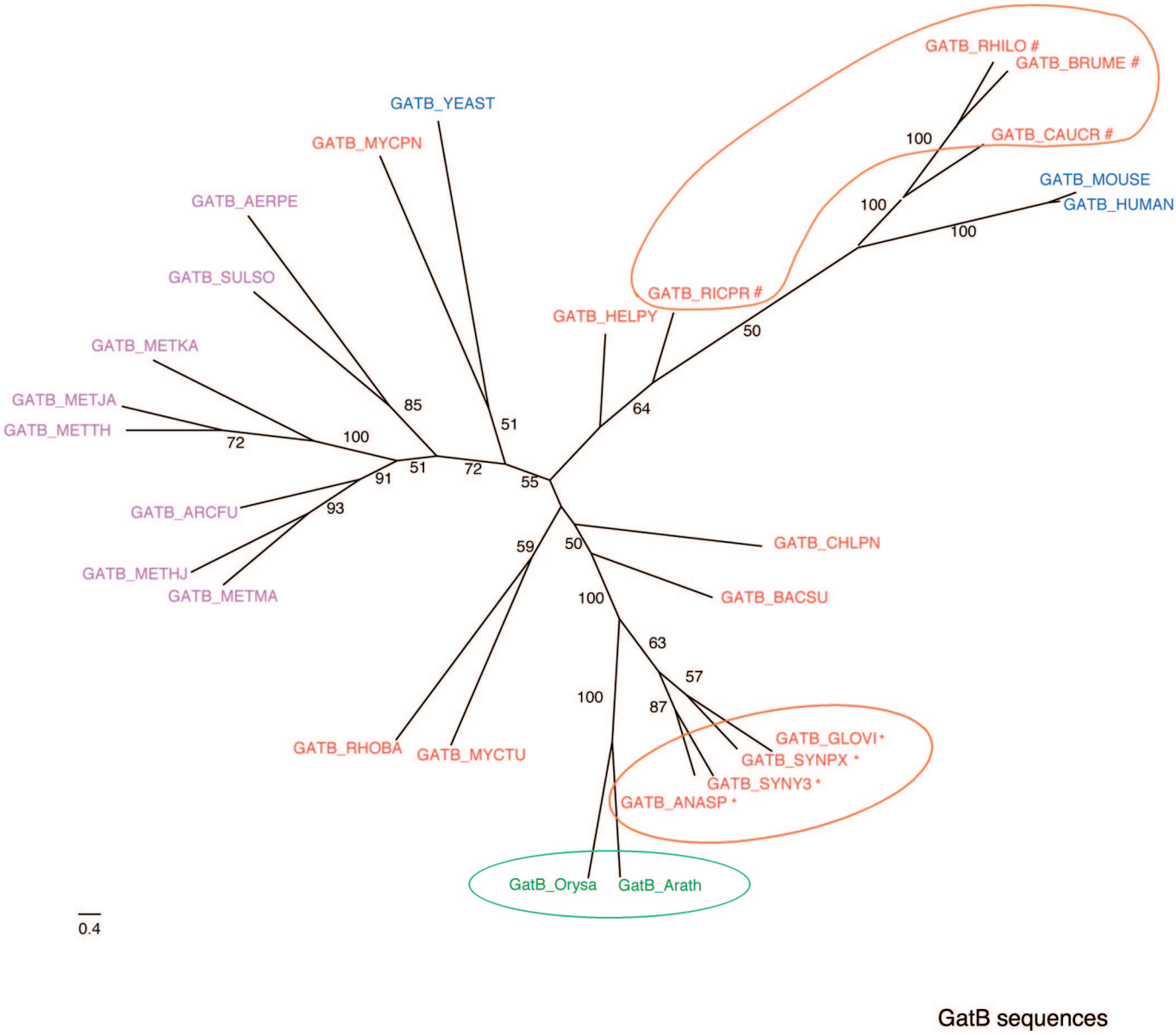

Fig. S3. Continued

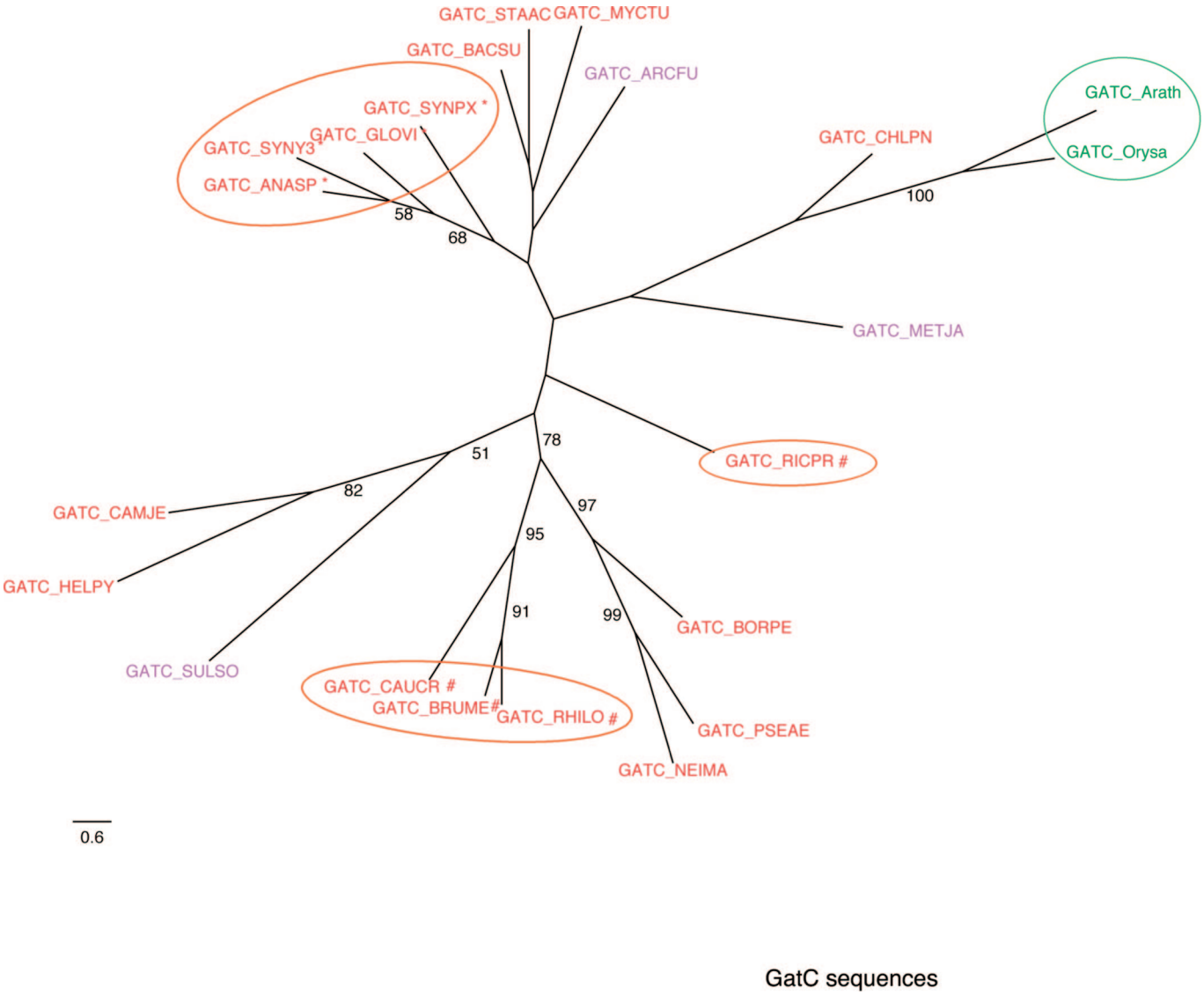

Fig. S3. Continued

Table S1. Species used for the phylogenetic analysis

Group		abbreviation	species
A		AERPE	Aeropyrum pernix.
A		ARCFU	Archaeoglobus fulgidus.
A		METJA	Methanococcus jannaschii.
A		METKA	Methanopyrus kandleri.
A		METMA	Methanosarcina mazei (Methanosarcina frisia).
A		METTH	Methanobacterium thermoautotrophicum.
A		SULSO	Sulfolobus solfataricus.
A		METHJ	Methanospirillum hungatei
B	cyano	ANASP	Anabaena sp. (strain PCC 7120).
B	firmicutes	BACSU	Bacillus subtilis.
B	beta	BORPE	Bordetella pertussis.
B	alpha	BRUME	Brucella melitensis.
B	epsilon	CAMJE	Campylobacter jejuni.
B	alpha	CAUCR	Caulobacter crescentus.
B	chlamydia	CHLPN	Chlamydia pneumoniae (Chlamydophila pneumoniae).
B	cyano	GLOVI	Gloeobacter violaceus.
B	epsilon	HELPY	Helicobacter pylori (Campylobacter pylori).
B	firmicutes	MYCPN	Mycoplasma pneumoniae.
B	actinomycetes	MYCTU	Mycobacterium tuberculosis.
B	beta	NEIMA	Neisseria meningitidis (serogroup A).
B	gamma	PSEAE	Pseudomonas aeruginosa.
B	alpha	RHILO	Rhizobium loti (Mesorhizobium loti).
B	plantomycetes	RHOBA	Rhodopirellula baltica.
B	alpha	RICPR	Rickettsia prowazekii.
B	firmicutes	STAAC	Staphylococcus aureus
B	cyano	SYNPX	Synechococcus sp. (strain WH8102).
B	cyano	SYNY3	Synechocystis sp. (strain PCC 6803).
E		HUMAN	Homo sapiens
E		MOUSE	Mus musculus
E		YEAST	Saccharomyces cerevisiae
P		ARATH	Arabidopsis thaliana
P		ORYSA	Orysa sativa

A, archae; B, eubacteria; E, eukaryotes; P, plants. cyano, cyanobacteria; alpha, α-proteobacteria; beta, β-proteobacteria; gamma, χ-proteobacteria; epsilon, ε-proteobacteria.

Table S2. Import into chloroplasts

Tube	1	2	3	4
Light/dark	Light	Light	Dark	Dark
Incubation 10 min at 25°C				
Translation lysate	5 μl	5 μl	5 μl	5 μl
Light/dark	Light	Light	Dark	Dark
GTP 0.33 mM final	+	+	-	-
Incubation 20 min at 25°C				
Proteinase K 0.033 mg/ml final 10 min on ice	-	+	-	+
PMSF 2.7 mM final	-	+	-	+
Percoll cushion				

Table S3. Import into mitochondria

Tube	1	2	3	4
Valinomycin 2μM final	−	−	+	+
Incubation 10 min at 25°C				
Translation lysate	5μl	5μl	5μl	5μl
Incubation 30 min at 25°C				
Proteinase K 0.2 mg/ml final 10 min on ice	−	+	−	+
PMSF 8 mM final	−	+	−	+
Sucrose cushion				