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Overlapping Destinations for Two Dual Targeted Glycyl-tRNA Synthetases in *Arabidopsis thaliana* and *Phaseolus vulgaris**

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In plant mitochondria, some of the tRNAs are encoded by the mitochondrial genome and resemble their prokaryotic counterparts, whereas the remaining tRNAs are encoded by the nuclear genome and imported from the cytosol. Generally, mitochondrial isoacceptor tRNAs all have the same genetic origin. One known exception to this rule is the group of $tRNA^{Gly}$ isoacceptors in dicotyledonous plants. A mitochondrion-encoded $tRNA^{Gly}$ and at least one nucleus-encoded $tRNA^{Gly}$ coexist in the mitochondria of these plants, and both are required to allow translation of all four GGN glycine codons. We have taken advantage of this atypical situation to address the problem of tRNA/aminoacyl-tRNA synthetase coevolution in plants. In this work, we show that two different nucleus-encoded glycyl-tRNA synthetases (GlyRSs) are imported into *Arabidopsis thaliana* and *Phaseolus vulgaris* mitochondria. The first one, GlyRS-1, is similar to human or yeast glycyl-tRNA synthetase, whereas the second, GlyRS-2, is similar to *Escherichia coli* glycyl-tRNA synthetase. Both enzymes are dual targeted, GlyRS-1 to mitochondria and to the cytosol and GlyRS-2 to mitochondria and chloroplasts. Unexpectedly, GlyRS-1 seems to be active in the cytosol but inactive in mitochondrial fractions, whereas GlyRS-2 is likely to glycylate both the organelle-encoded $tRNA^{Gly}$ and the imported $tRNA^{Gly}$ present in mitochondria.

Aminoacyl-tRNA synthetases (aaRSs)[1] play a crucial role in protein synthesis by catalyzing the addition of amino acids to their cognate tRNAs. In plants, protein synthesis occurs in three cellular compartments: the cytosol, the mitochondria, and the chloroplasts. All tRNAs and aaRSs necessary for mRNA translation have to be present in these three compartments. In photosynthetic plants, cytosolic tRNAs are all nucleus-encoded, and chloroplastic tRNAs are all chloroplast-encoded (1). By contrast, plant mitochondrial tRNAs have several genetic origins. Some are nucleus-encoded and imported from the cytosol. The others are mitochondrion-encoded and show 70–75% identity with their prokaryotic counterparts and less than 60% identity with their cytosolic counterparts. These mitochondrion-encoded tRNAs are of two types: the "native" tRNAs derived from authentic mitochondrial tRNA genes and the "chloroplast-like" tRNAs that are 97–100% identical to their chloroplast counterparts. The genes corresponding to the latter originated from the chloroplast and were inserted into the mitochondrial genome during evolution. The number of tRNAs in each category (imported, native, or chloroplast-like) and their identity can vary from one plant species to another (2, 3).

Higher plant aaRSs are all encoded by the nuclear genome and post-translationally addressed to the different subcellular compartments. The fidelity of translation relies in part on the specificity of the aminoacylation reaction catalyzed by the aaRSs. Thus, strong coevolution is expected between the aaRSs and their cognate tRNAs (4, 5). A few plant genes coding for mitochondrial aaRSs have been characterized, and it appears that the aaRSs used in mitochondrial translation have, in general, the same genetic origin as their substrate tRNAs. This is the case for $tRNAs^{Ala}$, $tRNAs^{Thr}$, and $tRNAs^{Val}$ and their cognate aaRSs in *Arabidopsis thaliana*. These tRNAs are most likely to be imported from the cytosol into mitochondria in *A. thaliana*, because the corresponding genes are absent from the mitochondrial genome (6), and they were shown to be imported into mitochondria in other plants such as potato (3). Similarly, cytosolic alanyl-tRNA synthetase (7), threonyl-tRNA synthetase, and valyl-tRNA synthetase (8) are also imported into mitochondria. In all three cases, the mitochondrial form and the cytosolic form of the enzyme are encoded by the same gene. Similarly, dual targeting to mitochondria and chloroplasts was observed for methionyl- (9), histidyl- (10), cysteinyl-, and asparaginyl-tRNA synthetase (11), whereas the corresponding mitochondrial tRNAs were shown to be encoded by native (initiator $tRNA^{Met}$, $tRNA^{Cys}$) or chloroplast-like (elongator $tRNA^{Met}$, $tRNA^{His}$, $tRNA^{Asn}$) genes present in the mitochondrial genome (6).

An organelle-encoded native $tRNA^{Gly}$(GCC) is present in mitochondria of the dicotyledonous plants *A. thaliana* (6, 12), potato (*Solanum tuberosum*) (13), and common bean (*Phaseolus vulgaris*) (14). Because this tRNA cannot read all four GGN glycine codons, at least one other $tRNA^{Gly}$ is required in mitochondria for translation to occur, and a $tRNA^{Gly}$(UCC) was shown to be imported from the cytosol into *S. tuberosum* and *P. vulgaris* mitochondria (14). Coexistence of imported and organelle-encoded isoacceptor tRNAs in mitochondria has been reported only in a very few cases, *i.e.* $tRNAs^{Ile}$ in higher plants (2) and $tRNAs^{Ile}$, $tRNAs^{Thr}$, and $tRNAs^{Val}$ in *Marchantia polymorpha* (15). The presence of isoacceptor tRNAs with different

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[1] The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; GlyRS, glycyl-tRNA synthetase; PCR, polymerase chain reaction; GFP, green fluorescent protein.

genetic origins in plant mitochondria raises the problem of the coevolution between tRNAs and aaRSs. In this work, we show that, along with the coexistence in the organelles of a cytosolic and a native mitochondrial $tRNA^{Gly}$, two different glycyl-tRNA synthetases are imported into mitochondria in dicotyledonous plants. Both are dual targeted proteins, one to the cytosol and mitochondria (called glycyl-tRNA synthetase-1 (GlyRS-1)) and the other to chloroplasts and mitochondria (called GlyRS-2). Unexpectedly, GlyRS-1 was shown to be active in the bean cytosol but not in mitochondria, whereas GlyRS-2 was able to aminoacylate either nuclearly or mitochondrially encoded $tRNAs^{Gly}$.

EXPERIMENTAL PROCEDURES

Inverse PCR—A. thaliana total DNA was extracted from whole plants according to Dellaporta *et al.* (16), digested with *Bgl*II, self-ligated, and used as a template for PCR amplification (17) with divergent oligonucleotides AM10 (5′-GCGGGATCCGGATCGGAGC-GATGGAGATTGGG-3′; the *Bam*HI site for cloning is underlined) and AM12 (5′-CGCTCTAGAGGCTTCCCGTGCAGCTAAACCAG-3′; the *Xba*I site for cloning is underlined) (see Fig. 1*A*).

Primer Extension—Total RNA was extracted from 3-week-old *A. thaliana* plants (18), and poly(A^+) RNA was prepared using a PolyATtract mRNA isolation system IV kit (Promega, Madison, WI). Primer extensions were performed with oligonucleotide AM16 (5′-GGATCG-GAGCGATGGAGATTGGG-3′) (see Fig. 1*A*) and with 1 μg of poly(A^+) RNA or 12 μg of total RNA (17). Sequencing reactions were primed with oligonucleotide AM16 (17).

Computer Predictions of Subcellular Targeting—Predictions of intracellular targeting of proteins were made using ChloroP, MITOPROT, Predotar, PSORT, or TargetP.

Isolation of Mitochondria and Chloroplasts—Isolation of organelles from *A. thaliana* plants gave a very poor yield, and extensive cytosolic contamination was observed. Organelles were therefore mainly isolated from bean, and to a lesser extent from wheat and potato. Mitochondria were extracted from 3-week-old *A. thaliana* plants, from 5-day-old etiolated bean hypocotyls or wheat plantlets, or from potato tubers in extraction buffers containing 0.6 M mannitol, 0.3 M sucrose, or 0.3 M mannitol, respectively, as an osmoticum (19) and were purified on continuous polyvinylpyrrolidone/Percoll gradients (20). For *A. thaliana* mitochondria, a second, discontinuous polyvinylpyrrolidone/Percoll gradient was necessary to reduce cytosolic contamination (14). Chloroplasts were extracted from leaves of 6-day-old bean plants (21).

For protease treatment, mitochondria were incubated in the presence of 100 μg/ml proteinase K for 5 min at room temperature and 10 min on ice. Upon addition of 2 mM phenylmethylsulfonyl fluoride, organelles were recovered by centrifugation through a cushion of 27% (w/v) sucrose, 20 mM Hepes-KOH, pH 7.5, 1 mM phenylmethylsulfonyl fluoride. For subfractionation, mitochondria were resuspended in 5 mM potassium phosphate buffer, pH 7.5, and incubated on ice for 20 min to disrupt the outer membrane. Gentle homogenizing with a plunger was applied several times during incubation to help release of the outer membrane. The suspension was subsequently loaded on a 15/32/45/52% sucrose step gradient in a 10 mM potassium phosphate buffer, pH 7.5, containing 2 mM EDTA and 0.2% (w/v) bovine serum albumin and centrifuged for 20 min at 125,000 × *g*. The outer membrane fraction and the mitoplasts were recovered at the 15/32% interface and the 45/52% interface, respectively. Both were washed in 10 mM potassium phosphate, pH 7.5, 0.3 M sucrose, 1 mM EDTA, 0.1% (w/v) bovine serum albumin, 5 mM glycine and pelleted for 10 min at 175,000 × *g*. Mitoplasts were resuspended in the same buffer and disrupted by three freeze/thaw cycles and two sonication cycles of 10 s. The suspension was successively centrifuged for 5 min at 2500 × *g*, to eliminate the remaining undisrupted material, and for 30 min at 175,000 × *g*, to separate the inner membrane fraction from the matrix.

Protein Extracts—Denatured protein extracts for SDS-polyacrylamide gel electrophoresis were prepared in 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.3% (w/v) SDS, 5% (v/v) β-mercaptoethanol. Enzymatic extracts for aminoacylation and chromatography were prepared according to Maréchal-Drouard *et al.* (22).

Aminoacylation Assays—Aminoacylations (19) were performed in the presence of 10^{-4} M [^{3}H]glycine and 3 μg/μl yeast or *Escherichia coli* total tRNA, 0.15 μg/μl bean total leaf, mitochondrial, or chloroplastic tRNA (22), or 0.04 μg/μl *in vitro* transcribed $tRNA^{Gly}$.

Green Fluorescent Protein (GFP) Targeting Analysis—The sequence corresponding to the N terminus of GlyRS-1 (nucleotides 616–810 in Fig. 1*A*) was amplified by PCR using the *A. thaliana* cDNA as a template and oligonucleotides SIGM.5 (5′-GATCTCTAGAAAAATGCG-CATCTTCTCTACA-3′; the *Xba*I site is underlined) and SIGM.3 (5′-CATGCTCGAGAGTTACCCTGAGCTTCGAC-3′; the *Xho*I site is underlined). To fuse this sequence with the GFP gene, the PCR products were cloned into the *Spe*I/*Sal*I sites of pOL-GFP-S65C (11), generating pSYGM in which the first AUG of the GlyRS-1 sequence was in a favorable context for initiation of translation. The sequence corresponding to the N terminus of GlyRS-2 (GenBank™ accession number AJ003069, nucleotides 32–275) was amplified by PCR using *A. thaliana* total DNA as a template and oligonucleotides SIGO.5 (5′-GATCTCTA-GAAAAATGGCCATCCTCCATT-3′; the *Xba*I site is underlined) and SIGO.3 (5′-CATGCTCGAGCCTGGAGGCGTTGAA-3′; the *Xho*I site is underlined). The PCR products were cloned into the *Spe*I/*Sal*I sites of pOL-GFP-S65C, generating pSYGO. The complete expression cassette containing the cauliflower mosaic virus 35S promoter, the GFP fusion, and the cauliflower mosaic virus 35S terminator was cut out of pSYGM and pSYGO with *Hin*dIII and cloned into the binary vector pGPTV-kan (23). The resulting plasmids, pNP7 and pNP8, respectively, were used to transform the *Agrobacterium tumefaciens* LBA4404 strain (Life Technologies, Inc.).

Transformation was performed by infiltration of *Nicotiana benthamiana* leaves (24) with a suspension of *A. tumefaciens* carrying pNP7 or pNP8. After 25–30 h, protoplats were prepared (25) from the infiltrated leaves. Protoplasts were stained with a mitochondria-specific dye (MitoTracker™, CMTMRos, Molecular Probes, Eugene, OR) and analyzed using an epifluorescence microscope (9).

In Vitro Import into Isolated Mitochondria—The sequence corresponding to the N terminus of GlyRS-1 (nucleotides 616–742 in Fig. 1*A*) was first amplified by PCR using the *A. thaliana* cDNA clone as a template and oligonucleotides AM26 (5′-CGCGCCATGGGCATCTTCT-CTACATTCGTCTTTCATCGC-3′; the *Nco*I site is underlined) and AM36 (5′-CGCGGATCCTCGGCGTCAATCGGAATCTGGATC-3′; the *Bam*HI site is underlined). This introduced a point mutation at position 735, replacing the second AUG codon with an AUU isoleucine codon. The PCR products were cloned into the *Nco*I/*Bam*HI sites of pCK-GFP3 (9), yielding a GFP fusion. The obtained plasmid was named pAM160. The *Eco*RI/*Hin*dIII fragments from pAM160 and pSYGO (see above), which contained the tobacco etch virus translation leader, the GlyRS-1 or GlyRS-2 N terminus, the GFP gene, and the cauliflower mosaic virus 35S terminator, were cloned into pBlueScript-KS (Stratagene, La Jolla, CA). These constructs were used as templates for *in vitro* transcription/translation carried out with a TNT™ coupled reticulocyte lysate system (Promega) in the presence of [^{35}S]methionine. Import of ^{35}S-labeled fusion proteins into purified potato mitochondria was performed according to Wischmann and Schuster (26) and analyzed by SDS-polyacrylamide gel electrophoresis.

Overexpression of GlyRS-1 in E. coli—The sequence of the cytosolic form (690 amino acids) of GlyRS-1 was amplified by PCR using the *A. thaliana* cDNA as a template and oligonucleotides AM19 (5′-CGCGC-CATGGACGCCACCGAGCAGTCTCTC-3′; the *Nco*I site is underlined) and AM20 (5′-GGCGGATCCGTCTGCAGCAGCAGAAGAATG-3′; the *Bam*HI site is underlined) and cloned into pQE60 (Qiagen, Hilden, Germany). The resulting plasmid, pAM147, was used to transform the *E. coli* TG2 strain (17). Overexpression in *E. coli* was induced with 2 mM isopropyl-1-thio-β-D-galactopyranoside. Protein extraction under denaturing conditions was performed according to the manufacturer's instructions. Extracts were fractionated by SDS-polyacrylamide gel electrophoresis. The polypeptide corresponding to GlyRS-1 was electroeluted from the gels (27) and injected into rabbits to raise antibodies. Alternately, native enzymatic extracts (22) were prepared from the *E. coli* strain overexpressing GlyRS-1 and used for liquid chromatography fractionation.

Western Blot Analysis—Proteins were separated by SDS-polyacrylamide gel electrophoresis, electrotransferred onto Immobilon™-P membranes (Millipore, Bedford, MA), and submitted to immunological detection following classical protocols (17). Antisera were used at a 1/5000 dilution. Antibodies against mitochondrial superoxide dismutase were a gift from Dirk Inzé (Gent, Belgium), and antibodies against α-tubulin were from Amersham Pharmacia Biotech. Antisera against bean chloroplastic leucyl-tRNA synthetase were obtained previously (28). Binding of the primary antibodies was revealed by chemiluminescence using peroxidase-conjugated secondary antibodies and ECL reagents (Amersham Pharmacia Biotech).

Liquid Chromatography—Enzymatic extracts were fractionated by medium pressure chromatography on a 1-ml POROS 20 PE hydrophobic column (PerSeptive Biosystems, Framingham, MA) driven by a BioLogic integrated system (Bio-Rad). The samples (1 mg of proteins)

adjusted to 1.5 M ammonium sulfate were loaded at 1 ml/min on the column equilibrated with a 20 mM Tris-HCl buffer, pH 7.5, containing 1.5 M ammonium sulfate, 1 mM $MgCl_2$, 5% (v/v) 1,2-propanediol, 0.1 mM EDTA, 5 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM diisopropylfluorophosphate. After washing with the same buffer (5 ml), elution was performed at 1 ml/min with a linear ammonium sulfate gradient (10 ml, 1.5 to 0 M). Fractions of 0.33 ml were collected, and aliquots were submitted to aminoacylation assays and Western blot analyses.

Enzymatic extracts were also fractionated on a 1.3-ml UNO™-Q1 anion exchange column (Bio-Rad). The samples were loaded at 0.5 ml/min on the column equilibrated with a 20 mM Tris-HCl buffer, pH 7.5, containing 10 mM NaCl, 1 mM $MgCl_2$, 5% (v/v) 1,2-propanediol, 0.1 mM EDTA, 5 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM diisopropylfluorophosphate. Elution was performed at 0.7 ml/min with a 15-ml linear gradient from 10 to 500 mM NaCl, followed by a 5-ml linear gradient from 500 to 1000 mM NaCl. Fractions of 0.5 ml were collected.

Preparation of tRNA Transcripts—Constructs encoding $tRNAs^{Gly}$ were amplified by PCR with the relevant primers (see below) so that the tRNA gene sequence was directly fused to the T7 RNA polymerase promoter at the 5′ terminus and to a *Bst*NI site at the 3′ terminus. PCR products were cloned into the *Eco*RI or *Eco*RI/*Bam*HI sites of pUC19. After *Bst*NI digestion, *in vitro* transcription of these constructs with T7 RNA polymerase yielded mature sized, unmodified tRNA transcripts with a 3′ CCA end (29). The following oligonucleotides were used as primers (*Eco*RI and *Bam*HI sites are underlined; the T7 RNA polymerase promoter or *Bst*NI site is in italics): for mitochondrial native $tRNA^{Gly}$(GCC), 5′ AGCAAGAATTC*GAATTGTAATACGACTCACTATA*GCGGAAATAGCTTAATGGTAG 3′ and 5′ GTACA*GAATTCCCTGG*AGCGGAAGGAGGGACTTGAAC 3′; for cytosolic $tRNA^{Gly}$(GCC), 5′ AGCAAGAATTC*GAATTGTAATACGACTCACTATA*GCACCAGTGGTCTAGTGGTAG 3′ and 5′ GTACAGAATTC*CCTGG*TGCACCAGCCGGGAATCGAAC 3′; for cytosolic $tRNA^{Gly}$(UCC), 5′ GCAAGAATTC*GAATTGTAATACGACTCACTATA*GCGTCTGTAGTCCAACGGTTAG 3′ and 5′ CGCGGATCC*TGG*TGCGTCTGCCGGGAGTCGAAC 3′.

RESULTS

Characterization of an A. thaliana Eukaryotic-type GlyRS, GlyRS-1—By analysis of cDNAs and inverse PCR products, we characterized a eukaryotic-type GlyRS gene in *A. thaliana* (GenBank™ accession number AJ002062) (30), and we termed this gene *GlyRS-1*. For this, an *A. thaliana* cDNA library (Strasbourg, France) was screened using as a probe an *A. thaliana* expressed sequence tag clone (GenBank™ accession number 117F7T7) (31) showing similarity with known GlyRS genes. Several incomplete cDNAs, lacking the 5′ end, were recovered from this screening. The 5′ region of the gene (Fig. 1*A*) was cloned from *A. thaliana* genomic DNA by using inverse PCR. Southern blot analysis (17) of total *A. thaliana* DNA digested with *Pst*I, *Bgl*II, or *Eco*RI suggested that the *GlyRS-1* gene is a single copy gene (data not shown), which was confirmed with the availability of the complete *A. thaliana* genomic sequence ((32); see the *Arabidopsis* Genome Initiative web site). A second gene showing similarities with *GlyRS-1* and other eukaryotic GlyRS sequences was previously identified in the *A. thaliana* genome (GenBank™ accession number AC002534). However, this second gene was considered to be a pseudogene, because the sequence is partial, it contains a number of frameshifts, and no corresponding expressed sequence tags could be detected.

The full *GlyRS-1* coding sequence is 729 amino acids long. It is similar to other known eukaryotic GlyRS sequences (more than 45% amino acid identity and 60% similarity with *Homo sapiens*, *Bombyx mori*, and *Caenorhabditis elegans* GlyRS sequences) and presents no obvious similarity with prokaryotic GlyRS sequences such as the *E. coli* GlyRS, suggesting a cytosolic localization. A multiple alignment of GlyRS sequences is available on our web site devoted to *A. thaliana* tRNAs and aminoacyl-tRNA synthetases (web site address available from the corresponding author). The high level of similarity between GlyRS-1 and eukaryotic GlyRSs suggests that the native enzyme probably has an α_2 quaternary structure, like other eukaryotic GlyRSs. The presence of a mitochondrial targeting peptide at the N-terminal end of the coding sequence was predicted by computer analysis. MITOPROT, Predotar, PSORT, and TargetP gave a score of 0.88, 0.90, 0.76, and 0.91, respectively, for a mitochondrial localization. Alignment of the GlyRS-1 amino acid sequence with that of the other GlyRS sequences starts around the end of the potential targeting sequence. It should also be noticed that a dual localization in the cytosol and in mitochondria was proposed for two homologs of GlyRS-1, in humans (33, 34) and in *Saccharomyces cerevisiae* (35).

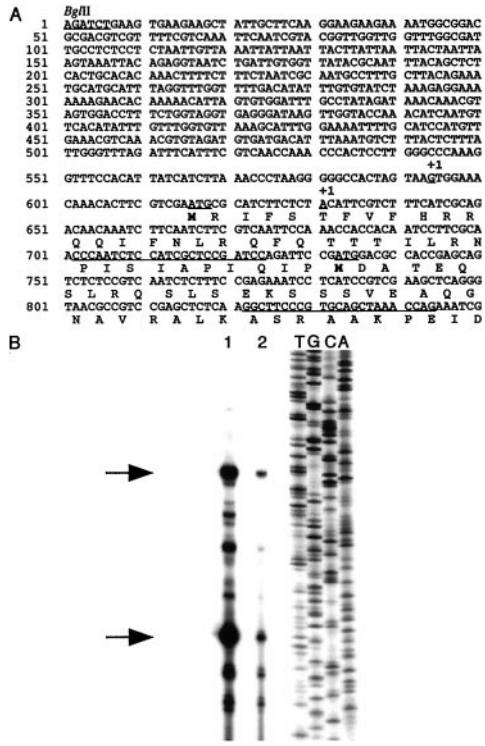

FIG. 1. **Structure and expression of the *A. thaliana GlyRS-1* gene.** The 5′ end and upstream region of the *GlyRS-1* gene are presented in *A*. Mapped 5′ ends of transcripts (*+1*) and positions of initiation codons (ATG) are *underlined*. The sequences corresponding to oligonucleotides AM12 and AM10/AM16 are also *underlined*. Primer extension analysis of the *GlyRS-1* mRNA (*B*) was performed with AM16 and with 12 μg of total (*lane 1*) or 1 μg of poly(A^+) (*lane 2*) *A. thaliana* RNA. *Arrows* indicate the major products synthesized. A sequencing ladder is shown on the *right*.

Expression of the *GlyRS-1* gene was investigated by primer extension. The results shown in Fig. 1*B* indicate that there are two major transcripts with different 5′ ends that map 22 nucleotides upstream and 15 nucleotides downstream, respectively, of the first putative AUG initiation codon. The sequence shows a second in-frame AUG, 40 codons downstream of the first initiator AUG. This second AUG maps near the position corresponding to the C-terminal end of the predicted mitochondrial targeting peptide. The 5′ end of the shortest mRNA is located upstream of the second in-frame AUG codon, which could thus be used to initiate translation on this transcript, leading to the synthesis of a short, cytosolic form of GlyRS-1 (690 amino acids, 76 kDa). Translation of the long transcript could generate a long protein (729 amino acids, 80 kDa) with a putative mitochondrial targeting peptide.

Reexamination of a Second A. thaliana GlyRS Gene (GlyRS-2)—An expressed GlyRS gene of prokaryotic-type (GenBank™ accession number AJ003069) was characterized by Uwer *et al.* (36) upon analysis of an *A. thaliana* embryo development mutant due to a unique insertion of a *Ds*A transposable element.

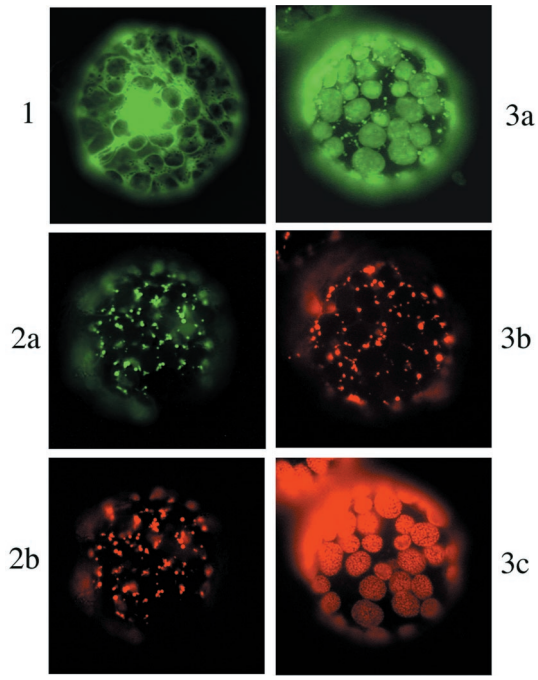

FIG. 2. **Expression of GFP fusions in *N. benthamiana* cells.** Fluorescence was observed at × 100 magnification with a Nikon Eclipse E800 epifluorescence microscope. In *panels 1*, *2a*, and *3a*, GFP fluorescence was observed using a GFP band pass filter set (excitation, 460–500 nm; band pass emission, 510–560 nm). In *panel 3c*, red chlorophyll autofluorescence was observed using a long pass filter set (excitation, 460–500 nm; long pass emission, 510 nm). In *panels 2b* and *3b*, fluorescence of the mitochondria-specific dye (MitoTracker™) was observed using a TRITC filter set (excitation, 540/25 nm; emission, 605/55 nm). *Panel 1* corresponds to unfused GFP. *Panels 2a* and *2b* correspond to the GlyRS-1 N terminus-GFP fusion. *Panels 3a*, *3b*, and *3c* correspond to the GlyRS-2 N terminus-GFP fusion.

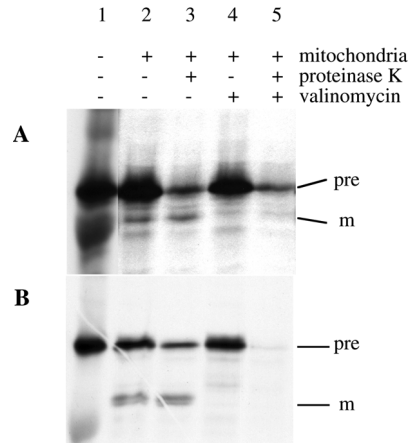

FIG. 3. **In vitro protein import into isolated mitochondria.** The GlyRS-1 N terminus-GFP fusion (*A*) and the GlyRS-2 N terminus-GFP fusion (*B*) were *in vitro* translated (*lane 1*) and incubated for 30 min at 25 °C with mitochondria (*lanes 2–5*). Mitochondria were treated with valinomycin prior to import (*lanes 4* and *5*) and/or submitted to proteinase K digestion after import (*lanes 3* and *5*). Migration of the chimeric preproteins (*pre*) and of the processed polypeptides (*m*) is indicated.

The corresponding protein, which is 1068 amino acids long, has no significant similarity with GlyRS-1 but presents regions similar to the α and β subunits of *E. coli* GlyRS (59% identity with the α subunit and 36% identity with the β subunit), so that a dimeric structure of GlyRS-2 would reflect the tetrameric $\alpha_2\beta_2$ structure of *E. coli* GlyRS. An alignment of the GlyRS-2 sequence with other GlyRS sequences is also available on our web site. Prediction of the subcellular localization run with ChloroP, Predotar, and TargetP gave a score of 0.57, 0.98, and 0.82, respectively, for a chloroplastic localization of GlyRS-2, and this enzyme was indeed shown to be imported into chloroplasts (36). However, the corresponding GlyRS mutant has an embryo lethal phenotype. Embryo growth was stopped between the globular and heart stages of embryonic development, and germination of mutant seeds was never observed. Such a phenotype was not expected for a plastid enzyme, and the function of this GlyRS during plastidic translation does not provide a direct hint to its role during embryogenesis (36). Indeed, Uwer *et al.* (36) did not exclude another localization, and further prediction analyses with MITOPROT gave a score of 0.95 for a mitochondrial localization of GlyRS-2. With PSORT, no clear prediction for mitochondrial or chloroplastic targeting was obtained.

Subcellular Localization of GlyRS-1 and GlyRS-2 as Implied by Targeting of GFP Fusions—As an *in vivo* approach to analyze the intracellular localization of GlyRS-1, the sequence encoding the first 65 amino acids of this enzyme was fused upstream to, and in frame with, an enhanced jellyfish (*Aequorea victoria*) GFP reporter gene. When transiently expressed in *N. benthamiana* cells, this construct yielded fluorescence associated with mitochondria (Fig. 2, *panels 2a* and *2b*), demonstrating that the N terminus of GlyRS-1 is an active mitochondrial targeting peptide *in vivo*.

Because potential dual targeting of GlyRS-2 to mitochondria and chloroplasts was predicted by computer analyses, a similar approach was developed for this enzyme. The sequence encoding the first 81 amino acids of the GlyRS-2 polypeptide was fused in frame to the 5′ end of the GFP gene. When this construct was expressed in *N. benthamiana* cells, fluorescence was associated with both chloroplasts and mitochondria (Fig. 2, *panels 3a*, *3b*, and *3c*), indicating that the GlyRS-2 presequence is able to promote dual targeting to both organelles *in vivo*.

Mitochondrial Localization of GlyRS-1 and GlyRS-2 as Implied by in Vitro Import into Isolated Mitochondria—The large size of the GlyRS-1 (80 kDa) and GlyRS-2 (117 kDa) polypeptides was expected to be unfavorable both for coupled *in vitro* transcription/translation and for *in vitro* import into mitochondria. Thus, these experiments were carried out with constructs corresponding to fusions between the GlyRS N-terminal sequences and GFP. *In vitro* transcription/translation of the chimeric genes yielded a 32-kDa fusion protein for the GlyRS-1 construct (Fig. 3*A*, *lane 1*) and a 35-kDa product for the GlyRS-2 construct (Fig. 3*B*, *lane 1*). In the presence of mitochondria, these products were partially processed into polypeptides of 27 and 30 kDa, respectively (Fig. 3, *A* and *B*, *lane 2*), which corresponded to the M_r of the fusion proteins upon cleavage of the predicted GlyRS targeting sequence. The addition of proteinase K to the import medium reduced the signals corresponding to the preproteins but did not affect the signals corresponding to the processed proteins that were protected (Fig. 3, *A* and *B*, *lane 3*). The addition of valinomycin, which is known to inhibit mitochondrial protein import, prevented the formation of the processed proteins (Fig. 3, *A* and *B*, *lanes 4* and *5*). When the same experiment was performed with unfused GFP, no interaction with mitochondria was observed, because no labeled protein was associated with mitochondria after incubation and centrifugation through a sucrose cushion (data not shown). These results showed that the GlyRS-1 and GlyRS-2 presequences can promote *in vitro* protein import into isolated mitochondria.

Subcellullar Localization of GlyRS-1 as Deduced from Western Blot Analysis—The cytosolic form of GlyRS-1 was overexpressed in *E. coli* and purified to raise polyclonal antibodies. Western blots were performed with *A. thaliana* total and mitochondrial proteins. Cross-contaminations were checked with

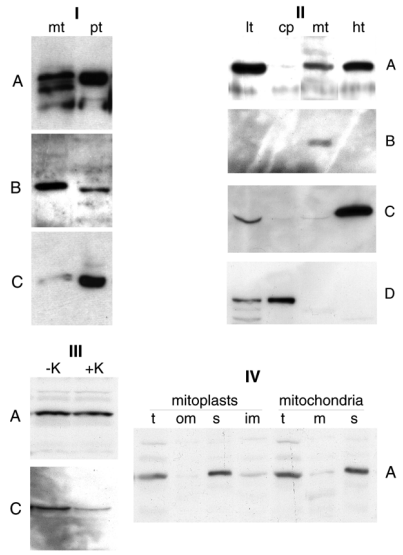

FIG. 4. **Western blot analyses.** Immunodetection of GlyRS-1 (*A*), mitochondrial superoxide dismutase (*B*), α-tubulin (*C*), and chloroplastic leucyl-tRNA synthetase (*D*) was performed with total (*pt*) and mitochondrial (*mt*) protein extracts from *A. thaliana* plantlets (*panel I*), with bean total leaf (*lt*), total hypocotyl (*ht*), chloroplastic (*cp*), and mitochondrial protein extracts (*panel II*), with protein extracts from untreated (−*K*) and proteinase K-treated (+*K*) bean mitochondria (*panel III*), and with total (*t*), soluble (*s*), membrane (*m*), inner membrane (*im*), or outer membrane (*om*) protein extracts from bean mitochondria or mitoplasts (*panel IV*). The chemiluminescence reaction in *panel IIIC* was developed for a much longer time to visualize residual contamination of mitochondrial proteins with α-tubulin.

antisera against mitochondrial superoxide dismutase (Fig. 4*IB*) for mitochondrial contamination or α-tubulin (Fig. 4*IC*) for cytosolic contamination. An immunoreactive protein of about 76 kDa was detected both in total and in mitochondrial protein extracts when using the antiserum against GlyRS-1 (Fig. 4*IA*). The size of the detected protein was in accordance with the calculated M_r of the cytosolic form and the processed mitochondrial form of the enzyme. Faster migrating polypeptides revealed with the GlyRS-1 antiserum in mitochondrial extracts (Fig. 4*IA*) might reflect some degradation.

An immunoreactive protein of the same size was also found in the cytosol and in mitochondria when using bean total and mitochondrial enzymatic extracts in Western blot analyses with the GlyRS-1 antiserum (Fig. 4*IIA*). This observation implies that a homolog of *A. thaliana* GlyRS-1 is present in bean and is dual targeted to the cytosol and to mitochondria, although it does not completely exclude the possibility that the cytosolic form and the mitochondrial form derive from two highly similar genes in bean. Cross-contaminations between cytosolic and mitochondrial extracts were checked as before (Fig. 4, *IIB* and *IIC*), whereas chloroplastic contamination was checked with an antiserum against chloroplast leucyl-tRNA synthetase (Fig. 4*IID*). Based on these observations, further experiments were developed with protein extracts from bean, because isolation of clean *A. thaliana* mitochondria was quite inefficient. Isolated bean mitochondria were treated with proteinase K before protein extraction. Such a treatment strongly reduced residual cytosolic contamination, as checked by immunodetection with the α-tubulin antiserum (Fig. 4*IIIC*), but did not affect the signal obtained with the antibodies against GlyRS-1 (Fig. 4*IIIA*), confirming that GlyRS-1 was present inside mitochondria. Submitochondrial localization of GlyRS-1 was then analyzed. Isolated bean mitochondria were lysed, and total membranes were separated from the soluble fraction by centrifugation. GlyRS-1 was essentially detected in the soluble fraction by Western blot analysis (Fig. 4*IV*). Finally, mitochondria were submitted to an osmotic shock to release the outer membrane, which was separated from the remaining mitoplasts on a step gradient. Lysis of the mitoplasts and centrifugation allowed us to recover the matrix and the inner membrane fraction. GlyRS-1 was found only in the matrix fraction and was not significantly associated with the outer membrane or the inner membrane (Fig. 4*IV*).

Subcellular Localization of GlyRS-1 and GlyRS-2 as Assessed by Chromatographic Fractionation—Following Western blot analysis (Fig. 4*II*), the enzymatic extracts from bean leaves, mitochondria and chloroplasts, as well as an enzymatic extract from an *E. coli* strain overexpressing the cytosolic form of *A. thaliana* GlyRS-1, were fractionated by chromatography on a hydrophobic column (POROS 20 PE) in standardized conditions. Proteins were eluted with a decreasing ammonium sulfate gradient. Collected fractions were tested for GlyRS activity using *E. coli* or yeast tRNAs and [^{3}H]glycine as substrates and submitted to immunodetection with the antiserum against GlyRS-1. The activity of the overexpressed GlyRS-1 reached its maximum in fractions 12–14 of the elution profile and was followed by a second peak corresponding to *E. coli* GlyRS (Fig. 5*A*). GlyRS-1 efficiently glycylated yeast tRNAs but very poorly glycylated *E. coli* tRNAs. Two peaks of GlyRS activity were also obtained with enzymatic extracts from bean leaves (Fig. 5*B*). The first peak matched the elution of the overexpressed GlyRS-1 (Fig. 5*A*), reaching its maximum with fractions 13/14 and showing efficient glycylation of yeast tRNAs *versus E. coli* tRNAs. The presence of the bean GlyRS-1 in these fractions was confirmed by Western blot analysis using anti-GlyRS-1 antibodies (Fig. 5*C*). It should be noted that the high ammonium sulfate concentrations present in the fractions at that point of the gradient were inhibitory for aminoacylation, and desalting of fractions 12–15 resulted in a 7-fold higher activity. The second peak of GlyRS activity, which reached its maximum with fractions 29/30, corresponded to glycylation of either yeast or *E. coli* tRNAs, with a more efficient recognition of the latter (Fig. 5*B*). Similar results were obtained with an enzymatic extract from green *A. thaliana* plantlets (data not shown), thus validating the use of bean for these analyses. Chromatographic fractionation of bean chloroplast enzymatic extracts, which were supposed to contain GlyRS-2 (36), on the hydrophobic column yielded only one peak of GlyRS activity (Fig. 5*D*), corresponding to the second peak in the elution profile obtained with enzymatic extracts from bean leaves. This peak, with a maximum GlyRS activity in fractions 28–30 and a better recognition of *E. coli* tRNAs *versus* yeast tRNAs, was therefore likely to be representative of GlyRS-2. Finally, the elution profile obtained upon fractionation of bean mitochondrial enzymatic extracts (Fig. 5*E*) was the same as for chloroplast extracts, showing only one peak of GlyRS activity, which matched the presumed GlyRS-2 elution. Together with the *in vivo* targeting analyses (Fig. 2) and the *in vitro* import experiments (Fig. 3), these results imply that GlyRS-2 is dual targeted to both chloroplasts and mitochondria. Unexpectedly, the peak of GlyRS-1 activity detected upon fractionation of enzymatic extracts from bean leaves or *A. thaliana* plantlets could not be recovered upon hydrophobic chromatography of bean mitochondrial extracts, although *in vivo* targeting studies (Fig. 2), *in vitro* import experiments (Fig. 3), and Western blot analyses (Fig. 4) had unambiguously shown the import of GlyRS-1 into mitochondria. Moreover, the GlyRS-1 protein was clearly detected in the bean mitochondrial enzymatic extracts prior to chromatographic fractionation (Fig. 4*IIA*). Probing with the anti-GlyRS-1 antiserum revealed that, during hydrophobic chromatography of mitochondrial extracts, elution of the

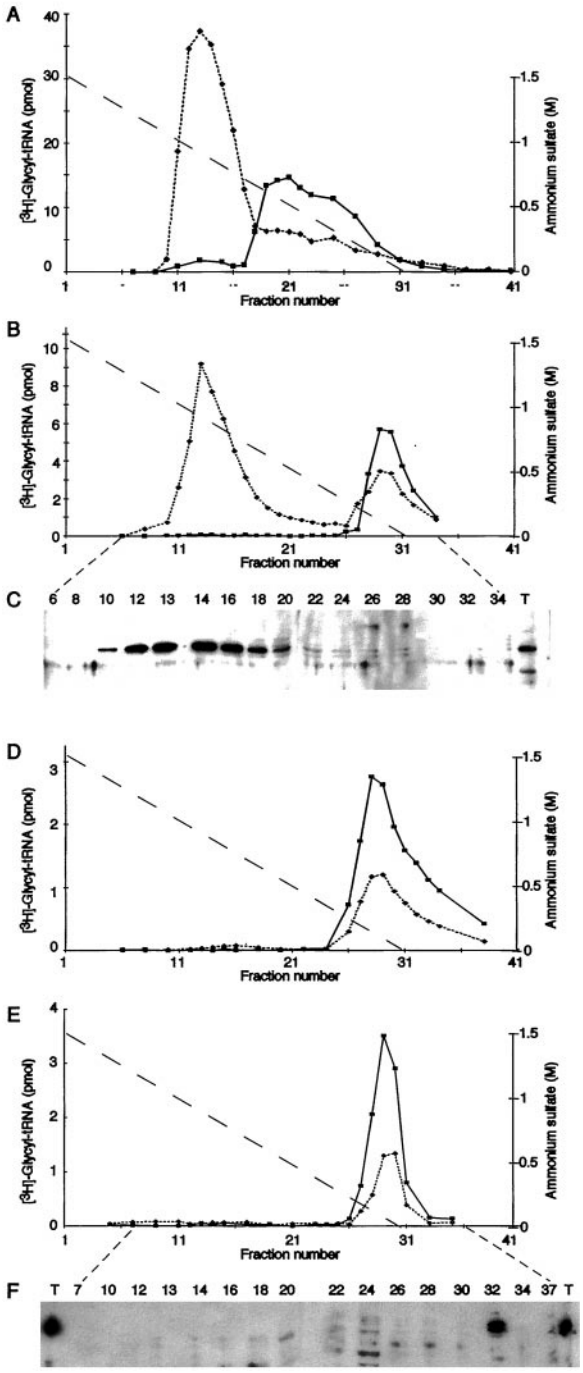

FIG. 5. **Hydrophobic column chromatography of GlyRS activities.** Fractionation is presented for enzymatic extracts from an *E. coli* strain overexpressing GlyRS-1 (*A*), bean leaves (*B* and *C*), bean chloroplasts (*D*), and bean mitochondria (*E* and *F*). Column fractions were analyzed for GlyRS activity (*A*, *B*, *D*, and *E*) in the presence of [^{3}H]glycine and yeast total tRNAs (*dotted lines*) or *E. coli* total tRNAs (*full lines*) and for reactivity with the antibodies against GlyRS-1 on Western blots (*C* and *F*). *T* corresponds to an unfractionated bean total leaf protein extract run as a control.

GlyRS-1 polypeptide occurred at the very end of the fractionation (around fraction 32; Fig. 5*F*), while washing the column with buffer devoid of ammonium sulfate, and not in fractions 13/14 as expected (see Fig. 5, *B* and *C*). Elution from this type of column occurs according to the hydrophobic characteristics of the proteins. From these experiments, it seems that GlyRS-1 is active and quite hydrophilic in cytosolic extracts but becomes inactive and hydrophobic in mitochondrial extracts.

To determine to what extent the above behavior of GlyRS-1 was an effect due to the hydrophobic column, enzymatic extracts from bean leaves and mitochondria were fractionated by chromatography on an anion exchange column (UNO-Q1). As done previously, an enzymatic extract from an *E. coli* strain overexpressing the cytosolic form of *A. thaliana* GlyRS-1 was used as a control. The overexpressed GlyRS-1 was eluted in fractions 9–13 (Fig. 6*A*). With the bean leaf extract, two peaks of GlyRS activity were again obtained (Fig. 6*B*). One peak reached its maximum with fraction 10, matching the elution of GlyRS-1 (Fig. 6*A*) and showing an efficient glycylation of yeast tRNAs. The second peak reached its maximum with fraction 16, with a more efficient glycylation of *E. coli versus* yeast tRNAs. Anti-GlyRS-1 antibodies gave a signal with fractions 10–12 on Western blots (Fig. 6*C*). This suggested that the first peak corresponded to GlyRS-1 and the second to GlyRS-2. With the bean mitochondrial extract, still only one peak of activity, corresponding to GlyRS-2, was obtained (Fig. 6*D*). In this case, the GlyRS-1 polypeptide was detected by Western blotting in the fractions around fraction 12 (Fig. 6*E*), as expected from the profile obtained with the total leaf extract, but no detectable activity was associated with these fractions even in the presence of bean total or mitochondrial tRNAs.

Based on quantitative analysis of the signals on Western blots, the absence of GlyRS-1 activity in the chromatographic profiles of mitochondrial extracts was unlikely to reflect a too weak concentration of the enzyme in the column fractions. The absence of GlyRS-1 activity could not be explained by an effect of the chromatography, because similar results were obtained with two different types of column. As a further step toward understanding these unexpected observations made with dicotyledons, we switched the analyses to another class of plants. According to the data available for wheat (*Triticum aestivum*) and maize (*Zea mays*), all mitochondrial $tRNAs^{Gly}$ seem to be imported from the cytosol in monocotyledonous angiosperms (3, 37). An active GlyRS of cytosolic type, like GlyRS-1, was therefore expected to be present in mitochondria of monocotyledons, considering substrate specificity and tRNA/aaRS coevolution. Wheat total (data not shown) and mitochondrial (Fig. 6, *F* and *G*) enzymatic extracts were prepared and fractionated on the anion exchange column. The same elution profiles were obtained for both extracts, showing two peaks of GlyRS activity. The first peak reached its maximum in fraction 12 (Fig. 6*F*), and the anti-GlyRS-1 antibodies recognized in these fractions a polypeptide of the expected size (Fig. 6*G*), suggesting the existence of an active equivalent of the bean and *A. thaliana* GlyRS-1 in wheat, both in the cytosol and in mitochondria. The inactivation of GlyRS-1 in mitochondrial extracts therefore appeared not to be a systematic behavior of the enzyme or an artifact produced by our experimental procedures. However, it was obvious from the column elution profiles (Fig. 6*F*) that, even in wheat, GlyRS-1 makes a very minor contribution to the total GlyRS activity present in mitochondria. The main activity showed up as a second peak having its maximum in fractions 15–17. The shouldered shape of this second peak, the aminoacylation ratio of yeast *versus E. coli* tRNAs along the different fractions, and the fact that recognition of a wheat-specific polypeptide smaller than GlyRS-1 by the anti-GlyRS-1 antibodies preferentially matched the second half of the peak (Fig. 6*G*) raised the possibility that at least two other forms of GlyRS are present in wheat mitochondria.

Substrate Specificity of GlyRS-1 and GlyRS-2—Fractions containing only GlyRS-1 activity obtained upon chromatography of a bean total enzymatic extract on either the hydrophobic or the anion exchange column were used to characterize the tRNA recognition specificity of GlyRS-1. A bean chloroplast enzymatic extract, which contained only GlyRS-2, was used to

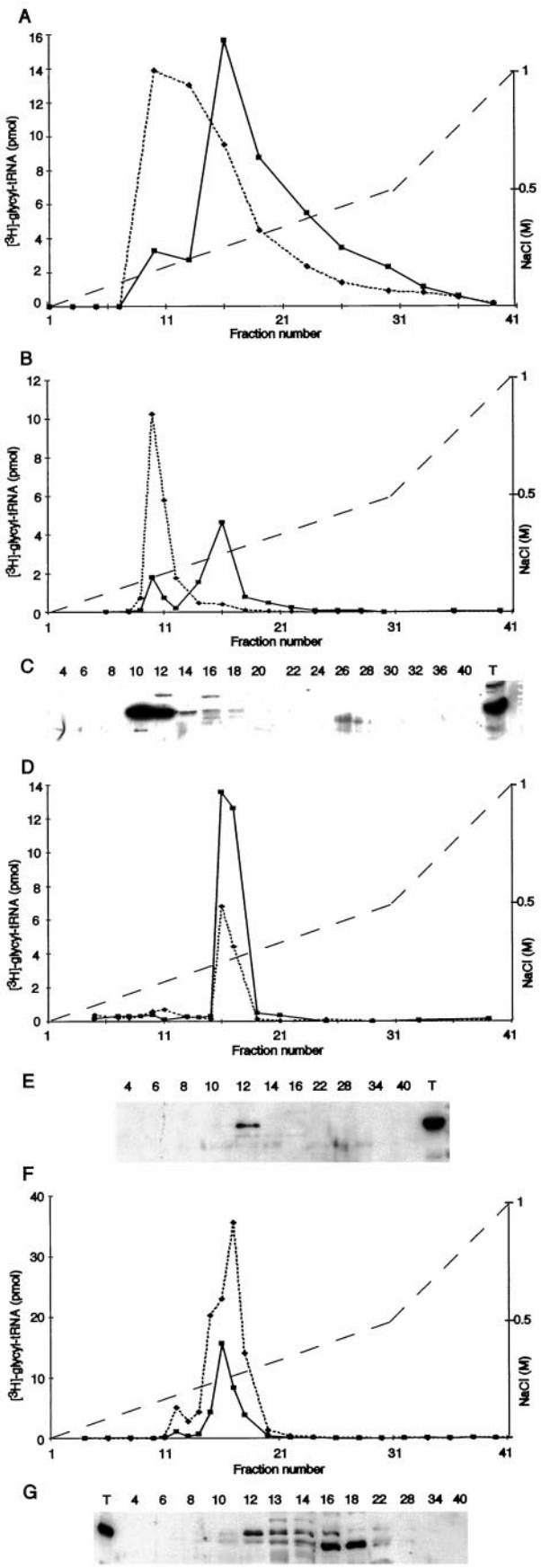

FIG. 6. **Anion exchanger column chromatography of GlyRS activities.** Fractionation is presented for enzymatic extracts from an *E. coli* strain overexpressing GlyRS-1 (*A*), bean leaves (*B* and *C*), bean mitochondria (*D* and *E*), and wheat mitochondria (*F* and *G*). Column fractions were analyzed for GlyRS activity (*A*, *B*, *D*, and *F*) in the presence of [^{3}H]glycine and yeast total tRNAs (*dotted lines*) or *E. coli* total tRNAs (*full lines*) and for reactivity with the antibodies against GlyRS-1 on Western blots (*C*, *E*, and *G*). *T* corresponds to an unfractionated bean leaf protein extract run as a control. For graphical convenience, GlyRS activity values obtained in the presence of yeast total tRNAs were divided by 3 in *A* and 10 in *B*.

A

tRNA from:	bean leaves	bean chloro.	bean mito.	*E. coli*	yeast
	(concentration: 0.15 μg/μl)			(3 μg/μl)	
GlyRS-1	1.00	0.17	0.18	0.23	1.43
GlyRS-2	1.20	1.00	0.76	1.01	0.66

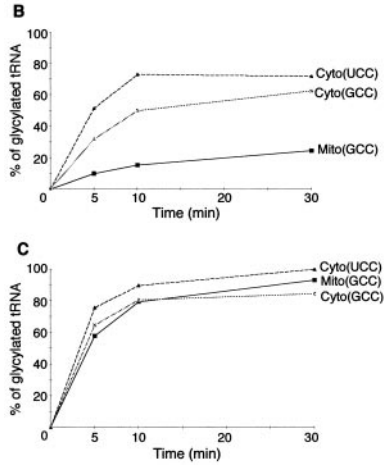

FIG. 7. **Aminoacylation assays with partially purified bean GlyRS fractions.** *A*, relative initial charging rate of different total tRNA samples (chloro., chloroplasts; mito., mitochondria) in the presence of an enzymatic extract containing either GlyRS-1 or GlyRS-2. Values are expressed relative to the rate obtained with total bean leaf tRNAs (1 = 1.7 pmol of [^{3}H]glycyl-tRNA/min) for the tests with GlyRS-1 and to the rate obtained with total bean chloroplast tRNAs (1 = 4.9 pmol of [^{3}H]glycyl-tRNA/min) for the tests with GlyRS-2. Because both enzymatic extracts were only enrichment intermediates, no conclusion could be drawn as to the relative specific activity of GlyRS-1 and GlyRS-2. *B* and *C*, the activity of GlyRS-1 (*B*) or GlyRS-2 (*C*) was tested in the presence of [^{3}H]glycine and cytosolic $tRNA^{Gly}$(UCC) transcript (*Cyto(UCC)*), cytosolic $tRNA^{Gly}$(GCC) transcript (*Cyto(GCC)*), or mitochondrial $tRNA^{Gly}$(GCC) transcript (*Mito(GCC)*).

characterize the tRNA recognition specificity of GlyRS-2. Despite its prokaryotic features revealed by a strong similarity to the bacterial GlyRS, GlyRS-2 was able to glycylate bean total (mainly cytosolic), chloroplastic, and mitochondrial tRNAs with little difference in efficiency (Fig. 7*A*). Moreover, the aminoacylation efficiency in the presence of GlyRS-2 was the same for *in vitro* transcripts of a mitochondrial $tRNA^{Gly}$(GCC), a cytosolic $tRNA^{Gly}$(UCC), and a cytosolic $tRNA^{Gly}$(GCC) (Fig. 7*C*). GlyRS-2 would therefore be sufficient to charge both the native, organelle-encoded $tRNA^{Gly}$(GCC) and the imported $tRNA^{Gly}$(UCC) in mitochondria of dicotyledonous plants, unless post-transcriptional modifications not present in our *in vitro* transcripts alter tRNA recognition *in vivo* (*e.g.* Ref. 38). By contrast, aminoacylation of bean chloroplastic or mitochondrial tRNAs in the presence of GlyRS-1 appeared to be five times less efficient, as compared with bean total tRNAs (Fig. 7*A*). GlyRS-1 was also three times less efficient in glycylating the *in vitro* mitochondrial $tRNA^{Gly}$(GCC) transcript *versus* the two cytosolic $tRNA^{Gly}$ transcripts.

DISCUSSION

In this work, we show that two different GlyRSs are imported into *A. thaliana* and *P. vulgaris* mitochondria. Moreover, these enzymes are both dual targeted, the first one, GlyRS-1, to the cytosol and to mitochondria and the second,

GlyRS-2, to chloroplasts and mitochondria, underlining the complexity of the relations between cell compartments. GlyRS-1 is similar to eukaryotic enzymes, whereas GlyRS-2 presents similarities with *E. coli* GlyRS. This situation is original and puzzling, especially because GlyRS-1 is imported but inactive in bean mitochondria, and GlyRS-2 appears to be sufficient to charge all $tRNAs^{Gly}$ in mitochondria of dicotyledonous plants. The latter results were unexpected, considering the identity elements previously defined for $tRNA^{Gly}$, *i.e.* position N73, the first three base pairs of the acceptor stem (1:72, 2:71, 3:70), as well as positions C35 and C36 in the anticodon (39). The most striking difference between prokaryotic and eukaryotic $tRNAs^{Gly}$ is the discriminator base at position 73, which is phylogenetically conserved as U in prokaryotes and as A in eukaryotes. In *A. thaliana*, N73 is a U in the native mitochondrial $tRNA^{Gly}$ and an A in the three cytosolic $tRNAs^{Gly}$. Because GlyRS-2 is similar to *E. coli* GlyRS, the same specificity was expected for these two enzymes. *E. coli* GlyRS was shown to aminoacylate *E. coli* $tRNA^{Gly}$ 18-fold more efficiently than yeast $tRNA^{Gly}$ (40), whereas both tRNAs were good substrates for plant GlyRS-2. A 73U→A mutation in the *E. coli* $tRNA^{Gly}$ transcript was responsible for an 11-fold loss in the charging efficiency by *E. coli* GlyRS (40), but GlyRS-2 does not seem to be sensitive to the nucleotide at position 73. Plant GlyRS-2 therefore appears to be a prokaryotic-like enzyme with new specificities. So far, in plant mitochondria, the genetic origin of aaRSs seemed to coincide with that of their cognate tRNAs, and cytosolic-like mitochondrial aaRSs were associated with imported cytosolic tRNAs (7, 8). The prokaryotic-type GlyRS-2 potentially charges a mitochondrion-encoded $tRNA^{Gly}$ and a nucleus-encoded $tRNA^{Gly}$, and the presence of this somehow atypical enzyme in mitochondria of dicotyledonous plants reveals new and complex aspects of tRNA/aaRS coevolution.

Several tentative explanations can be considered to account for the absence of GlyRS-1 activity in mitochondrial extracts. Among these, misfolding, or post-translational modification, of the protein during or after mitochondrial import is a possible cause. Phosphorylation (*e.g.* Refs. 41 and 42), acetylation (*e.g.* Refs. 43 and 44), isoprenylation (45), disulfide bond formation (46), and unidentified covalent modifications (47) have been shown to be associated with higher plant mitochondria. Changes in phosphorylation or acetylation should lead to the addition or lack of charges and to a shift in the elution from the anion exchanger, as compared with that of the cytosolic GlyRS-1 detected in total extracts. Significant glycosylation would affect the electrophoretic behavior, whereas addition of lipid derivatives would tend to promote anchoring into the membrane fraction, which is clearly not the case. Intersubunit disulfide bond formation looks plausible but has no obvious reason to increase the hydrophobicity of the protein *versus* the putative cytosolic GlyRS-1 α_2 dimer. Alternatively, it is possible that the lack of significant GlyRS-1 activity observed in bean hypocotyl mitochondria is specific for this plant tissue or this plant species or that GlyRS-1 is only active in mitochondria upon certain developmental conditions. However, the fact that wheat mitochondrial extracts contain significant but limited GlyRS-1 activity strengthens the idea that, whatever the reasons are for its lack of activity in the organelles of some species, the role of GlyRS-1 is not to make a major contribution to the aminoacylation of mitochondrial $tRNAs^{Gly}$. Therefore, one wonders why GlyRS-1 is imported into the organelles. An attractive hypothesis would be that this enzyme is required for import of cytosolic $tRNAs^{Gly}$ into mitochondria. Two different mechanisms have been proposed for mitochondrial tRNA import. The first one, which seems to apply for trypanosomatids, is a direct import of the tRNAs through the mitochondrial membranes via receptor(s) and a specific channel (48). The second mechanism, which has been proposed to account for the mitochondrial import of the single cytosolic $tRNA^{Lys}$(CUU) in yeast, is a co-import of the tRNA with protein factors, and in particular the corresponding aminoacyl-tRNA synthetase (49). In plants, it was shown that a point mutation in a normally imported tRNA, $tRNA^{Ala}$(UGC), blocked both the aminoacylation of this tRNA by alanyl-tRNA synthetase and its import into mitochondria *in vivo* (29). Although recent studies in *Xenopus laevis* and in yeast imply that proofreading might prevent or impair nuclear export of inactive tRNAs (50, 51), these observations suggest a role of alanyl-tRNA synthetase, and more generally of aaRSs, in plant mitochondrial tRNA import. One can speculate that mitochondrial import of $tRNA^{Gly}$ isoacceptors is perhaps mediated by GlyRS-1. Considering that unfolding steps are implicated in mitochondrial protein import (*e.g.* Refs. 52–54), the conformation of GlyRS-1 might be modified during these processes, so that tRNA import would rely on tRNA/aaRS interactions that differ from those occurring during aminoacylation. Such a mechanism has already been proposed for the lysyl-tRNA synthetase-mediated mitochondrial import of $tRNA^{Lys}$(CUU) in yeast (49). Conservation of this alternative conformation would also be a possibility to explain the lack of GlyRS-1 aminoacylation activity in the mitochondria of some plant species. Finally, the possibility also remains open that GlyRS-1 has another role than recognition of tRNAs during import or inside mitochondria. Indeed, there is growing evidence that aaRSs are involved in cellular processes other than tRNA aminoacylation, for example in transcription (55), intron splicing (56), and mRNA 3′ end processing (57).

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