

Involvement of a chloroplast homologue of the signal recognition particle receptor protein, FtsY, in protein targeting to thylakoids

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Abstract We isolated an *Arabidopsis thaliana* cDNA whose translated product shows sequence similarity to the FtsY, a bacterial homologue of SRP receptor protein. The *Arabidopsis* FtsY homologue contains a typical chloroplast transit peptide. The in vitro-synthesized 37 kDa FtsY homologue was imported into chloroplasts, and the processed 32 kDa polypeptide bound peripherally on the outer surface of thylakoids. Antibodies raised against the FtsY homologue also reacted with a thylakoid-bound 32 kDa protein. The antibodies inhibited the cpSRP-dependent insertion of the light-harvesting chlorophyll *a/b*-binding protein into thylakoid membranes suggesting that the chloroplast FtsY homologue is involved in the cpSRP-dependent protein targeting to the thylakoid membranes.

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Key words: Signal recognition particle; Thylakoid import; FtsY; Protein export; *Arabidopsis thaliana*

1. Introduction

Biogenesis of the photosynthetic apparatus in higher plant chloroplasts requires the assembly in a coordinated fashion of the thylakoid proteins encoded by the two distinct genetic systems, the plastid and the nuclear genomes. The nuclear-encoded thylakoid proteins are synthesized in the cytosol as precursors with an amino-terminal extension called a transit peptide, imported into the chloroplast stroma, and further directed to the thylakoid membranes [1–5]. At least four distinct routes are known to be involved in the protein targeting to the thylakoids: (i) a Sec-dependent pathway, (ii) a signal recognition particle (SRP)-dependent pathway, (iii) a Δ pH-dependent pathway, and (iv) a spontaneous pathway. Recent results clearly indicated that all pathways including the Δ pH-dependent pathway have evolved from their bacterial counterparts [4–10].

SRP was first identified as a ribonucleoprotein complex required for a co-translational protein targeting to the eukaryotic endoplasmic reticulum membrane [11]. Mammalian SRP is composed of six polypeptides and a 7S RNA. Among these components, only SRP54 binds to the hydrophobic endoplasmic reticulum-targeting signal sequences [12–14]. The eukaryotic SRP interacts with the SRP receptor, SR α , to target the

polypeptides to the translocation channel in the membrane. Cycles of GTP binding and hydrolysis by SRP54 and SR α have been shown to regulate the SRP-mediated protein translocation [15–17]. Bacterial cells contain the SRP homologue consisting of Ffh and the 4.5S RNA and an SR α counterpart, FtsY, as well [18–21]. The bacterial SRP pathway has been suggested to target primarily hydrophobic inner membrane proteins [22,23].

Chloroplast SRP (cpSRP) has been proposed to mediate the targeting of a specific subset of precursors to the thylakoid membranes [24–27]. The light-harvesting chlorophyll *a/b*-binding protein (LHCP) is one of the major thylakoid membrane proteins in higher plant chloroplasts. The LHCP is synthesized in the cytosol as a larger precursor with an envelope transit peptide only. After being processed to its mature polypeptide in the stroma, the LHCP is integrated into the thylakoid membrane by means of information contained in the mature moiety [28–30]. The integration process has been shown to require GTP and the stromal cpSRP [25,31,32]. Recently, the cpSRP was found to consist of cpSRP54 and cpSRP43 instead of an RNA [33,34]. The cpSRP54 was shown to specifically recognize the third transmembrane domain of mature LHCP [26]. Although cpSRP54 and cpSRP43 were sufficient to form a transit complex with LHCP, an additional soluble factor(s) appeared to be required for the proper targeting of LHCP to the thylakoid membrane [33]. Moreover, a cpSRP receptor protein which might mediate the targeting of cpSRP-bound LHCP to the thylakoid membrane has not yet been identified.

In the present study, we have cloned the *Arabidopsis thaliana* cDNA encoding an FtsY homologue, a most probable candidate for the cpSRP receptor. We have immunologically identified the FtsY homologue in the thylakoid membranes of higher plant chloroplasts. Its possible involvement in targeting of LHCP into the thylakoid membranes has also been demonstrated.

2. Materials and methods

2.1. Cloning of the *ftsY* homologous gene from the *A. thaliana* cDNA library

When we started this project, only a partial genomic sequence of *A. thaliana* (GenBank accession number B25715) was known, whose translated product partly showed close similarity to a portion of bacterial FtsY proteins. Two oligonucleotide primers which correspond to the tentative FtsY sequence were used for the PCR amplification of 5'- and 3'-flanking sequences from the *A. thaliana* cDNA library. The amplified cDNA fragments were subcloned into pMOSblue T-vector (Amersham), and their nucleotide sequences were determined with a Taq Dye Terminator Cycle Sequencing Kit (Applied Biosystems).

2.2. In vitro import into isolated chloroplasts and thylakoids

The entire *cpftsY* gene was constructed from the obtained cDNA

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Abbreviations: SRP, signal recognition particle; GTP, guanosine 5'-triphosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LHCP, the light-harvesting chlorophyll *a/b*-binding protein

segments and was inserted into pGEM-4Z (Promega). An expression plasmid for the LHCP precursor was constructed by inserting a PCR-amplified pea LHCP cDNA into the same vector. In vitro transcription was performed with the RiboMax Kit (Promega). The synthesized mRNAs were purified and subjected to in vitro translation either with a wheat germ or with a reticulocyte cell-free system in the presence of [4,5-³H]leucine (Amersham). In vitro import of the radiolabeled precursor proteins into isolated pea chloroplasts and thylakoids and suborganellar fractionation after import were performed as described previously [35–37]. For the experimental details, see the figure captions, also.

2.3. Production of antisera

The PCR-amplified cpFtsY cDNA fragment was inserted into pET21b (Novagen), and the resulting plasmid was used for the expression of cpFtsY in *Escherichia coli* BL21 (DE3) cells. The overexpressed protein was purified and used for the generation of antibodies in rabbits.

2.4. GTP photoaffinity crosslinking

The GST (glutathione *S*-transferase)-cpFtsY fusion protein was produced by fusing amino acids 41–366 of cpFtsY in-frame to the carboxy-terminus of GST in the pGEX-KG vector. The purified GST-cpFtsY samples (0.7 μM final concentration) were incubated in 10 mM HEPES-KOH, pH 8.0, 10 mM MgCl₂ and 0.33 μM [α-³²P]GTP (3000 Ci/mmol; Amersham) for 30 min at 0°C. Crosslinking was induced by irradiation with UV light (254 nm) at a distance of 5 cm for 15 min on ice. Proteins were subjected to 12.5% SDS-PAGE and visualized by autoradiography.

2.5. Miscellaneous methods

Published procedures were used for growing plants, recombinant DNA technique, subcellular and suborganellar fractionations, SDS-PAGE, fluorography, and immunoblotting analyses [35–37].

3. Results and discussion

Higher plant-derived nucleotide sequences registered in the GenBank/EMBL/DDBJ data library were searched using the sequences for bacterial FtsY protein, and an *A. thaliana* genomic survey sequence (GenBank accession number B25715) that was close in predicted protein sequence to a portion of bacterial FtsY was identified. To obtain a cDNA corresponding to the genomic sequence, 5' RACE and 3' RACE experiments were performed. The amplified cDNA fragments most likely cover the entire FtsY homologous cDNA, which potentially encodes 366 amino acids with a calculated molecular mass of 39 678 (Fig. 1). The alignment of the predicted amino acid sequence with those of the previously identified bacterial FtsY and SRα [38–42] reveals low but significant sequence similarity throughout the sequences including the three consensus motifs for GTP binding (Fig. 1, boxed). The *Bacillus subtilis* FtsY has similar size and also shows the highest degree of identity (35%) [39]. Like the *B. subtilis* FtsY and *Mycoplasma hominis* FtsY [42], the *Arabidopsis* FtsY homologue seems to consist of only the so-called NG domain which is common in all SRP-type GTPases [43], and has no highly acidic amino terminal domain found in the *E. coli* FtsY (Fig. 1) which has been suggested to be essential for its membrane targeting [44]. The *Arabidopsis* FtsY homologue shows 21% identity with the yeast SRα [41] and 22% identity with *Arabidopsis* cpSRP54 [24]. After we had completed determining the nucleotide sequence of the cDNA, a genomic sequence encompassing the entire gene corresponding to the cDNA was deposited in the

FtsY (<i>Esch.</i>)	1	MAKEKKRGFFSWLGFQKEQTPEKETEVQNEQPVVEEIVQAQEPVKASEQAVEEQPQAHT
FtsY (<i>Esch.</i>)	61	EAAEETFAADVVEVTEQVAESEAQPEAEVVAQPEPVVEETPEPVAIEREELPLPEDVNA
cpFtsY (<i>Arab.</i>)	1	-----MATSSAHL SFLAGRISPFSSEIRIGLFLRGEFRPRMTRFRCSAGPSGF
FtsY (<i>Bac.</i>)	1	-----MSE
FtsY (<i>Esch.</i>)	121	EAVSPEEWQAEAEETVEIVEAEEEEAAKEEITDSELETALAAEAEEAVMVVPPAEEEQPV
cpFtsY (<i>Arab.</i>)	49	FTRLGRLIKEKAKSDVEKVFSCFSKTRENLAVIDELLLEWNLAEETDRLVLELEELIVSD
FtsY (<i>Bac.</i>)	4	EKKLKEKITKQTDVSEKFKDGLKTFNSFQNKVNDLVSRKVDDEFEELVVISAD
FtsY (<i>Esch.</i>)	181	EEIAQEQEKPTKEGFFARLKRSLLKTKENLG-SGFISLFRGKKIDDDLEELVQLLIAD
cpFtsY (<i>Arab.</i>)	109	FGPKITVRIIVEREDRIMSGKLKSGSEIKDALKEVLEMLAKKNSK-TELOLGFRKPAVI
FtsY (<i>Bac.</i>)	64	VGFTTVMELTDEKKEVKERNIQDPKEVQSVISEKLVEITYNSGDEQISELNIQDGRNLVI
FtsY (<i>Esch.</i>)	240	VGVEITRKIIITNLTEGASRKQLRDAEALYGLLKEBEMGEILLAKVDE---PLNVEGKAPFVI
cpFtsY (<i>Arab.</i>)	168	MIVGVNCGGKTTSLGKLAHRLKNEGTVLMAAGDTFRAAASDOLEIWAERTGCEIVVAEG
FtsY (<i>Bac.</i>)	124	LLVGVNGVGKTTTIGKLAHMKOEKSVVLAAGDTFRACATEOLEVWGERTGVPIKOTA
FtsY (<i>Esch.</i>)	297	LMVGVNGVGKTTTIGKLARQFEQGGKSVMLAAGDTFRAAAVEQLQVNGQRNNIPVIAOHT
cpFtsY (<i>Arab.</i>)	228	DKAKAATVLSKAVKRGKEEGYDVVLCDTSGRLHTNYSLMEEIACKKAVGKIIVSCAPNEI
FtsY (<i>Bac.</i>)	184	G-SDPAAVIYDAVHAAKARNADVLICDTAGRLONKVNLMKELEKVKRVIERYEPETPHEV
FtsY (<i>Esch.</i>)	357	G-ADSASVIFDAIQAAKARNIDVLIADTAGRLONKSHLMEEIKKIVRMKKLDVEAPHEV
cpFtsY (<i>Arab.</i>)	288	LLVLDGNTGLNMLPOARENEVVGITGLILTKLDGSARGGCVSVVDELGIPVKEFIGVGE
FtsY (<i>Bac.</i>)	243	LLALDAITGONAMAOAKEFSKATNVTGIALTKLDGTAKGGITVLAIKRELHIPPVKLVGLGE
FtsY (<i>Esch.</i>)	416	MLTIDASTGONAVSOAKLEHVAEVLGTITLTKLDGTAKGGVIFSVADQFGIPYRITGVGE
cpFtsY (<i>Arab.</i>)	348	AVEDLOFPDPEAFVNAIFS-----
FtsY (<i>Bac.</i>)	303	KVDDLOBFDPESYVYGLFSDLVEKADD
FtsY (<i>Esch.</i>)	476	RIEDLRPEKADDETFEALFARED----

Fig. 1. Deduced amino acid sequence of an *Arabidopsis* cDNA encoding a cpFtsY homologue (*Arab.*). The *Bacillus subtilis* (*Bac.*) and *Escherichia coli* (*Esch.*) sequences are representative of bacterial FtsY. Identical amino acid residues are fulltone-inverted. Three consensus motifs for a GTP-binding domain are boxed. The putative chloroplast transit peptide found in cpFtsY is underlined. Positions of introns are indicated by filled triangles.

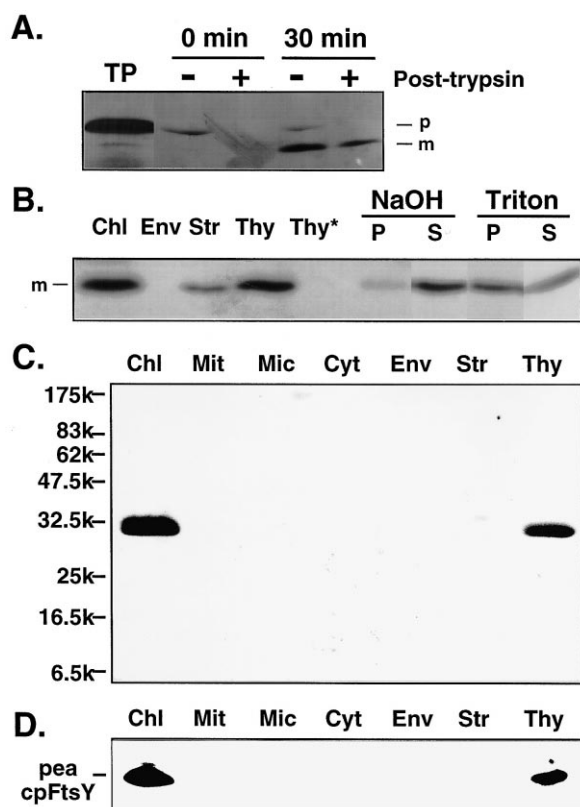


Fig. 2. A: In vitro import of cpFtsY into isolated pea chloroplasts. The cpFtsY precursor protein was synthesized in the reticulocyte lysate cell-free translation system (TP) and then transported into isolated pea chloroplasts for 0 min or 30 min at 25°C. After the incubation, chloroplasts were reisolated and divided into two aliquots. One aliquot was further treated with 60 µg/ml trypsin in the import buffer for 5 min on ice. Protein samples were analyzed by SDS-PAGE followed by fluorography. B: Suborganellar localization and solubilization of the imported cpFtsY. After the in vitro import reactions were carried out as in A, chloroplasts were reisolated (Chl) and fractionated into the envelope membranes (Env), the stroma (Str), and the thylakoids (Thy). Isolated thylakoids were further treated either with 60 µg/ml trypsin, 200 mM NaOH, or 1% Triton X-100 for 30 min on ice. Reisolated trypsinized thylakoids (Thy*) and membrane pellets (P) and supernatant (S), which were obtained by ultracentrifugation after the NaOH or Triton X-100 treatment, were analyzed as described above. Western analysis of the various cellular fractions prepared from leaves of *A. thaliana* (C) and *Pisum sativum* (D) using anti-cpFtsY antibodies. Chl.: chloroplasts; Mit.: mitochondria; Mic.: microsomes; Cyt.: cytosol; Env.: envelope; Str.: stroma; Thy.: thylakoids.

data library (GenBank accession number AC004665). By comparison of the genomic sequence with the cDNA sequence we determined, the nuclear gene for the FtsY homologue appears to contain 10 introns as indicated in Fig. 1 (filled triangles).

Instead of the highly acidic amino terminal domain which is usually found in the bacterial FtsY, the *Arabidopsis* FtsY homologue has an amino-terminal extension more than 40 residues longer than the *B. subtilis* FtsY (Fig. 1, underlined). The amino-terminal extension appears to have characteristic features of transit peptides for intracellular targeting to chloroplasts (i.e. rich in basic and hydroxylated amino acids) [45]. In order to confirm directly that the *Arabidopsis* FtsY homologue is a chloroplast protein, we performed in vitro chloroplast import experiments. Although the FtsY protein synthe-

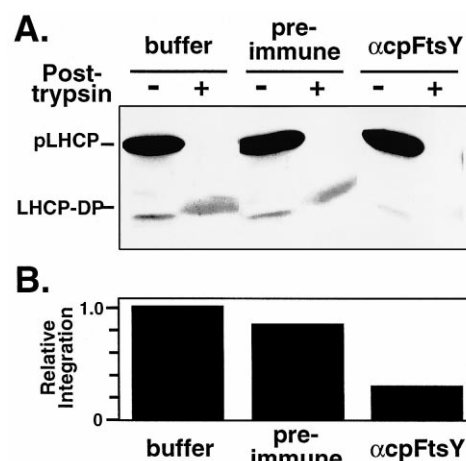


Fig. 3. Anti-cpFtsY antibody inhibited the integration of pLHCP into isolated pea thylakoid membranes. Isolated pea thylakoids were treated with either the import buffer, preimmune serum, or anti-cpFtsY antiserum at 0°C for 60 min and then were reisolated by centrifugation. After washing with the import buffer once the reisolated thylakoids were suspended with the stromal fraction prepared from pea chloroplasts and were subjected to in vitro integration experiments of pLHCP. After incubating with radio-labeled pLHCP for 30 min at 25°C the thylakoids were recovered and analyzed directly or treated with 60 µg/ml trypsin for 15 min on ice. Analysis was by quantitative SDS-PAGE/fluorography. A photograph of a fluorogram of the gel is shown in A. The extent of LHCP integration was determined by trypsin post-treatment of recovered thylakoids and quantitation of the characteristic protease resistant fragment of LHCP (LHCP-DP) [28–30]. Each treatment is expressed relative to the buffer control as shown in B.

sized in the wheat germ extracts was unable to be imported into the isolated pea chloroplasts (data not shown), that synthesized in the reticulocyte lysate was efficiently transported into the chloroplasts and converted to 32 kDa protein (Fig. 2A). Because the translated polypeptides in both cell-free translation systems seemed to be very similar in size (37 kDa), the import incompetence of the FtsY synthesized in the wheat germ extracts might be due to unexpected phosphorylation(s) of the transit peptide in the extracts as reported previously [46]. Suborganellar fractionation of the chloroplasts after import revealed that the processed FtsY was mainly associated with the stromal side of the thylakoid membranes (Fig. 2B). Although a small fraction of the processed FtsY was also observed in the stromal fraction, chase experiments indicated that the stromal FtsY could be converted to the thylakoid-bound form (data not shown). Whereas the thylakoid-associated FtsY protein was hardly solubilized with 1% Triton X-100, alkali treatment with 200 mM

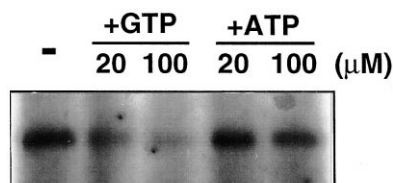


Fig. 4. cpFtsY was specifically crosslinked to GTP. Purified GST-cpFtsY was incubated with [α - 32 P]GTP and then UV-irradiated to crosslink bound GTP to protein as described in Section 2. Unlabeled GTP or ATP was included in the incubation at the indicated concentration. The reaction products were separated by SDS-PAGE followed by autoradiography.

NaOH could easily liberate the FtsY from thylakoid membranes suggesting that the FtsY protein localizes in the outer surface of the thylakoid membranes as a peripheral membrane protein.

In order to investigate whether the FtsY homologue is actually expressed and localized in the same cellular location in vivo, antibodies were raised against the FtsY homologue which had been expressed in and purified from *E. coli* cells. The anti-FtsY antibodies specifically reacted with a 32 kDa protein which was exclusively localized in the thylakoids prepared from *Arabidopsis* leaves (Fig. 2C). The antibodies also reacted with a 32 kDa thylakoid protein of pea chloroplasts (Fig. 2D). Features of the membrane association of the 32 kDa proteins were quite similar to that found in the in vitro import study as described above (data not shown). These results indicate that higher plant chloroplasts have the FtsY homologue which peripherally binds to the stromal side of the thylakoid membranes, and thus we named it cpFtsY.

We then investigated whether or not the cpFtsY is involved in the cpSRP-mediated protein targeting to the thylakoids. To address this question, in vitro import experiments of the LHCP precursor into isolated thylakoids in the presence of stromal extracts was performed, and the proper integration of the LHCP into the thylakoid membranes was assessed as the amount of LHCP-DP, a protease-protected LHCP fragment [28]. As shown in Fig. 3A,B, preincubation of the isolated pea thylakoids with the anti-cpFtsY antibodies led to significantly decreased insertion of the LHCP precursor in the membranes. In the absence of stroma containing cpSRP, no proper integration of the LHCP precursor into the thylakoid membranes could be observed as reported previously (data not shown) [28–30]. In contrast, the anti-cpFtsY antibodies did not block the thylakoidal transport of OE23, the 23 kDa subunit of oxygen-evolving complex whose transport into thylakoids has been shown to be independent on cpSRP (data not shown). Therefore, involvement of the thylakoid-bound cpFtsY in the cpSRP-mediated targeting of LHCP to the thylakoid membranes is strongly suggested.

In the case of the bacterial FtsY, GTP has been shown to be important for its proper function in protein targeting, and the cpFtsY also possesses consensus motifs for GTP binding. To test whether the cpFtsY is able to bind GTP, a photoaffinity crosslinking approach was taken. Recombinant cpFtsY was purified as a fusion protein with glutathione *S*-transferase (GST-cpFtsY). The purified GST-cpFtsY was incubated in the presence of radiolabeled GTP and irradiated with UV light. As shown in Fig. 4 (lane 1), cpFtsY is indeed a GTP-binding protein. The addition of unlabeled GTP reduced the amount of the GTP-crosslinked GST-cpFtsY (lanes 2 and 3), whereas the presence of the excess unlabeled ATP did not affect the GTP-binding capacity of cpFtsY (lanes 4 and 5), suggesting that the nucleotide binding to this protein is specific for GTP.

In summary, we identified the thylakoid-bound cpFtsY, which was strongly suggested to participate in the cpSRP-dependent protein targeting to the thylakoid membranes. We are now investigating whether cpFtsY interact directly with cpSRP on targeting of LHCP to the thylakoid membranes. We are also attempting to determine whether cpFtsY is involved in the integration of other thylakoid membrane proteins.

One remarkable feature of the cpFtsY is its exclusive mem-

brane-bound property. Although the bacterial FtsY has been shown to act as a peripheral membrane protein, a large fraction of FtsY is found to be soluble in the cytoplasm [20]. Zelazny et al. showed that *E. coli* FtsY must target to the membrane for its proper function and that the membrane localization function might be mediated by the amino terminal acidic domain [44]. This acidic domain could be changed to an unrelated integral membrane polypeptide [44]. Interestingly, cpFtsY lacks the amino terminal acidic domain and also other hydrophobic potential membrane anchoring segments. Nevertheless, most of cpFtsY is found as a thylakoid-bound peripheral protein in chloroplasts. Alkali treatment could liberate the membrane-bound cpFtsY suggesting that cpFtsY might bind on the surface of the thylakoid membranes via a protein-protein interaction.

Mammalian SR α , a counterpart of bacterial FtsY, is also known to be peripherally associated with the endoplasmic reticulum membrane, and its membrane association has been shown to be mediated by another SRP receptor component, SR β [47–49]. SR β is a 30 kDa type I integral membrane protein whose carboxy-terminal portion resides on the cytoplasmic face of the endoplasmic reticulum. SR β belongs to another GTP-binding protein family which is distinct from the SRP-type GTP-binding protein family including FtsY and SR α . Although any bacterial counterparts corresponding to SR β have not yet been identified, it remains possible that far related SR β homologous protein might mediate the membrane association of FtsY and cpFtsY. In this respect, it might be noteworthy that thylakoid membranes contain another unidentified 34 kDa GTP-binding protein on the stromal surface in addition to cpFtsY (Kogata et al., unpublished results). We are currently investigating the nature of membrane association of cpFtsY in more detail.

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