Isolation and characterization of the cDNA for pea chloroplast SecA Evolutionary conservation of the bacterial-type SecA-dependent protein transport within chloroplasts

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Abstract We report here the isolation of the cDNA for pea chloroplast SecA. Pea SecA encodes a polypeptide of 1,011 amino acids and shows high sequence similarity with cyanobacterial SecA. Pea SecA was synthesized as a larger precursor and was imported into isolated chloroplasts in vitro. The purified pea SecA, which was expressed in *Escherichia coli* cells, stimulated the in vitro import of the 33 kDa protein of the oxygen-evolving complex into thylakoids. These results indicate that higher plant chloroplasts contain a bacterial-type SecA protein-dependent system for the intraorganellar protein transport into thylakoids.

Key words: SecA; Chloroplast; Protein import; Thylakoid

1. Introduction

According to the endosymbiont hypothesis, plant chloroplasts may have derived from oxygen-evolving photosynthetic bacteria that resembled cyanobacteria [1,2]. In prokaryotic cells, Sec proteins including SecA and SecY mediate protein transport across the cytoplasmic membrane [3–5]. Cyanobacteria have, in addition to the cytoplasmic membrane, the internal thylakoid membrane network, where oxygenic photosynthesis is performed. Two Sec proteins, SecY and SecA, have been found in both the cytoplasmic and thylakoid membranes in the cyanobacterium *Synechococcus* PCC7942 and therefore the same Sec proteins appear to mediate protein transport across both membranes in cyanobacterial cells [6–8].

The membrane structures of chloroplasts resemble those of cyanobacteria. They are surrounded by the two-membrane envelope and have internal thylakoids. However, most of the chloroplast proteins are encoded by the genes in the nucleus, synthesized outside the chloroplasts, and then imported back into the chloroplasts [9]. Once inside the chloroplasts, proteins are transported to their final destination such as thylakoids [9,10]. Since this intraorganellar protein transport process in the chloroplasts resembles the intracellular protein transport process in cyanobacteria, it is interesting to ask if chloroplasts have cyanobacterial-type Sec proteins for protein transport within organelles. Indeed recently, a part of the *secA* homologous gene was cloned from pea cDNA and the antibodies against the partial pea SecA recognized a 110-kDa protein of the chloroplast stroma [11]. The 110-kDa stromal protein appeared to mediate intraorganellar transport of some thylakoidal proteins, which are imported from the cytosol [11,12].

Here we report the isolation of the entire gene (cDNA) for pea chloroplast SecA. Pea SecA was synthesized as a larger precursor and was imported into chloroplasts. The purified pea SecA, which was expressed in *Escherichia coli* (*E. coli*) cells, stimulated the in vitro import of the 33-kDa protein of the oxygen-evolving complex (33K) into thylakoids. These results indicate that higher plant chloroplasts contain a bacterial-type SecA protein-dependent system for the intraorganellar protein transport into thylakoids.

2. Materials and methods

2.1. Cloning of the secA cDNA

Starting from the part of pea (*Pisum sativum*) *secA* cDNA [11], the 5′-flanking sequence was PCR-amplified with the 5′-Amplifinder RACE kit (Clonetech) and the 3′-flanking sequence by the 3′-RACE method by Frohman [13]. Briefly, to clone the 5′-end sequence, 5′-CTGCATTACTCGACCAGTAAACTCATCAAC-3′ and 5′-CTTTGCAGCCTGAAATATTGATCACTGGG-3′ were used as primers for cDNA synthesis and for the subsequent amplification, respectively. To clone the 3′-end sequence, 5′-GTAGAGAGGGAAGCAGAAATTGTCGCACAA-3′ was used as a primer for amplification. These primers were designed on the basis of the nucleotide sequence of the previously isolated internal cDNA segment [11]. The amplified cDNA fragments were subcloned into pUC119 [14], and their nucleotide sequences were determined with Taq Dye Primer Cycle Sequencing Kit (Applied Biosystems).

2.2. Construction of the genes encoding SecA derivatives

The entire *secA* gene was constructed from the obtained *secA* cDNA segments by ligation, and was inserted into pGEM-4Z (Promega) for in vitro transcription. To construct the in-frame fusion genes encoding S(1–129)-DHFR and S(62–129)-DHFR, nucleotides corresponding to residues 1–129 and residues 62–120 of pea SecA were PCR-amplified, respectively, and were inserted into pDHFR/SP containing the mouse dihydrofolate reductase (DHFR) gene and the promoter sequence for SP6 RNA polymerase. The initiator ATG codon was introduced just in front of the fusion gene for S(62–129)-DHFR. The gene encoding SecA(1–303) was constructed by inserting the cDNA fragment encoding residues 1–303 of SecA, which had been prepared by the 5′-RACE method, into pGEM-4Z.

2.3. In vitro import into chloroplasts and thylakoids

In vitro transcription was performed with the Megascript system (Ambion). The synthesized mRNAs were purified and were subjected to in vitro translation with a wheat germ cell-free system in the presence of [^{35}S]methionine. In vitro import of the radiolabeled proteins into isolated chloroplasts and suborganellar fractionation after import were

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Abbreviations: E. coli, Escherichia coli; DHFR, dihydrofolate reductase; S(1–129)-DHFR, a fusion protein consisting of residues 1–129 of pea SecA followed by DHFR; S(62–129)-DHFR, a fusion protein consisting of residues 62–129 of pea SecA followed by DHFR; SecA(1–303), a truncated protein corresponding to residues 1–303 of pea SecA; 33K, 33-kDa protein of the pea photosystem II oxygen-evolving complex; Ni-NTA, Ni-nitrilo-triacetic acid.

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performed as described previously [15]. In vitro import of the radiolabeled proteins into isolated thylakoids were carried out after the procedures described previously [11]. For the experimental details, see the figure captions, also.

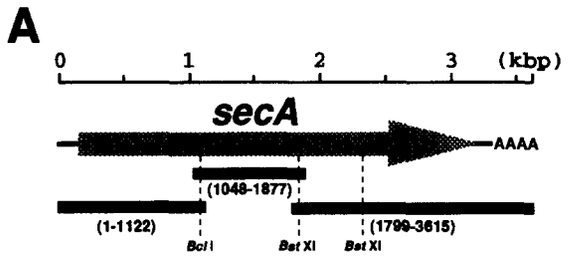
2.4. Purification of the recombinant pea SecA

The pea *secA* gene was introduced into pET-21d (Novagen) to construct a gene encoding a modified SecA (lacking residues 1–60) with a C-terminal hexa-histidine tag [16]. The resulting plasmid, pET-21d/secA was transformed into the *E. coli* BL21 (DE3) cells (Novagen). Expression of the $(His)_6$-tagged SecA was induced by cultivating the transformed cells in the presence of 1 mM isopropyl-β-thiogalactoside. Cells, which were harvested by centrifugation for 5 min at $3,000 \times g$ at 4°C, were suspended in 10 mM $NaKP_i$ (pH 7.2) and were disrupted by sonication. The soluble fraction was prepared by centrifugation for 20 min at $8,000 \times g$ followed by centrifugation for 20 min at $100,000 \times g$ at 4°C. The $(His)_6$-tagged SecA was partly recovered in the soluble fraction (~20%) while the rest of the fraction remained in the pellet (~80%). The soluble fraction containing the soluble form of the $(His)_6$-tagged SecA was applied onto the Ni-nitrilo-triacetic acid (NiNTA) column according to the instruction manual (Novagen). The bound materials were eluted with buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl) containing increasing concentrations of imidazole (4, 50, 100 and 200 mM). Each fraction was analyzed for the $(His)_6$-tagged SecA by SDS-PAGE and Coomassie blue staining. The fractions containing only the $(His)_6$-tagged SecA (those eluted with 100 and 200 mM imidazole) were combined and concentrated.

3. Results and discussion

3.1. Isolation of the cDNA encoding pea SecA

A part of the pea *secA* cDNA was PCR-amplified with pea cDNA as template as described previously [11]. The isolated *secA* cDNA fragment encoded a polypeptide of 276 amino acids [11], which corresponds to residues 217–509 of the *E. coli* SecA protein [17]. We now obtained the flanking parts of the *secA* cDNA by the 5′- and 3′-rapid amplification of cDNA ends



C

		SY	PA	AN	ES	BA	OL
Pisum sativum	PI	58	43	45	48	52	39
Synechococcus PCC7942	SY		47	48	43	40	45
Pavlova lutherii	PA			47	37	36	40
Antithamnion sp.	AN				38	38	43
Escherichia coli	ES					51	41
Bacillus subtilis	BA						39
Olisthodiscus luteus	OL						

B

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PI   1:MATSSLCSSFTSQTCNPHSRPHRKTLTLPGSVFLCRQFHLNSPSVSKTRRIRTRQSGPVASLGGLLGGIFKGTDTGEATRKQYAAIVNTINGLEPKISALSDSELRDMTFASRERAQKGE
SY   1:-------------------------------------------------------MLNLLLGDPNVRKVKKYKPLVTEINLLEEDIEPLSDKDLIAKTAEFRQKL-DKVSHSPA-AEK-E
AN   1:-------------------------------------------------------------MFN-FLFSNKI-MQYQRIVKQINSLDSTYNKYSDQELKQQTNKLKDQIIATQ
ES   1:-----------------------------------------------------------MLIKLLTKVF-GSRNDRTLRRM-RKVVNIINAMEPEMEKLSDEELKGKTAEFRARLEKGE
                                                                                                                *

PI 121:SLDSLLPEAFAVVREASKRVLGLRPFDVQLIGGMVLHKGEIAEMRTGEGKTLVAILPAYLNALVGKGVHVVTVNDYLARRDCEWVGQVFRFLGMKVGLIQQNMTSEQKRENYLCDITYVT
SY  63:LLAELLPEAFAVMREASKRVLGLRHFDVQMIGGMILHDGQIAEMKTGEGKTLVATLPSYLNALSGKGAHVVTVNDYLARRDAEWMGQVHRFLGLSVGLIQQGMSPEERRRNYNCDITYAT
AN  51:DIDTILPKAFAITKEAIKRATGLLLFDVQLIGAIILNQGKIAEMRTGEGKTLVAMLTAYLNSLFNKGVHIVTVNEYLAKRDATLAKQIFEYLNIHIGIIDQSMHSQERRKQYSCDITYLT
ES  59:VLENLIPEAFAVVREASKRVFGMRHFDVQLLGGMVLNERCIAEMRTGEGKTLTATLPAYLNALTGKGVHVVTVNDYLAQRDAENNRPLFEFLGLTVGINLPGMPAPAKRRAYAADITYGT
                * ***   ** **  *   ****  *   *     **** ******** * *  *** *  ** * **** *** **          *    *     *          * **** *

PI 241:NSELGFDFLRDNLATSVEELVIRGFNYCVIDEVDSILIDEARTPLIISGPAEKPSDQYFKAAKIADAFER----DI-------HYTVDEKQKSVLLSEQGYEDAEEIL---AVKD----L
SY 183:NSELGFDYLRDNMAAVIEEVVQRPFNYAVIDEVDSILIDEARTPLIISGQVDRPSEKYMRASEVAALLQRSTNTDSEE-EPDGDYEVDEKGRNVLLTDQGFINAEQLL---GVSD----L
AN 171:NSELGFDYLRDNMAIQKEDLVQRDFFFAIIDEIDSILIDEARTPLIISGPANMKLTEYLEAAKVANLLNQ--NTD---------YEIDEKNKNIILNEDGIKKSENIL--D-INN----L
ES 179:NNEYGFDYLRDNMAFSPEERVQRKLHYALVDEVDSILIDEARTPLIISGPAEDSSEMYKRVNKIIPHLIRQEKDSETFQGEGHFSVDEKSRQVNLTERGLVLIEELLVKEGIMDEGESL
         * * *** **** *   *  * *      ** ***************      *                *          ***     *  *   * *          *

PI 343:YD-PREQWASFVINAIKAKELFLRDVNYIIRGKEVLIVDEFTGRVMQGRRWSDGLHQAVEAKEGLPIQNETVTLASISYQNFFLQFPKLCGMTGTAATEITEFESIYKLKVTIVPTNKPM
SY 295:FD-SNDPWAHYIFNAIKAKELFIKDVNYIVRGGEIVIVDEFTGRVMPGRRWSDGLHQAVESKEGVEIQPETQTLASITYQNFFLLYPKLSGMTGTARTEELEFEKTYKLEVTVVPTNRVS
AN 273:YD-IQKPWIKYILNALKAKEIFIKNKDYIVKNMEIVIVDEFTGRIMEGRRWSDGLHQAIEAKEKQKIQQENKTLASITYQNLFLLYEKLSGMTGTARTEEAAELEQIYKLKVVEIPTNKLN
ES 299:YSPANIMLMHEVTAALRAHALFTRDVDYIVKDGRVIIVDEHTGRTMQGRRWSDGLHQAVEAKEGVQIQNENQTLASITFQNYFRLYEKLAGMTGTADTEAFEFSSIYKLDTVVVPTNRPM
          *  *   *      **    *  **** *** * ********** * **   ** *  *****  ** *    ** ****** **  *    ***      ***

PI 462:IRKDESDVVFRATTGKWRAVVVEISRMNKTGRPVLVGTTSVEQSDSLSQQLKEAGILHEVLNAKPENVEREAEIVAQSGRLGAVTIATNMAGRGTDIILGGNAEFMARLKLREIMMPRVV
SY 414:RRRDQPDVVYKTEIGKWRAIAADCAELHARGRPVLVGTTSVEKSEFLSQLLNEQGIPHNLLNAKPENVEREAEIVAQAGRRGAVTISTNMAGRGTDIILGGNADYMARLKLREYWMPQLV
AN 392:QRKDLSDLVYKTEYVKWKAVANECFDMYQIGRPTLVGTTSIEKSELLAKILKELQVFYNLLNAKPENITRESEIITQAGRKYTITISTNMAGRGTDIILGGNPQIIAKTALT--------
ES 419:IRKDLPDLVYMTEAAEKIQAIIEDIKERTAKGQPVLVGTISIEKSELVSNELTKAGIKHNVLNAK-PHAN-EAAIVAQAGYPAAVTIATNMAGRGTDIVLGGS--------------------
         * *  * *      * *              * * **** * * *       **  *       * *  * *     ** ********** ***

PI 582:KLVAEGEFV-SV-----KKPP-----------PSKTWKVNEKLFPCQLSNQNTELAEKAVQLAVKTWGKRSLTELRAHERLSYSCHKGPAQDEVIARLRNAFLEISKEYKVPTEEERKKV
SY 534:SFEEDGFGIAGVAGLEGGRPAAQGFGSPNGQKPRKTWKASSDIFPAELSTEAEKLLKAAVDLGVKTYGGNSLSELVAEDKIATAAEKAPTDDFVIQRLREAYQQVRKEYEAVTKQEQAEV
AN 504:IHINKILNLTQYN-TNYKIENEITY-ILNSINNT-----------LLINN-ID--INSQDISQSINNII--NNNMI--QD-AKSYKISNIYKIVL-----------NKYKQLCHNEKQEI
ES 519:-------------------------------------------------------------------WQAEVAALENPTAEQI------------EKIKADWQVRHDAV

PI 685:VAAGGLHVVGTERHESRRIDNQLRGRSGRQGDPGSSRFFLSLEDNIFRIFGGDRIQGLMRAFRV-EDLPIDSQMLTKALDEAQRKVENYFFNIRKQLFEYDEVLNSQRDRFYTERRRALQ
SY 654:VELGGLHVIGTERHESRRVDNQLRGRAGRQGDPGSTRFFLSLEDNLLRIFGGDRVAKLMNAFRVEEDMPIESGMLTRSLESAQRKVESTYYYDIRKQVFEYDEVMNNQRRAIYAERRRVLE
AN 592:ITLGGLYVIGTERHESRRIDNQLRGRSGRQGDLRSSRFFLSLQDNLLKIFGGDKISDFMQNLNIDEDMPIESSILNKSLSSAQRKIEAYFYDVRKQLFEYDEVLNNQRQAIYIERKRLLK
ES 549:LEAGGLHIIGTERHESRRIDNQLRGRSGRQGDAGSSRFYLSMEDALMRIFASDRVSGMQRKLGMKPGEAIEHPWVTKAIANAQRKVESRNFDIRKQLLEYDDVANDQRRAIYSQRNELLD
         ***  ********** ******* *****  * ** **  *   **  *     *          *          ** * *    ***  *** * * * **   *  *   *

PI 804:SVNLQSLLIEYAELTIDDILEANIGSDAPKESWDLDKLIAKIQQYCYLLTDLTPDLLLNECSD-YE-GLRSYLRLRGKEAYLQKRDIVEQQAPGLMREAERFILLSNIDRLWKEHLQALK
SY 774:GRELKEQVIQYGERTMDEIVDAHINVDLPSEEWDLEKLVNKVKQFVYLLEDLEAK-QLSDLS-P-E-AIKIPLHEQLRIAYDLKEAQIDQIQPGLMRQAERYFILQQIDTLWREHLQAME
AN 712:SNYTRDCILEYAE--STIEEM-LVTYNQQTDISRKTKI-LSKILKLLNL-NIYINNNILLNME--ENDIKSPLFEQLRITYDLRESYLEQLRPGLIRQLEKYYLLQQDIDYAWQEHINKIS
ES 669:VSDVSETINSIREDVFKATIDAYIPPQSLEEMWDIPGLQER-LKNDFDL-DLPIAEWLDKEPELHEETLRDGILAQSIEVYQRK--EEV-VGAEMMRHFRKGVMLQTLDSLWKEHLAAMD
                *                                                                                                  *  *  * * **

PI 922:FVQQAVGLRGYAQRDPLIEYKLEGYNLFLEMMAQIRRNVIYSIYQFKPVLLKQDQDKMENQKSGKRNARPPTDTNPDPVGTVEPSTSASS
SY 890:ALRESVGLRGYQQKDPLLEYKSEGYELFLEMMTAIRRNVIYSMFMFDPQPQARPQAEVV
AN 825:ILKESIGWRSYGQQDPLIEYKNEAFNLFINMVTYIRQTVIYLTMRSRLIVNIDN
ES 784:YLRQGIHLRGYAQKDPKQEYKRESFSMFAAMLESLKYEVISTLSKVQVRMPEEVEELEQQRRMEAERLAQMQQLSHQDDDSAAAAALAAQTGERKVGRNDPCPCGSGKKYKQCHGRLQ
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Fig. 1. The PCR-amplified cDNA fragments that cover the entire pea *secA* cDNA (A), the amino-acid sequences of pea SecA (*PI*) and the previously identified SecA proteins from *Synechococcus* PCC7942 (*SY*), *Antithamnion* sp. (*AN*), and *E. coli* (*ES*) (B), and percentage of identical amino acids of SecA proteins [8,17–21] (C). In (B), the conserved amino acids are indicated with asterisks, and the proposed ATP-binding site is underlined. The nucleotide sequence of the pea SecA has been deposited in the EMBL/GenBank/DDBJ Data Library under the accession number X82404.

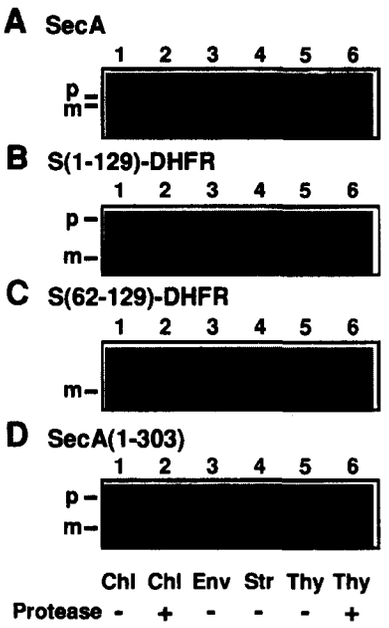

Fig. 2. In vitro import of pea SecA (A), S(1–129)-DHFR (B), S(62–129)-DHFR(C) and SecA(1–303) (D) into isolated pea chloroplasts. The radiolabeled proteins (translation products) were incubated with isolated pea chloroplasts in the light for 30 min at 25°C in 8 mM ATP, 17 mM $MgCl_2$, 0.1% bovine serum albumin, 330 mM sorbitol and 50 mM HEPES–KOH, pH 8.0. After import, intact chloroplasts were re-isolated and were subjected to suborganellar fractionation [15]. For protease treatment, the chloroplasts after import were incubated with 0.1 mg/ml thermolysin and 1 mM $CaCl_2$ for 30 min on ice, or the thylakoids were incubated with 0.4 mg/ml thermolysin and 4 mM $CaCl_2$ for 30 min on ice. Lanes 1 = chloroplasts (Chl) without further treatment; lanes 2 = chloroplasts after protease treatment; lanes 3 = envelope membranes (Env); lanes 4 = a soluble fraction containing the stroma (Str); lanes 5 = thylakoids (Thy) without protease treatment; lanes 6 = thylakoids after protease treatment. p, precursor forms; m, mature-sized forms.

(RACE) methods [14]. The amplified cDNA fragments were analyzed for their nucleotide sequences and were found to overlap partly with the previously isolated *secA* cDNA fragment (Fig. 1A). A Southern hybridization analysis confirmed that the obtained *secA* fragments derived from the pea genome (data not shown). The three cDNA fragments (Fig. 1A) cover the entire pea *secA* cDNA, which encodes a polypeptide of 1,011 amino acids with a calculated molecular mass of 114,130 (Fig. 1B). The alignment of the predicted amino acid sequence of pea SecA with those of the previously identified bacterial and algal SecA proteins [8,17–21] reveals homology throughout the sequences including the ATP-binding region (Fig. 1B). Cyanobacterial SecA shows the highest degree of identity (58%), whereas *E. coli* SecA shows 48% identity [8,17]. Pea SecA is 43%, 45% and 39% identical with SecA encoded by the plastid DNA of the chromophytic alga (*Pavlova lutherli*), the red alga (*Antithamnion* sp.), and another chromophytic alga (*Olisthodiscus luteus*), respectively (Fig. 1C) [19–21].

3.2. Pea SecA is a chloroplast protein

The predicted amino acid sequence of pea SecA shows the presence of an N-terminal extension of about 60 residues as compared with the other SecA proteins (Fig. 1B). This N-terminal extension is rich in hydrophilic amino acids (especially hydroxylated amino acids) but lacks acidic ones, and therefore resembles chloroplast transit peptides that guide the proteins to chloroplasts [22]. In order to confirm directly that pea SecA is a chloroplast protein, we performed in vitro chloroplast import experiments for pea SecA, S(1–129)-DHFR consisting of residues 1–129 of pea SecA followed by DHFR, S(62–129)-DHFR consisting of residues 62–129 of pea SecA followed by DHFR, and SecA(1–303) corresponding to residues 1–303 of pea SecA (Fig. 2). Incubation with chloroplasts converted pea SecA, SecA(1–303) and S(1–129)-DHFR to lower molecular-mass forms (Fig. 2A–C, lanes 1), which were resistant to digestion by externally added protease (Fig. 2A–C, lanes 2), while S(62–129)-DHFR was not converted to a lower molecular mass form or sequestered to the protease-protected compartments. Pea SecA was thus imported into chloroplasts and proteolyti-

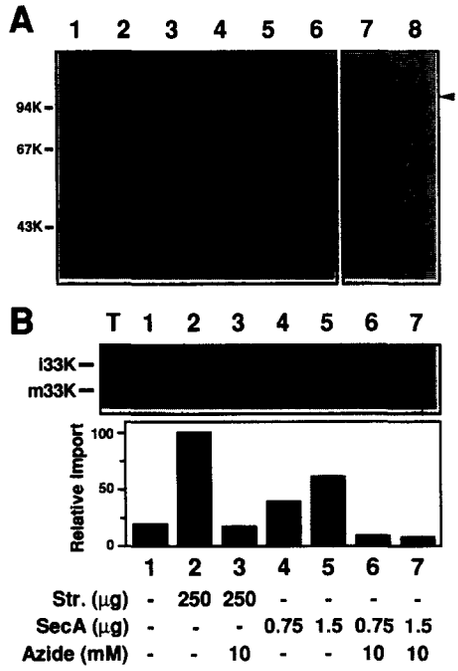

Fig. 3. Purification of the recombinant pea SecA expressed in *E. coli* (A) and reconstitution of the import of the intermediate form of 33K into isolated pea thylakoids (B). In (A), lane 1 = the soluble cell extract prepared from the BL21(DE3) cells harboring the pea SecA expression plasmid; lane 2 = flow-through from the Ni-NTA column; lanes 3, 4, 5 and 6 = the fractions eluted with buffer containing 4, 50, 100 and 200 mM imidazole, respectively; lanes 7 and 8 = 1 and 2 μg of purified, recombinant pea SecA, respectively. SecA is denoted with arrowheads. In (B), chloroplasts (2 mg/ml chlorophyll) were disrupted by osmotic shock by incubation for 10 min on ice in 8 mM ATP, 26 mM $MgCl_2$, 0.1% bovine serum albumin, 10 mM HEPES–KOH, pH 8.0. The disrupted chloroplasts (chloroplast lysates) were fractionated into the thylakoids and the stroma by centrifugation for 10 min at 7,500 × *g*. The protein concentration of the stromal fraction was determined by the Bradford methods (BioRad). The thylakoids were washed twice with 10 mM $MgCl_2$ and 10 mM HEPES&nash;KOH, pH 8.0, and used for import experiments. For in vitro import into thylakoids, the in vitro translated intermediate form of 33K was incubated with the thylakoids in the light for 20 min at 25°C in the import buffer (8 mM ATP, 18 mM $MgCl_2$, 10 mM NaCl, 0.055% bovine serum albumin, 80 mM sorbitol and 17 mM HEPES–KOH, pH 8.0). The incubation was carried out in the absence or presence of the indicated concentrations of the stromal fraction (Str), the recombinant SecA (SecA), and/or sodium azide (Azide). i33K and m33K, the intermediate and mature forms of pea 33K, respectively.

cally processed to the mature form with an apparent molecular mass of 110 kDa, and residues 1–129 including the N-terminal extension of pea SecA was sufficient to direct a foreign protein (DHFR) to chloroplasts. Since the size of the processed form of S(1–129)-DHFR is close to that of S(62–129)-DHFR, the transit peptide of pea SecA is likely cleaved at around residues 61/62. These results suggest that the isolated pea *secA* cDNA encodes the 110-kDa chloroplast protein previously identified by immunological analyses [11,12].

Suborganellar fractionation of the chloroplasts after import revealed that the processed pea SecA was mainly recovered in the stromal fraction, and that a fraction of the imported pea SecA was also associated with the thylakoids (Fig. 2A, lanes 3–6). This is consistent with the results of immunological analyses that the authentic chloroplast 110-kDa protein was detected mainly in the stroma and partly in the thylakoids [11]. Interestingly, neither S(1–129)-DHFR nor SecA(1–303) was associated with the thylakoids (Fig. 2B,D, lanes 3–6), suggesting that the mature part of pea SecA is necessary for its binding to the thylakoids.

3.3. *Pea SecA stimulates in vitro transport of 33K into thylakoids*

The intraorganellar transport of thylakoid lumenal proteins can be reconstituted in vitro using the artificial intermediate forms of the thylakoidal proteins and isolated thylakoids. Such in vitro thylakoidal transport experiments have revealed that thylakoid lumenal proteins are transported into thylakoids by at least two different mechanisms. The transport of the 33K (33-kDa protein of the oxygen evolving complexes) and plastocyanin requires the presence of ATP, stromal factor(s), and the thylakoidal ΔpH, whereas transport of 23- and 16-kDa proteins of the oxygen evolving complexes requires only the thylakoidal ΔpH and is independent of ATP or stromal factors [11,12,23,24].

Pea SecA without the N-terminal extension was tagged with hexa-histidines [16] and was expressed in *E. coli*. The $(His)_6$-tagged pea SecA was partly recovered in the soluble fraction, although it partly formed insoluble aggregates. The soluble form of the recombinant pea SecA was purified to homogeneity by binding to Ni-NTA beads (Fig. 3A). The purified pea SecA was tested for its ability to stimulate the in vitro import of the artificial intermediate form of 33K into isolated thylakoids (Fig. 3B). The import of the 33K intermediate into isolated thylakoids required the stromal fraction (Fig. 3B, lanes 1 and 2) but was inhibited by sodium azide, a potential inhibitor of SecA (Fig. 3B, lane 3). The recombinant pea SecA could be substituted for the stromal fraction in the import of the 33K intermediate into thylakoids (Fig. 3B, lanes 4 and 5), and the import stimulation by pea SecA was inhibited by sodium azide (Fig. 3B, lanes 6 and 7). The import of the intermediate of another thylakoidal protein, the 23-kDa protein of the oxygen-evolving complex, into isolated thylakoids was not stimulated by the stromal fraction or the recombinant pea SecA (not shown). Therefore pea SecA in the stroma mediates intraorganellar transport of a subset of thylakoidal proteins including 33K, which are imported from the cytosol.

3.4. *Conclusion*

In the present study, we have isolated the entire cDNA encoding pea SecA. Pea SecA was synthesized as a larger precursor, was imported into chloroplasts, and reached the stroma and the thylakoid surface. The purified, recombinant pea SecA stimulated the in vitro import of the intermediate form of 33K into isolated thylakoids.

These results indicate that higher plant chloroplasts contain a bacterial-type SecA protein-dependent system for the intraorganellar protein transport into thylakoids. Since cyanobacterial cells contain SecA and SecY in the thylakoid membrane as well as the cytoplasmic membrane [7,8], higher plant chloroplasts have most likely inherited the bacterial-type SecA-dependent system for thylakoidal protein transport from the ancestral endosymbiont that resembled cyanobacteria. This system appears to be employed for the intraorganellar transport of thylakoidal proteins including 33K that have also derived from those of the cyanobacterial-type progenitor.

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