Communication

Characterization of the Nuclear- and Plastid-Encoded secA-Homologous Genes in the Unicellular Red Alga Cyanidioschyzon merolae

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SecA is an ATP-driven motor for protein translocation in bacteria and plants. Mycobacteria and listeria were recently found to possess two functionally distinct secA genes. In this study, we found that Cyanidioschyzon merolae, a unicellular red alga, possessed two distinct secA-homologous genes; one encoded in the cell nucleus and the other in the plastid genome. We found that the plastid-encoded SecA homolog showed significant ATPase activity at low temperature, and that the ATPase activity of the nuclear-encoded SecA homolog showed significant activity at high temperature. We propose that the two SecA homologs play different roles in protein translocation.

Key words: secA; Cyanidioschyzon merolae; ATPase activity; temperature dependence

Protein translocation via Sec translocase is a well-studied protein transport mechanism. In bacteria, the bulk of protein export across the cytoplasmic membrane is carried out by Sec translocase. Its components have been well-characterized in studies with Escherichia coli and Bacillus subtilis.1) Bacterial Sec translocase consists mainly of a highly conserved protein-conducting channel, SecYEG, and a peripheral ATPase motor SecA. ATPase SecA couples ATP hydrolytic energy to protein translocation.2) Through repeated cycles of ATP binding and hydrolysis, SecA undergoes dynamic conformational changes that drive the stepwise translocation of pre-protein through the SecYEG channel.3)

Sec translocase is conserved also in protein translocation pathways into the endoplasmic reticulum (ER) and the thylakoid lumen or membrane of the chloroplast in eukaryotic cells.4–6) The post-translational translocation pathway into the ER consists mainly of Sec61αβγ (a homolog of SecYEG), the tetrameric Sec62/63 complex, and luminal chaperone BiP. It is believed that this pathway does not include a peripheral “pushing” motor protein, like SecA.7)

In plants and algae, SecYE homologs exist on thylakoid membranes and probably form the protein-conducting channel,8,9) and a SecA homolog exists in the chloroplast stroma.9) Chloroplast SecA is responsible for the translocation of thylakoid proteins, such as the 33-kDa polypeptide of the oxygen-evolving complex of photosystem II, plastocyanin, the F subunit of photosystem I, and cytochrome f.10,11) Chloroplast SecA is believed to drive the translocation of the precursor proteins into thylakoid lumen or membrane from stroma in a manner similar to that employed by bacterial SecA,12) and is thought to play an essential role in chloroplast biogenesis.13)

Most bacteria possess only a single essential secA gene, but mycobacteria and listeria possess two non-redundant secA-homologous genes.14,15) One is essential for viability (secA1) like the secA genes of many bacteria, but the other (secA2) is not. It has been suggested that SecA2 is dedicated to exporting specific subsets of proteins related to bacterial virulence.16,17) It shows intrinsic ATPase activity. Hence it has been suggested that it functions in a manner similar to E. coli SecA in undergoing cycles of ATP hydrolysis.18)

Cyanidioschyzon merolae is an ultra-small unicellular red alga living in acidic hot springs. Most algae investigated to date encode only a single secA-homologous gene, which is found in the plastid genome. In contrast, complete genome sequencing identified two secA-homologous genes in C. merolae, one in the cell nucleus and the other in the plastid genome (http://merolae.biol.s.u-tokyo.ac.jp).)19–21) The differences between and the significance of the two secA genes in mycobacteria and listeria have been well investigated, but they have not received attention in plants until recently. Here we report the first characterization of a pair of secA-homologous genes in a single plant species, and we include an examination of the ATPase activity of the two corresponding SecA homologs. In this paper, we refer to the nuclear-encoded secA-homologous gene (C. merolae genome project ID: CMQ9393C) as secA(nuc), and to the plastid-encoded secA-homologous gene (C. merolae genome project ID: CMV071C) as secA(pt) to distinguish them. We conducted ATPase assay at various temperatures and found that SecA(nuc) and SecA(pt) can be distinguished by the way its activity depends on temperature. The data provided here give clues to understanding the functions of the two secA-homologous genes in C. merolae.

The protein encoded by secA(nuc) is a 113-kDa protein of 1,011 amino acids, 46% identical to the chloroplast pre-protein translocase of Arabidopsis thali-
ana (AtcpSecA) and 44% identical to the SecA of Synechocystis sp. PCC 6803 (SySecA). The other homologous gene, secA (pt), encodes a 90-kDa protein of 774 amino acids, 59% and 58% identical to AtcpSecA and the SySecA respectively. SecA(nuc) was 46% identical to SecA(pt). Figure 1A shows the amino acid sequence alignments of the N-terminal regions, including the SecA DEAD-like domains of AtcpSecA, SecA(nuc), SecA(pt), and SySecA. The conserved Walker A and B motifs, which are required for optimal ATPase activity, were found in the amino acid sequences of both SecA homologs of C. merolae. The conserved lysine residue in the Walker A motif was also found in both SecA homologs (Fig. 1A, boldface). Many genes that function in chloroplasts are encoded in cell nuclei and include a sequence coding for N-terminal transit peptides. The secA gene of A. thaliana is also encoded in the cell nucleus. A possible cleavage site of the transit peptide was found between positions 22 and 23 in AtcpSecA and between 41 and 42 in SecA(nuc) (Fig. 1A, arrow) by SignalP. The subcellular localizations of AtcpSecA and SecA(nuc) led to an inference of 'Chloroplast' by the WoLF PSORT program (http://wolfpsort.org/).

Fig. 1. Multiple Alignment and Putative Domains of C. merolae SecA Homologs.
A, Multiple alignment of amino acid sequences of N-terminal regions including the SecA DEAD-like domains of SecA proteins. The amino acid sequences of the SecA homologs, deduced from the nucleotide sequences of A. thaliana, C. merolae nuclear-encoded, C. merolae plastid-encoded, and the Synechocystis sp. PCC 6803 secA genes, were aligned with the Clustal X program. Amino acid residues shared by all the SecA homologs are shown by asterisks. Putative Walker A and B motifs are enclosed by squares. The conserved lysine residues in the Walker A motif are indicated in boldface. The arrow indicates a possible cleavage site of the C. merolae nuclear-encoded SecA homolog predicted by SignalP. B, Putative protein domains of C. merolae SecA homologs. Three domains (SecA DEAD-like, pre-protein cross-linking, and Wing and Scaffold) characteristic of SecA proteins are shown. These domains were predicted by the Pfam database (http://pfam.sanger.ac.uk/).
of SecA(nuc) includes the transit peptide for plastid targeting. GFP from vector plasmid pUC18-CaMV35S-sGFP(S65T)-NOS was observed in the cytoplasm of the epidermal cells (Fig. 2B). 

The SecA protein includes several domains required for protein translocation (Fig. 1B). Putative domains characteristic of SecA proteins, the SecA DEAD-like domain (Pfam ID: PF07517) representing the N-terminal region of SecA(nuc) includes the transit peptide for plastid targeting, and the wing and scaffold domain (Pfam ID: PF01043), the SecA cross-linking domain (Pfam ID: PF07516) were found in the cytoplasm of the epidermal cells (Fig. 2B). 

To determine whether the SecA(nuc) and SecA(pt) functions as SecA, we performed complementation tests using E. coli strain MM52, a temperature-sensitive secA mutant. Full-length SecA(nuc) and SecA(pt) did not complement the temperature-sensitive secA at 42 °C. Hence we tested constructs encoding chimeric proteins whose C-terminal regions SecA(nuc) or SecA(pt) were replaced with the C-terminal regions of E. coli SecA. Only the chimeric protein composed of the N-terminal region of SecA(pt) and the C-terminal region of E. coli SecA partially complemented temperature-sensitive secA(nuc) genes in C. merolae as examined by RT-PCR. Total RNA isolated from C. merolae cells was used as the template for cDNA synthesis. PCR products amplified with specific primers for various secA-homologous genes were detected with ethidium bromide. No bands were detected in the absence of reverse transcriptase, indicating that the reaction mixtures were uncontaminated by genomic DNA (Supplemental Fig. S1A, +RTase; see Biosci. Biotechnol. Biochem. Web site). In contrast, the bands derived from transcripts of the secA-homologous genes appeared clearly when reverse transcriptase was added (Supplemental Fig. S1A, −RTase), indicating that the two homologous genes are not pseudogenes and have certain functions. The full open reading frames of secA(nuc) and secA(pt) and their expression were confirmed by these results and previous DNA microarray analyses. 

In order to determine the expression patterns of secA(nuc) and secA(pt) during light-dark cycles, relative transcript levels were assessed by real-time RT-PCR. C. merolae was cultivated under a 12-h light/12-h dark cycle, and total RNA was isolated every 4 h under light and every 2 h under dark (Supplemental Fig. S1B). In the first half of the dark period (from L12 to D6), the transcript levels of both secA(nuc) and secA(pt) decreased gradually and consequently showed minimal levels in the middle of the dark period (D6). In the latter half of the dark period (from D6 to D12), the transcript levels appeared remain at the minimal level or to increase gradually toward the light period. There did not appear to be obvious differences in the expression patterns of secA(nuc) and secA(pt) under this light-dark cycle. The transcript level of secY in the dark period decreased slightly. This decrease in transcript level did not appear to be remarkable under the light-dark cycle, compared with that of the secA-homologous genes. This decrease and increase of the transcripts might have been due to the periodic switching of the lighting conditions. The gradual decrease may serve to keep a minimal required transcript level, and the gradual increase may be preparation for the adaptation to the light period. Indeed, chloroplast SecA is responsible for the translocation of proteins related to photosynthesis, and the secA gene of A. thaliana is light-inducible and is expressed in green tissues.

In order to gain an understanding of the evolutionary relationship between secA(nuc) and secA(pt), we constructed a phylogenetic tree inferred from the putative SecA proteins by the neighbor-joining method (Fig. 3). Homologous sequences were obtained by BLAST against the NCBI database with E-values of <1 × 10−10. Alignments of homologous sequences were made with the Clustal W program in MEGA 4.0 software using the default parameter sets (http://www.megasoftware.net/). In the phylogenetic tree, the bacterial SecA proteins were used as the out-group. The plant and cyanobacterial SecA contributes to the protein import into the thylakoid lumen, whereas the bacterial SecA contributes to protein secretion.
C. merolae SecA(nuc) constituted a cluster distinct from that of SecA(pt) (the node is indicated by an arrow). In Phaeodactylum tricornutum, the nuclear-encoded SecA homolog also constituted a cluster distinct from that of the plastid-encoded SecA homolog. The phylogenetic tree suggests that the nuclear-encoded secA-homologous genes belong to the plant/cyanobacterial cluster, and may have branched off in an early divergent evolutionary step or may have been acquired through endosymbiotic/horizontal gene transfer events. On the other hand, two SecA homologs of Chlamydomonas reinhardtii (here denoted SecA1 and SecA2 for expedience), which adjoin in the cell nucleus, were closely related. The tree led us to assume that the two nuclear-encoded secA-homologous genes of C. reinhardtii are closely related phylogenetically and arose in a gene duplication event. Additionally, we investigated the evolutionary relationship between bacterial SecA1 and SecA2. The SecA2 proteins of M. tuberculosis and L. monocytogenes, which have been reported to function distinctly from the canonical SecA1,17–20) formed clusters distinct from those of canonical SecA1 proteins (data not shown). Considering our phylogenetic analysis, the distant relationship between secA(nuc) and secA(pt) suggests distinct functions of the two SecA homologs in C. merolae.

To determine whether SecA(nuc) and SecA(pt) possess intrinsic ATPase activities, the recombinant proteins were overexpressed as N-terminal His-tag fused proteins and purified with a Ni²⁺-charged column close to homogeneity. Overexpression of the proteins was confirmed by Western blotting using anti-His9 monoclonal antibody (Supplemental Fig. S2A). The purity of the eluted fraction was checked by Coomassie Brilliant Blue (CBB) staining (Supplemental Fig. S2B).

We performed ATPase assays of these purified SecA homologs using malachite green. The purified SecA homologs showed ATPase activities. When the conserved lysine residues in the Walker A motif of the proteins were replaced with arginine residues by site-directed mutagenesis, the mutated SecA homologs, SecA(nuc)K88R and SecA(pt)K87R, showed little ATPase activity (Fig. 4A). These results indicate that SecA(nuc) and SecA(pt) indeed possess intrinsic ATPase activities and that the conserved lysine residue is required for the ATPase activities of both C. merolae SecA homologs. Substitutions at the invariable lysine residue in the Walker A motif block the translocation activity of E. coli and B. subtilis SecA.31) The ATPase activity is thus almost certainly essential to the protein translocation activity of SecA. Based on these observations and our data, we surmise that both SecA(nuc) and SecA(pt) function in protein translocation.

The intrinsic ATPase activity of the purified SecA homologs was measured at various temperatures (Fig. 4B). SecA(pt) showed high ATPase activity at 30, 35, and 40 °C (approximately 100 pmol Pi/μg/min), but showed very low activity at 45, 50, 55, and 60 °C (less than 5 pmol Pi/μg/min). The ATPase activity of SecA(nuc) increased gradually as the temperature rose, and it was highest at 55 °C (approximately 70 pmol Pi/μg/min). Even at 60 °C, SecA(nuc) showed significant ATPase activity (approximately 20 pmol Pi/μg/min).

C. merolae lives in acidic hot water, as in hot springs, at about 45 °C. Temperatures of less than 40 °C might be low for C. merolae. Since SecA(pt) showed high
ATPase activity at low temperatures (at 30, 35, and 40°C), it appears to possess protein translocation activity at low temperatures, but it had low ATPase activity at high temperatures (at 45, 50, 55, and 60°C). In contrast, SecA(nuc) showed significant ATPase activity at the higher temperatures. Based on this difference in the temperature dependence of the ATPase activity, we assume that SecA(nuc) is required for protein translocation at high temperatures. Chloroplast SecA is essential for photosynthetic development in *A. thaliana*.13) *C. merolae* is an obligate photoautotrophic organism that can grow at 50°C. Although there is a possibility that SecA(nuc) transports a subset of proteins distinct from the substrate proteins of SecA(pt), such as mycobacterial and listerial SecA2, we suppose that *C. merolae* preserved (or acquired) and evolved the secA(nuc) gene in adapting to high temperature environments. A recent study indicates that *A. thaliana* possesses two secA genes with distinct functions.32) The studies described here are intended to lay the groundwork for further investigation of the two SecA homologs in plants.

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