

Translocation of a Phycoerythrin α Subunit across Five Biological Membranes*

Received for publication, March 2, 2007, and in revised form, August 16, 2007 Published, JBC Papers in Press, August 16, 2007, DOI 10.1074/jbc.M701869200

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Cryptophytes, unicellular algae, evolved by secondary endosymbiosis and contain plastids surrounded by four membranes. In contrast to cyanobacteria and red algae, their phycobiliproteins do not assemble into phycobilisomes and are located within the thylakoid lumen instead of the stroma. We identified two gene families encoding phycoerythrin α and light-harvesting complex proteins from an expressed sequence tag library of the cryptophyte *Guillardia theta*. The proteins bear a bipartite topogenic signal responsible for the transport of nuclear encoded proteins via the ER into the plastid. Analysis of the phycoerythrin α sequences revealed that more than half of them carry an additional, third topogenic signal comprising a twin arginine motif, which is indicative of Tat (twin arginine transport)-specific targeting signals. We performed import studies with several derivatives of one member using a diatom transformation system, as well as intact chloroplasts and thylakoid vesicles isolated from pea. We demonstrated the different targeting properties of each individual part of the tripartite leader and show that phycoerythrin α is transported across the thylakoid membrane into the thylakoid lumen and protease-protected. Furthermore, we showed that thylakoid transport of phycoerythrin α takes place by the Tat pathway even if the 36 amino acid long bipartite topogenic signal precedes the actual twin arginine signal. This is the first experimental evidence of a protein being targeted across five biological membranes.

Cryptophytes are an unusual group of flagellate algae common in marine and fresh water. Whereas plant plastids derive from endosymbiosis of a cyanobacterium, cryptophytes acquired their plastid by engulfing and stably integrating a red algal cell, leading to a eukaryote-eukaryote chimera (1, 2). In cryptophytes, the eukaryotic endosymbiont is remarkably reduced, but, unlike most other secondarily evolved algae, the endosymbiont nucleus persists in a highly vestigial form (3). The plastid is located within the host endoplasmic reticulum

(ER)² and four membranes separate the cytosol from the stroma: (i) the ER membrane, (ii) the periplastidial membrane, the former cytoplasm membrane of the red alga, (iii) the outer plastid envelope membrane, and (iv) the inner plastid envelope membrane (Fig. 5). Between the outer and inner membrane pair, remnants of the cytosol and the nucleus of the red alga endosymbiont are still present and are referred to as the periplastidial compartment and nucleomorph, respectively. Together with the thylakoid membrane system that is located within the plastid stroma, this makes five distinct membranes separating the thylakoid lumen from the host cytosol (Fig. 5), barriers that have to be dealt with by all nuclear-encoded plastid proteins with a thylakoid destination.

One further peculiarity of cryptophytes is their unusual thylakoid ultrastructure. Electron microscopic analyses of the thylakoid lumen have shown that this compartment is filled with electron dense material in cryptophytes. It was suggested that this material might represent photosynthetic pigments located on the lumenal, rather than the stromal, side of the thylakoid membrane (4, 5). This makes the cryptophytes phycobilin-based antenna system very different from others such as that of red algae, glaucophytes and cyanobacteria. In these organisms the light-harvesting pigments such as phycoerythrin, phycocyanin, or allophycocyanin, are arranged into so-called phycobilisomes, which are associated to the thylakoid membrane from the stromal or cytosolic side (9).

The unusual localization of phycobiliproteins was confirmed by immunolocalizing studies of phycoerythrin (PE) in *Rhodomonas lens* (6), indicating that in cryptophytes phycobiliproteins are located inside the thylakoid lumen and not arranged into phycobilisomes. Sequencing the plastid genome of the cryptophyte *Guillardia theta* supported the latter observation, as no genes-encoding linker proteins, which are common in plastid genomes of rhodophytes, are expressed in the plastid (7). However, the lack of a topogenic signal in the phycoerythrin β subunit, encoded by the plastid *cpeB* gene, stands in contrast to the observed localization of the molecule (7, 8).

To investigate this phenomenon, focusing on the transport across five membranes into the thylakoid lumen of cryptophytes, we screened our EST data base of *G. theta* and identified two separate gene families: one encoding the canonical light-harvesting apoproteins (LHCP) and one, the α subunits of phy-

* This work was supported by grants from the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 648 and 695) and the state Sachsen-Anhalt (Exzellenzcluster Biowissenschaften). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

New and relevant EST data were deposited using EMBL WebIn under the following accession numbers: AM183804 and AM491779-AM491800.

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² The abbreviations used are: ER, endoplasmic reticulum; PE, phycoerythrin; EST, expressed sequence tag; BTS, bipartite topogenic signal; LHCP, light-harvesting apoproteins; GFP, green fluorescent protein; Tat, twin arginine transport.

coerythrin (PE α). All genes encode preproteins containing a bipartite topogenic signal (BTS), composed of an N-terminal signal peptide for co-translational import into the ER lumen via the Sec61 complex, followed by a transit peptide-like region mediating transport across the remaining three membranes into the plastid stroma (10, 11). Additionally, more than half of the PE α subunits carried a twin pair of arginine residues, followed by a hydrophobic stretch, between the transit peptide and mature protein region.

We have examined the organellar localization of one member of the PE α family using *in vitro* import assays and *in vivo* localization with different GFP fusion constructs. Our results demonstrate that the PE α subunit is transported into the thylakoid lumen and that the twin arginine translocase (Tat) catalyzes this transport. This is the first example in which experimental proof demonstrates transport of proteins across five biological membranes within one cell and the processing of the precursor protein three times during the translocation.

EXPERIMENTAL PROCEDURES

cDNA Library of *G. theta*—A cDNA library of *G. theta* CCMP327, was generated from three liters of culture ($1\text{--}5 \times 10^9$ cells/ml) harvested at three different time points every 8 h (one liter, respectively), starting 1 h before the light was turned on (12 h day/night cycle). The cells were shock frozen in liquid nitrogen and packed in dry ice for shipping. The cDNA library was generated by the Vertis Biotechnology AG (Freising, Germany). The normalized and 5' full-length enriched cDNA library was ligated via the EcoRI and NotI restriction site into the pExCell plasmid and supplied as a λ -ExCell phagemid library by the company.

GFP Fusion Constructs and Analysis—Two different GFP fusion constructs were generated for the analysis of the subcellular localization in the diatom *Phaeodactylum tricornutum*. In the first construct only the signal peptide encoding region of the PE α was fused to the GFP gene using the oligomers PhySP-5': GAATTCATGCTCCGTGCTACCGTCGCTGCCGCCGCTCGTTGCCTCCGCCGCTGCCATGGTGAGCAAGGGCG and GFP-3': CTAAGCTTACCTGTACAGCTCGTCCATGC for amplification of the complete fusion construct. The second construct included both, signal and transit peptide encoding region, using PhycoBTS-5': AAGAATTCATGCTCCGTGCTACCGTCGCTGCCG and PhycoBTS-3': CCATGGACTCGGCCTGCATGGCGACGCC oligomers for amplification of the topogenic signal. In case of the SP-GFP fusion construct the vector pKS-eGFP (Kroth Lab, Konstanz Germany) was used as a template for standard PCR. Oligonucleotides introduced a 5' EcoRI and 3' HindIII restriction site for cloning the fragment into pPhaT1. For the second construct a standard PCR reaction was performed using genomic DNA from *G. theta* CCMP327 as a template. Oligonucleotides introduced a 5' EcoRI and 3' NcoI restriction site and PCR-products were digested with EcoRI and NcoI, the plasmid pPhaT1 with EcoRI and HindIII and the GFP-encoding fragment with NcoI and HindIII. All fragments were ligated and subsequently transformed into *Escherichia coli* MRF in a single step. Fidelity of amplification and cloning was checked via sequencing of the con-

struct. Diatom transformations were performed as described previously by Apt *et al.* (12). For ER staining of wild-type cells, 1 ml of dense grown culture was incubated for 15 min with 0.5 μM ER-TrackerTM Green BODIPY[®] FL glibenclamide (Molecular Probes, Invitrogen Detection Technologies). Cells were washed once with PBS and analyzed directly. Analysis of the diatoms was performed with a confocal laser-scanning microscope Leica TCS SP2 at room temperature in f/2 culture medium, using a PL APO 63x/1.32–0.60 oil Ph3 CS objective. GFP, ER-TrackerTM Green BODIPY[®] FL glibenclamide (Molecular Probes, Invitrogen Detection Technologies) and chlorophyll fluorescence was excited at 488 nm, filtered with beam splitter TD 488/543/633 and detected by two different photomultiplier tubes with a bandwidth of 500–520 and 625–720 nm for GFP and chlorophyll fluorescence, respectively. Image processing was done using the LCS Lite software from Leica (Wetzlar, Germany) and Adobe Photoshop.

Import Constructs—For analyzing the import of the PE α subunit into thylakoids and chloroplasts, four constructs were made. Three different 5'-oligonucleotides (PhyThy-5': 5'-AAGAATTCATGCTCCGTGCTACCGTCGCTGCCG-3', PhyThyTP-5': 5'-AAGAATTCATGTTTCAGCCCAGCC-CCCCAGATGGG-3' and PhyThyRR-5': AAGAATTCATGTCTGTCTCCCGCCGTGAGATCGTCC) of which the latter two introduced new translation starts in order to delete the signal peptide in the second and the whole bipartite topogenic signal in the third construct, were used together with one 3' oligonucleotide (PhyThy-3': TTCTAGATTACTTGCTGTATCCGACGTCGCGG) for PCR amplification using total DNA of *G. theta* as template. Amplification products were purified from preparative 1% agarose gels, digested with EcoRI/XbaI and cloned into the pBAT plasmid (13) that was digested the same way.

Import Analysis—Isolation of chloroplasts and thylakoids from pea and subsequent protein transport experiments with radiolabeled precursor proteins in the presence and absence of competitor proteins followed the protocols described in Marques *et al.* (14) and Hou *et al.* (15).

RESULTS

Cloning of Nuclear-encoded Plastid Proteins of *G. theta*—Our EST data base from *G. theta* was the source for our analyses, in which we identified ESTs encoding proteins belonging to the two distinct light-harvesting machineries present in cryptophytes. We detected 139 ESTs assembling into 17 independent contigs encoding LHCP proteins, which are found also in land plant chloroplasts. Another 206 ESTs assembled into 14 contigs encoding PE α subunits, the second system capable of transferring excitation energy to the photosystems chlorophyll. This is peculiar, because in red algae phycobiliproteins are encoded by a very small gene family (16). Alignment of ESTs encoding PE α sequences identified two groups with respect to the predicted targeting signals, because only nine of the entries encode for preproteins that are specified by an additional twin arginine motif (Fig. 1) that is indicative for transport by the ΔpH -dependent Tat pathway across the thylakoid membrane (17). In a recent publication on the cryptophyte *Rhodomonas* CS24, a

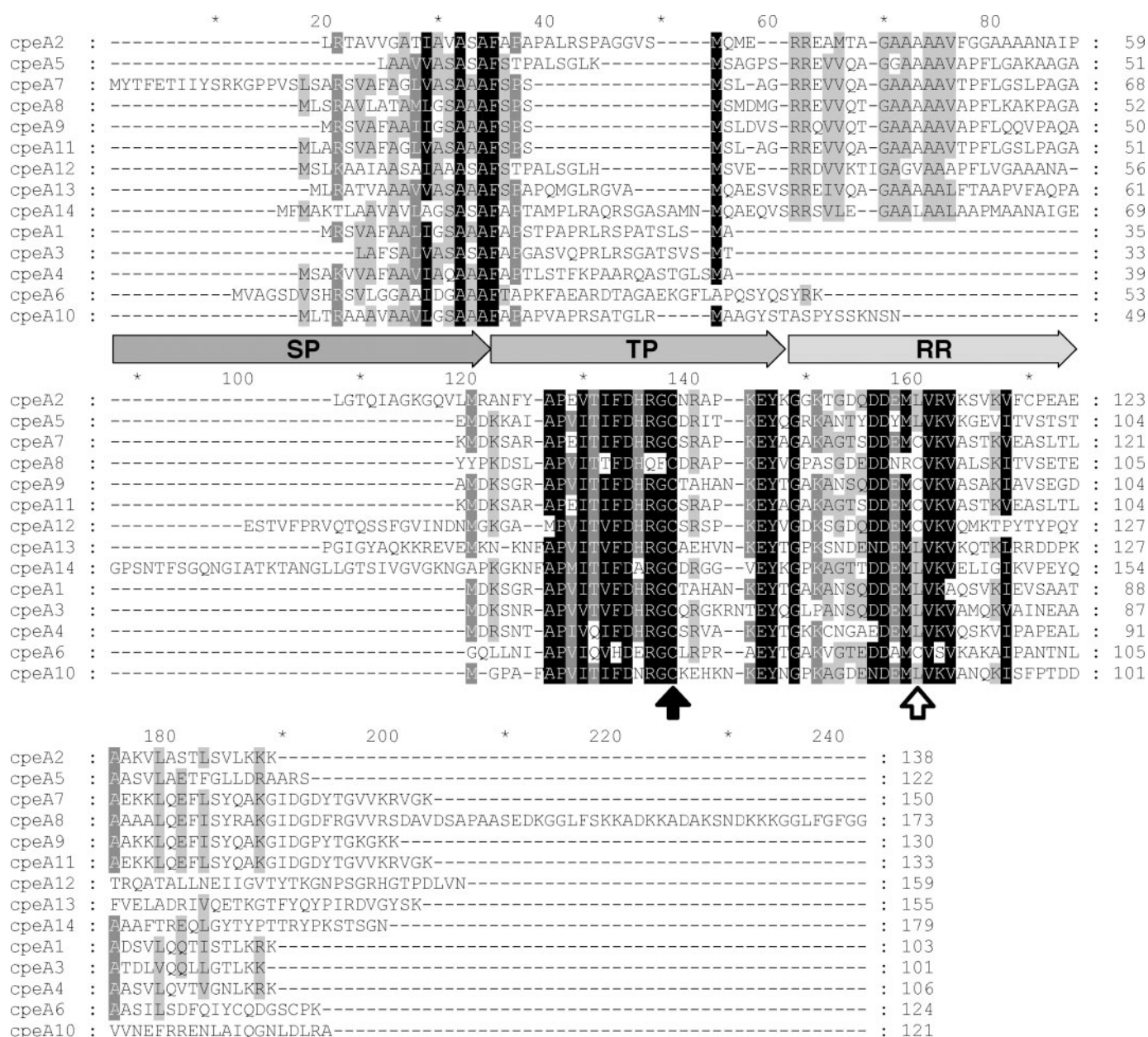


FIGURE 1. Alignment of phycoerythrin- α subunits (PE α) of *G. theta*. In the first upper block, the entire topogenic signals are displayed. The conserved AxAF cleavage site separates the ER-targeting signal peptides (SP) and the chloroplast-targeting transit peptides (TP). In the top nine sequences an additional Tat topogenic signal (RR) is present, which is missing in the last five subunits. All subunits contain at least one conserved DXXXC-motif for the attachment of the chromophore group to the cysteine residue (solid black arrow). Some, no matter whether with or without RR, have a second DXXXC-motif further downstream (open black arrow). Aligned amino acids highlighted in black represented a conservation of at least 90%, gray 80%, and light gray 60%. Note that cpeA2, 3 and 5 do not represent full-length ESTs.

similar finding was reported (18): six PE α -encoding genes were described of which three apparently show a similar tripartite topogenic signal arrangement as the nine PE α proteins identified of *G. theta* described above. A clear sorting into PE α_1 and PE α_2 subunits, as shown for *Rhodomonas* CS24, was not possible for the PE α sequences from *G. theta*, because the protein sequences are too divergent (Fig. 1). However, taking the twin arginine motif as the discriminating signal, the nine ESTs with tripartite signals may be homologous to the PE α_1 of *Rhodomonas* CS24, whereas the five entries with a bipartite signal may have a similar history (see below) as PE α_2 from *Rhodomonas* CS24.

To examine the localization of PE α within plastids in detail, we have performed *in vivo* and *in vitro* import experiments using a diatom transformation system and intact chloroplasts and thylakoid vesicles isolated from pea, respectively. Because a transformation system for cryptophytes is lacking, we chose an existing protocol for the transformation of a phylogenetic sister group, the diatom *P. tricornutum*. It has been shown to serve as an excellent substitution when analyzing the topogenic signals of cryptophytes (10, 19).

For the analysis, various derivatives of one arbitrarily chosen member of the PE α cDNAs (see Fig. 1, cpeA13) were generated by *in vitro* mutagenesis and cloned into suitable vectors. Two

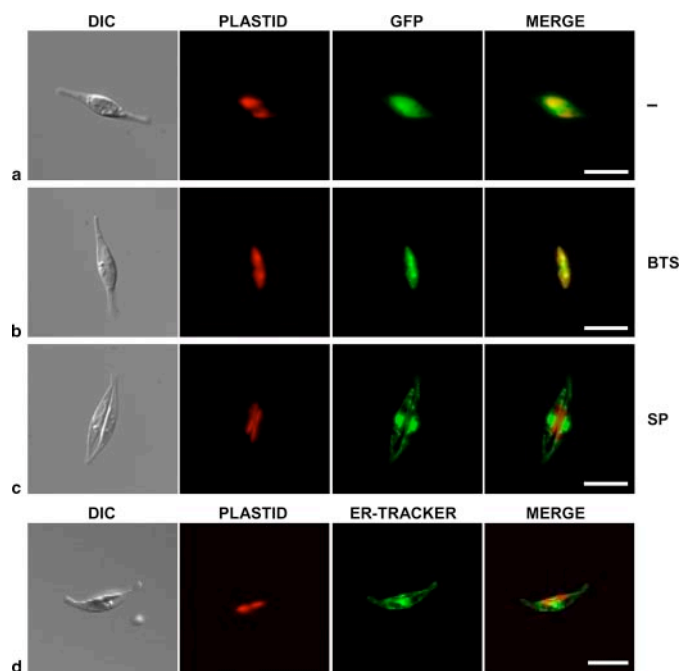


FIGURE 2. *In vivo* localization of GFP fusion proteins in the diatom *P. tricornutum*. In *P. tricornutum* GFP expressed without a topogenic signal accumulates within the cytosol marked by a diffuse distribution all over the cell (panel a). The bipartite topogenic leader of PE α , which consists of a signal peptide followed by a transit peptide, directs GFP to the plastid stroma of *P. tricornutum* as it can be seen in the merge of part b, where as the signal peptide of PE α alone targets GFP to the ER of the cell (panel c). ER localization of the GFP fusion construct was confirmed by using ER-Tracker Green[®] on wild-type cells (panel d). ER staining is observed as a network distributed all over the cell with the nucleus envelope prominently marked. Plastid autofluorescence is shown in red, GFP fluorescence and ER-Tracker in green. Scale bar represents 10 μ m.

constructs were generated for the *in vivo* localization of GFP fusion proteins in the diatom. In the first one, only the sequence encoding the first 16 amino acids, representing the signal peptide (SP-GFP) was fused to the GFP gene, whereas in the second construct the first 29 amino acids preceding the RR motif, representing the BTS (BTS-GFP), were used. For the *in vitro* analyses, three clones were generated, which encoded either (i) the full-length PE α (PE α C) predicted to harbor a tripartite topogenic signal for transport across all five membranes, (ii) a deletion derivative (PE α TP) lacking the ER-targeting signal peptide at the N terminus, or (iii) a deletion derivative (PE α RR) lacking both the N-terminal ER-targeting signal and the transit peptide. In this case, only the putative Tat-specific thylakoid transport signal is present at the N terminus of the mature PE α protein. All three proteins were generated in radiolabeled form by *in vitro* transcription and subsequent *in vitro* translation in reticulocyte lysate in the presence of [³⁵S]methionine.

In Vivo* Localization in the Diatom *P. tricornutum—Analyzing the targeting properties of the first two of the three N-terminal topogenic signals of PE α revealed the same results as with similar constructs from recent reports (10, 19, 20). The signal peptide alone targets GFP to the ER, which is distributed throughout the diatom cell (Fig. 2, panel c), as confirmed by the staining of the cells with ER-Tracker Green[®] (Fig. 2, panel d). This is in line with earlier results obtained from targeting GFP to the diatom ER with different signal peptides (10, 19, 20). The BTS-GFP construct with the additional 13 amino acids

FSPAPQMGLRGVA reassembling the transit peptide, then leads to import of GFP into the plastid, *i.e.* across four membranes, as shown by the colocalization with the chlorophyll autofluorescence (Fig. 2, lane b).

Import of PE α into Intact Chloroplasts Isolated from *Pea*—Incubation of the three PE α precursor proteins with intact pea chloroplasts under import conditions showed that both, PE α C and PE α TP, were efficiently imported into the plastids and processed by organellar peptidases to a protein of \sim 12 kDa (Fig. 3A), which corresponds well to the size expected for mature PE α . These putative mature PE α proteins are protected by the plastid membranes against externally added protease, which proves that both proteins were internalized into the organelles. Thus, irrespective of the presence or absence of an additional ER-targeting signal peptide at the N terminus, the chloroplast transit peptide of PE α from *G. theta* is capable of mediating transport of the protein into chloroplasts from higher plants. This strongly suggests that the transport information of chloroplast transit peptides was conserved during secondary endosymbiosis, demonstrated also for other chromalveolates (11, 21, 22). In contrast, PE α RR, which lacks both the ER-targeting signal peptide and the chloroplast-targeting transit peptide, was apparently not imported since no processing product corresponding in size to the mature polypeptide could be detected (Fig. 3A). This is in line with the assumption that the putative thylakoid-targeting transport signal of PE α is not sufficient for organelle import. However, it should be noted that this truncated precursor protein showed significant association with the isolated chloroplasts and was even protected to some extent against proteolysis, although proteolysis of the translation product in the absence of organelles led to complete degradation under these conditions (Fig. 3B). It remains unclear, whether this association is caused by binding of the protein to the import machinery of the chloroplast envelope or whether it is the result of unspecific interactions between the targeting signal and the lipid bilayer.

Localization of PE α within Chloroplasts—Fractionation of the chloroplasts after import of PE α C and PE α TP showed that in both instances the presumed mature protein of \sim 12 kDa is found exclusively in the thylakoid fraction, where it is resistant to protease added from the stromal side to the vesicles (Fig. 3A). The same import behavior is observed for authentic precursors of thylakoid lumen proteins of higher plants, like the 23-kDa subunit of the oxygen evolving complex associated with photosystem II (OEC 23), which was analyzed in parallel (Fig. 3C).

This clearly demonstrates that PE α was translocated across the membrane into the thylakoid lumen, which is in line with the recent *in silico* analysis of Broughton *et al.* (18) who described indications for a localization of PE α in the thylakoid lumen of *Rhodomonas* CS24. In addition to the mature proteins in the thylakoid lumen, polypeptides of \sim 13 kDa are found in the stromal fractions of the import assays analyzing PE α C and PE α TP (Fig. 3A). They might represent stromal intermediates in which the chloroplast-targeting transit peptides have been removed, while the thylakoid targeting transport signals are still present. However, the size difference of only \sim 1 kDa to the mature protein argues against such a transport intermediate, because the thylakoid targeting Tat-signal comprises at least 24

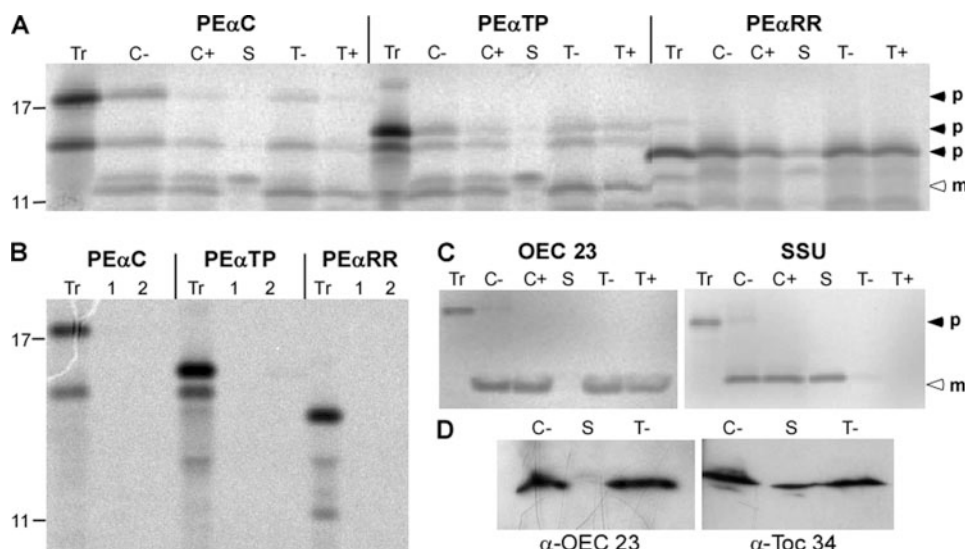


FIGURE 3. Import of phycocerythrin derivatives into isolated intact pea chloroplasts. *A*, full size phycocerythrin precursor protein (PEαC) as well as its deletion derivatives PEαTP and PEαRR were obtained by *in vitro* transcription/translation in the presence of [³⁵S]methionine and incubated with isolated pea chloroplasts for 20 min at 25 °C in the light. After the import reaction, the chloroplasts were either treated with thermolysin (150 μg/ml) for 30 min on ice (*lanes C+*) or mock-treated (*lanes C-*), and then re-isolated by centrifugation through a Percoll cushion. Aliquots of the protease-treated chloroplasts were additionally fractionated into stroma (*lanes S*) and thylakoids. The thylakoid fractions were treated with either thermolysin (200 μg/ml, 30 min on ice, *lanes T+*), or mock-treated (*lanes T-*). Stoichiometric amounts of each chloroplast fraction, corresponding to 12.5 μg of chlorophyll (and 3 μl of *in vitro* translation product added), were separated on 10–17.5% SDS-polyacrylamide gradient gels and visualized by phosphorimaging. In *lanes Tr*, 1 μl of the respective *in vitro* translation products were loaded. Positions of the precursor (*p*) and mature proteins (*m*) are indicated by closed and open arrowheads, respectively. *B*, control of protease sensitivity of PEαC, PEαTP, and PEαRR. The respective *in vitro* translation products were subjected to import buffer lacking chloroplasts and treated with thermolysin (150 μg/ml, 30 min on ice, *lanes 1*, or 200 μg/ml, 30 min on ice, *lanes 2*). *C*, control import of authentic chloroplast proteins from higher plants. Precursor proteins of the 23-kDa subunit of the oxygen evolving complex associated with photosystem II (OEC 23, *left panel*) and the small subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (SSU, *right panel*) were obtained by *in vitro* transcription/translation and analyzed as described in *A*. *D*, Western analysis of chloroplast fractions. Stoichiometric amounts, corresponding to 12.5 μg chlorophyll, of mock-treated chloroplasts (*C-*), stroma (*S*), and mock-treated thylakoids (*T-*) were separated on 10–17.5% SDS-polyacrylamide gradient gels and subjected to Western analysis using polyclonal antisera raised against the 23-kDa subunit of the oxygen evolving complex associated with photosystem II (α-OEC23, *left panel*) or the 34 kDa receptor component of the protein translocase of the outer envelope membrane of chloroplasts (α-Toc 34, *right panel*).

residues (Fig. 1). Furthermore, because a polypeptide of similar size is sometimes found also in the stromal fraction of the import assay analyzing PEαRR (Fig. 3A), which is not imported into chloroplasts, they might instead represent distinct degradation products that artificially appear only in the presence of organelles (Fig. 3B). Anyhow, they are not the result of partial insertion of the proteins into the chloroplast envelope membranes, because envelope proteins are found in both, the stroma and the thylakoid fraction, upon lysis of the organelles (Fig. 3D).

To examine thylakoid transport of PEα in an independent approach, *in vitro* transport experiments were additionally performed with isolated thylakoid vesicles from pea. In these experiments, all three derivatives of PEα were successfully imported into the thylakoid vesicles where they were processed to the presumed mature polypeptide of ~12 kDa (Fig. 4A). These processing products were resistant against externally added protease proving that they had been fully translocated across the thylakoid membrane into the lumen. Thus, neither the bipartite topogenic signal of the full-length precursor protein nor the residual chloroplast targeting transit peptide present at the N terminus of the PEαTP derivative seem to impair the transport of the protein across the thylakoid membrane.

This appears surprising at first glance but similar observations have been described already earlier (23). It is further support for the recently proposed model that after direct loop insertion of the Tat substrate into the lipid bilayer with both, the N and C terminus, exposed to the stroma, the C-terminally located passenger protein is translocated across the thylakoid membrane, while the N terminus of the precursor protein remains on the stromal side of the membrane (15) until the thylakoid targeting transport signal is proteolytically removed (24). It should be noted though, that it cannot formally be ruled out that the substrates that were actually recognized by the thylakoidal protein transport machinery in the assays analyzing PEαC and PEαTP were not the respective precursor proteins but instead N-terminally truncated polypeptides that are the result of translation initiation at position 23 of the amino acid sequence of the PEα precursor. A second methionine residue is present at this position (Fig. 1, *cpeA13*) that could give rise to a polypeptide with a calculated mass of 14.8 kDa, which corresponds well to the translation products of ~15 kDa observed in the translation assays of PEαC and PEαTP (Figs. 3 and 4). In the translation assay of the PEαRR derivative no such product is found, in line with the fact that the corresponding methionine codon is not present in the respective clone (data not shown).

Irrespective of the actual size of the N-terminal extension in the various PEα derivatives, thylakoid transport was presumably mediated in all instances by the RR signal peptide. In order to examine if this thylakoid transport signal is indeed a Tat-specific transport signal, as suggested by the twin arginine motif, thylakoid transport experiments were performed in the presence of competitive amounts of the OEC 23 precursor protein. This protein is exclusively transported by the ΔpH-dependent Tat pathway across the thylakoid membrane (25, 26) and it was frequently used in such competition experiments (15, 27–29). Raising the concentration of competitor in the assays gradually decreases the thylakoid transport of each of the three PEα derivatives (Fig. 4B), which unequivocally proves that they are translocated by the Tat pathway across the thylakoid membrane.

DISCUSSION

Red algae, glaucophytes, and cyanobacteria use phycobiosomes as a light-harvesting complex. These are composed of

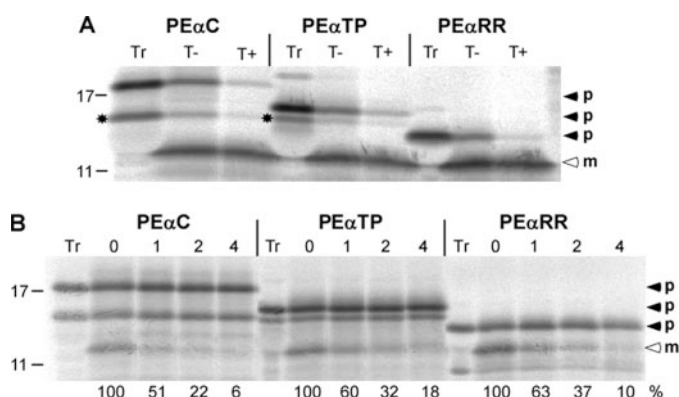


FIGURE 4. Import of phycocerythrin derivatives into thylakoid vesicles isolated from pea chloroplasts. A, isolated thylakoids were incubated with radiolabeled precursor proteins for 15 min at 25 °C in the light. After the import reaction, thylakoids were treated with either thermolysin (200 μg/ml, 30 min on ice, lanes T+), or mock-treated (lanes T-). In lanes Tr, 1 μl of the respective *in vitro* translation assays were loaded. The asterisks indicate N-terminally truncated translation products, which presumably derive from translation initiation at an internal start codon (see also text). B, saturation of the Tat-dependent pathway inhibits transport of PE α across the thylakoid membrane. Thylakoid transport experiments were performed in the presence and absence of increasing amounts of precursor of the 23-kDa subunit of the oxygen-evolving system that were obtained by overexpression in *E. coli*. The concentration of competitor protein (in μM) present in each assay is indicated above the lanes. After the import reaction, the assays were loaded without further treatment to 10–17.5% SDS-polyacrylamide gradient gels. Mature PE α accumulating in the thylakoid lumen was quantified for each protein, and the relative amounts (in terms of percentage of mature PE α accumulating in the absence of competitor protein) are given below the lanes. For further details, see the legend to Fig. 3.

different phycobiliproteins and linker proteins, which together assemble into a higher order structure (9). Phycobilisomes are attached to the stromal side of the thylakoid membrane in red algae and the cytoplasmic side in cyanobacteria. The Chromalveolates, a unifying kingdom suggested by Cavalier-Smith (2), include as the phototrophic members cryptophytes, heterokontophytes, haptophytes, and some dinoflagellates. They originated by the engulfment and intracellular reduction of a red algal-like cell within a heterotrophic eukaryote (3). Interestingly, the phycobilisomes and associated proteins, which were introduced with the red algal endosymbiont, were lost without traces in heterokontophytes, haptophytes and dinoflagellates. In cryptophytes though, phycobiliproteins such as phycocerythrin or phycocyanin are still present. However, unlike cyanobacteria and red algae, the phycobiliproteins are located in the thylakoid lumen and are not assembled into phycobilisomes (6, 30). As phycobiliproteins are restricted to the thylakoid lumen in cryptophytes, at least some of the phycobiliproteins had to develop new targeting information mediating their transport across the intraorganellar membranes. The experimental aim of our study presented here was to elucidate *in silico*, *in vitro* and *in vivo* the pathway(s) and components of thylakoid import in cryptophytes.

Translocation and Assembly—The *in vivo* localization analysis with GFP fusion proteins show that the signal peptide of the tripartite leader of a PE α family member is responsible for the first step in the transport chain, notably the targeting to the ER (Fig. 2, panel c). The following transit peptide guides the precursor across the next three membranes into the plastid stroma (Fig. 2, panel b). Performing import experiments with isolated

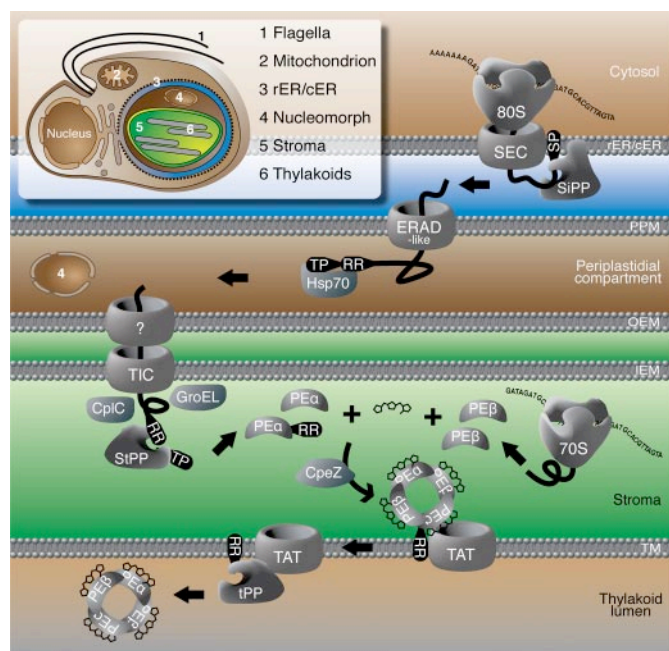


FIGURE 5. Current working model of phycocerythrin transport, processing and assembly in cryptophytes. Nuclear-encoded phycocerythrin α subunit (PE α) crosses the first of the five membranes in a cotranslational manner via the Sec-complex. The rough ER (rER) surrounding the endosymbiont is also known as the chloroplast ER (cER, in blue). After removal of the signal peptide (SP) by the signal peptide peptidase (SiPP), the second topogenic signal, the transit peptide (TP), mediates the transport of the protein across the following three membranes. In the periplastidial membrane (PPM), the former red algal cytoplasmic membrane, a recently described ER-associated degradation-like machinery (ERAD-like) might serve as the translocase. While for the outer plastid envelope membrane (OEM) no translocase has yet been identified in cryptophytes, transport across the inner envelope membrane (IEM) of the plastid probably involves those TIC components, for which genes have been identified in both the nucleus and the nucleomorph. Within the stroma, assembly of the nuclear-encoded PE α , the plastid encoded PE β and the chromophores is assumed to take place, involving the CpeZ lyase. Finally, the entire PE-heterotetramer is translocated by the Tat translocase across the thylakoid membrane (TM), making use of the tat topogenic signal of one single PE α subunit, which is cleaved after translocation by a thylakoid processing peptidase (tPP) releasing the mature complex into the thylakoid lumen.

chloroplasts and thylakoids from pea we could show that the thylakoid translocation of PE α takes place by the Δ pH-dependent Tat pathway and is mediated by the third part of the leader containing the RR motif (Figs. 3 and 4). The latter result strongly suggests that a protein transport pathway corresponding to the Tat pathway of higher plant chloroplasts exists also in cryptophyte plastids.

Thus, together with our recently published findings (10, 19), the here presented results imply that in cryptophytes, PE α crosses five membranes by courtesy of three topogenic signals and is processed three times (Fig. 5). The N-terminal ER targeting signal and the Sec61 complex catalyze guided import into the lumen between the two outermost membranes, in which the preprotein is processed leading to the exposition of the transit peptide (10, 11). The next step, the passage across the second outermost membrane, is most likely dependent on the transit peptide and an ERAD-like mechanism as proposed recently (31). By entering the periplastidial compartment, the next barrier, being the third membrane, has to be crossed by a still unknown mechanism, whereas for the innermost membrane Tic-like components were identified. Within

the stroma, the second processing step leads to the removal of the transit peptide and exposure of the topogenic signal carrying the twin arginine motif. This signal mediates Tat-dependent transport across the thylakoid membrane. In its final destination, the protein is then processed to its mature form.

Recently, Broughton *et al.* (18) presented their analyses of light-harvesting proteins in the cryptophyte *Rhodomonas* CS24. In line with our results, they report a gene family for phycoerythrin and describe that some but not all members of this family encode proteins harboring tripartite signal sequences including putative Tat signal peptides. Our analyses in which we show for the first time thylakoid import of PE encoded with a tripartite signal sequence support the suggestion of Broughton *et al.* (18) that those proteins lacking signal peptides might enter the thylakoids in a piggyback manner similar to that described already for the bacterial Tat substrate hydrogenase. Additionally, the results obtained by us explain earlier findings of Mörschel and Wehrmeyer (33) who noticed a ratio of 1:1 between PE α and PE β and that the heterotetramer complex PE(α)₂(β)₂ was always found to migrate at around 44.5 kDa. However, the identification of three different isoelectric points (pI) for the isolated PE α -subunits is inconsistent. In the light of the detection of a gene family encoding PE α -subunits, the observation from Mörschel and Wehrmeyer (33) indicates that different PE α are assembled to different complexes, but always with an observed mass of around 44.5 kDa.

The Tat machinery is capable of transporting folded proteins of different molecular weight and, thus, also different size (17, 34–36). It was suggested that the translocation pore can adapt to the size of the substrate that has to be translocated (37), presumably by oligomerization of TatA subunits (38). The diameter of a fully assembled phycobilisome within a rod of cyanobacteria (six subunits, hence two more than observed in cryptophytes) is about 120 Å (9). This is the diameter of the ring-like complex. Because of the flexibility of the translocation pore and the non-static form of the smaller PE(α)₂(β)₂ heterotetramer, only the Tat might mechanistically be able to translocate the whole complex.

Thus, the model suggests that the assembly of phycobiliproteins to a higher order structure occurs in the same cellular localization as in cyanobacteria and red algae, namely in the plasmatic/stromal compartment. Further support for this assumption came from a putative phycolyase with a HEAT domain and homology to the PBS lyase of *Crocospira watsonii* WH 8501, which was detected in our EST data base of *G. theta*. In this case, the protein is encoded as a precursor with a typical stroma-targeting transit peptide (data not shown). This suggests that in cryptophytes the attachment of the chromophore to the phycoerythrin apoprotein takes place in the stroma like in red algae (9).

Because of the properties of the Tat pathway regarding transport, even complexes in which only one partner carries a Tat signal peptide are translocated (32). Hence, the bipartite topogenic signal harboring PE α and topogenic signal lacking PE β subunits might be imported by the use of the Tat signals of the PE group encoded as tripartite preproteins. Thus, all of the components of the initial progenitor of cryptophytes are reused and only a tripartite signal on one subunit was needed for thy-

lakoid lumen targeting of phycoerythrin complexes. This system furthermore allows the assembly of the heterotetramers inside the stroma, making it gratuitous for assembly factors to be translocated into the thylakoid lumen.

These results and the deduced import model (Fig. 5) furthermore raise the question of how, and in what order, the cell manages to assemble the complexes such that all have their chromophore groups attached and contain one PE α with a Tat signal for the translocation of the whole heterotetramer. At first and most important, the cell must be able to recognize single PE α components having a Tat signal and which have not yet been assembled to a heterotetramer. One must propose a way the Tat signal, within the cryptophyte stroma, is masked until the whole heterotetramer is fully assembled and ready for translocation. If the cells were lacking a coordinated assembly one would need to conclude that, firstly, complexes lacking the necessary Tat signal are degraded inside the stroma and, secondly, that single PE α subunits are imported into the thylakoid lumen at the same time. The latter, at least, seems unlikely as it would contradict *e.g.* results of Mörschel and Wehrmeyer (33).

Why Secondary Endosymbiosis Relaxes the Functional Constraints in the Need for a New Targeting Mechanism of Phycoerythrin in Cryptophytes—But why, in cryptophytes, are the phycobiliprotein heterotetramers targeted to the thylakoid lumen at all, and why are there so many nuclear gene copies of PE α -subunits present in cryptophytes? One early model, suggesting that cryptophyte thylakoids are maybe inside out, proved to be wrong as freeze-fracture and freeze-etch analyses have shown that the thylakoid architecture is the same as in land plants (39, 40). Ludwig and Gibbs (6) thought the unusual localization of PE α can possibly be explained by the loss of phycobiliprotein linker-polypeptides after secondary endosymbiosis, hence leading to the loss of the ability to generate phycobilisomes in parallel. If so, the PE(α)₂(β)₂ heterotetramers then needed to be targeted to the thylakoid lumen in order to more efficiently transfer excitation energy to the photosystem, where they are captured and not freely floating in the stroma. In any case, this model cannot yet fully explain the step-by-step evolution, which is necessary to create Tat signal harboring PEs and loss of the linker proteins.

If one tries to reconstruct the evolution of the progenitor of cryptophytes (or chromalveolates) one has to predict that, initially, the secondary endosymbiont had phycobilisomes similar to those in red algae. The initial genetic compartmentalization of PE is still seen in modern cryptophytes, which synthesize the β subunit in the plastid and the α subunits in a eukaryotic genome. Thus, the α subunit genes, introduced into the secondary cellular merger, were transferred from the endosymbiont nucleus into that of the host. There are good reasons to believe that several copies originated in the cell nucleus, either by multiple transfers or by gene duplications in the cell nucleus of the host. All these genes, which already possessed a transit peptide, had to acquire an ER targeting sequence, thus composing a gene encoding preproteins with a bipartite signal sequence. In our model not only preproteins with a bipartite signal sequence originated, but also genes encoding beside the mature protein a tripartite signal sequence with a Tat signal. Several scenarios could explain the origin of such a protein with

a tripartite signal sequence. Either it has already originated in the ancestor of the red algal endosymbiont and was successfully transferred into the cell nucleus, or the C-terminal amino acids of the transit peptide or N-terminal ones of the mature protein were converted into an efficient Tat signal. Another possibility may be the tripartite signal originated *de novo* in the cell nucleus. In any case, co-existence of proteins with and without a Tat signal could lead to phycobilisomes in the stroma and phycoerythrin complexes within the thylakoids. If so, a window of opportunity to evolve a thylakoid luminal phycoerythrin complex was created. The linker proteins were then lost in the absence of selection pressure once light harvesting was occurring inside the thylakoid lumen.

Light Harvesting and Energy Transfer within the Thylakoid Lumen—In addition to the evolutionary aspects concerning the development of tripartite targeting signals, the localization of the components of the light harvesting apparatus within the thylakoid lumen rather than on the stromal side of the membrane has also significant physiological consequences. In the ordered antenna-like structure of phycobilisomes, the absorbed light energy is transferred to the chlorophyll in a highly controlled manner. For the situation found in cryptophytes this is difficult to imagine, as one must assume that the heterotetramers can freely diffuse within the thylakoid lumen. However, an attachment of the phycobiliprotein complexes to the luminal face of the thylakoid membrane cannot be ruled out.

Furthermore, the thylakoid lumen is a highly variable environment depending on the light conditions. Whereas the chloroplast stroma shows a relatively stable pH value of ~7.5–8 over a wide range of light conditions, the pH value of the thylakoid lumen is strongly dependent on the light intensity the chloroplast is exposed to. At low light, the lumen is only mildly acidic, whereas at high light intensities the pH is assumed to reach values of ~4 to 5. It is obvious to assume that such variability in proton concentration by several orders of magnitude in the environment of the light harvesting apparatus has an impact on the properties of the chromophores to capture the light energy and to transfer it to the photosystems. So far, it is entirely unsolved how this is achieved, but it is tempting to speculate that such variation in acidity might even be used for regulation of light harvesting efficiency.

Acknowledgment—We thank Geoff I. McFadden for critically reading this manuscript.

REFERENCES

- Bhattacharya, D., Yoon, H. S., and Hackett, J. D. (2004) *Bioessays* **26**, 50–60
- Cavalier-Smith, T. (1999) *J. Eukaryot. Microbiol.* **46**, 347–366
- Hjorth, E., Hadfi, K., Gould, S. B., Kawach, O., Sommer, M. S., Zauner, S., and Maier, U. G. (2005) *Endocytobios. Cell Res.* **15**, 459–468
- Wehrmeyer, W. (1970) *Arch. Mikrobiol.* **71**, 367–383
- Dodge, J. D. (1969) *Arch. Mikrobiol.* **69**, 266–280
- Ludwig, M., and Gibbs, S. P. (1989) *J. Cell Biol.* **108**, 875–884
- Douglas, S. E., and Penny, S. L. (1999) *J. Mol. Evol.* **48**, 236–244
- Reith, M., and Douglas, S. (1990) *Plant Mol. Biol.* **15**, 585–592
- MacColl, R., and Guard-Friar, D. (1987) *Phycobiliproteins*, CRC Press, Boca Raton
- Gould, S. B., Sommer, M. S., Hadfi, K., Zauner, S., Kroth, P. G., and Maier, U. G. (2006) *J. Mol. Evol.* **62**, 674–681
- Wastl, J., and Maier, U. G. (2000) *J. Biol. Chem.* **275**, 23194–23198
- Apt, K. E., Kroth-Pancic, P. G., and Grossman, A. R. (1996) *Mol. Gen. Genet.* **252**, 572–579
- Annweiler, A., Hipskind, R. A., and Wirth, T. (1991) *Nucleic Acids Res.* **19**, 3750
- Marques, J. P., Dudeck, I., and Klösgen, R. B. (2003) *Mol. Genet. Genomics* **269**, 381–387
- Hou, B., Frielingsdorf, S., and Klösgen, R. B. (2006) *J. Mol. Biol.* **355**, 957–967
- Matsuzaki, M., Misumi, O., Shin, I. T., Maruyama, S., Takahara, M., Miyagishima, S. Y., Mori, T., Nishida, K., Yagisawa, F., Nishida, K., Yoshida, Y., Nishimura, Y., Nakao, S., Kobayashi, T., Momoyama, Y., Higashiyama, T., Minoda, A., Sano, M., Nomoto, H., Oishi, K., Hayashi, H., Ohta, F., Nishizaka, S., Haga, S., Miura, S., Morishita, T., Kabeya, Y., Terasawa, K., Suzuki, Y., Ishii, Y., Asakawa, S., Takano, H., Ohta, N., Kuroiwa, H., Tanaka, K., Shimizu, N., Sugano, S., Sato, N., Nozaki, H., Ogasawara, N., Kohara, Y., and Kuroiwa, T. (2004) *Nature* **428**, 653–657
- Müller, M., and Klösgen, R. B. (2005) *Mol. Membr. Biol.* **22**, 113–121
- Broughton, M. J., Howe, C. J., and Hiller, R. G. (2006) *Gene (Amst.)* **369**, 72–79
- Gould, S. B., Sommer, M. S., Kroth, P. G., Gile, G. H., Keeling, P. J., and Maier, U. G. (2006) *Mol. Biol. Evol.* **23**, 2413–2422
- Kilian, O., and Kroth, P. G. (2005) *Plant J.* **41**, 175–183
- DeRocher, A., Hagen, C. B., Froehlich, J. E., Feagin, J. E., and Parsons, M. (2000) *J. Cell Sci.* **113**, 3969–3977
- Nassoury, N., Cappadocia, M., and Morse, D. (2003) *J. Cell Sci.* **116**, 2867–2874
- Fincher, V., McCaffery, M., and Cline, K. (1998) *FEBS Lett.* **423**, 66–70
- Frielingsdorf, S., and Klösgen, R. B. (2007) *J. Biol. Chem.* **282**, 24455–24462
- Cline, K., Ettinger, W. F., and Theg, S. M. (1992) *J. Biol. Chem.* **267**, 2688–2696
- Mould, R. M., Shackleton, J. B., and Robinson, C. (1991) *J. Biol. Chem.* **266**, 17286–17289
- Berghöfer, J., and Klösgen, R. B. (1999) *FEBS Lett.* **460**, 328–332
- Molik, S., Karnauchov, I., Weidlich, C., Herrmann, R. G., and Klösgen, R. B. (2001) *J. Biol. Chem.* **276**, 42761–42766
- Cline, K., Henry, R., Li, C., and Yuan, J. (1993) *EMBO J.* **12**, 4105–4114
- Gantt, E., Edwards, M. R., and Provasoli, L. (1971) *J. Cell Biol.* **48**, 280–290
- Sommer, M. S., Gould, S. B., Lehmann, P., Gruber, A., Przyborski, J. M., and Maier, U. G. (2007) *Mol. Biol. Evol.* **24**, 918–928
- Rodrigue, A., Chanal, A., Beck, K., Muller, M., and Wu, L. F. (1999) *J. Biol. Chem.* **274**, 13223–13228
- Mörschel, E., and Wehrmeyer, W. (1977) *Arch. Microbiol.* **113**, 83–89
- Marques, J. P., Schattat, M. H., Hause, G., Dudeck, I., and Klösgen, R. B. (2004) *J. Exp. Bot.* **55**, 1697–1706
- Clark, S. A., and Theg, S. M. (1997) *Mol. Biol. Cell* **8**, 923–934
- Hynds, P. J., Robinson, D., and Robinson, C. (1998) *J. Biol. Chem.* **273**, 34868–34874
- Gohlke, U., Pullan, L., McDevitt, C. A., Porcelli, I., de Leeuw, E., Palmer, T., Saibil, H. R., and Berks, B. C. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 10482–10486
- Dabney-Smith, C., Mori, H., and Cline, K. (2006) *J. Biol. Chem.* **281**, 5476–5483
- Spear-Bernstein, L., and Miller, K. R. (1985) *Protoplasma* **129**, 1–9
- Dwarte, D., and Vesik, M. (1983) *Protoplasma* **117**, 130–141