A Pentapeptide Motif Related to a Pigment Binding Site in the Major Light-Harvesting Protein of Photosystem II, LHCII, Governs Substrate-Dependent Plastid Import of NADPH:Protochlorophyllide Oxidoreductase A$^{1,2}$

Christiane Reinbothe*, Stephan Pollmann, Phetaphine Phetsarath-Faure, Françoise Quigley, Peter Weisbeek, and Steffen Reinbothe

Universität Bayreuth, Lehrstuhl für Pflanzenphysiologie, D–95447 Bayreuth, Germany (C.R.); Ruhr-Universität Bochum, Lehrstuhl für Pflanzenphysiologie, D–44801 Bochum, Germany (S.P., S.R.); Université Joseph Fourier et Centre National de la Recherche Scientifique, F–38041 Grenoble cedex 9, France (P.P.-F., F.Q., S.R.); and Department of Molecular Cell Biology, Utrecht University, 3584 Utrecht, The Netherlands (P.W.)

NADPH:protochlorophyllide (Pchlide) oxidoreductase (POR) A is the only known example thus far of a nucleus-encoded plastid protein that is imported to its final destination in a substrate-dependent, Pchlide-regulated manner. Previous work has shown that the cytosolic PORA precursor (pPORA) does not utilize the general import site but uses a distinct translocon designated the Pchlide-dependent translocon complex. Here we demonstrate that a pentapeptide motif, threonine-threonine-serine-proline-glycine (TTSPG) in pPORA's transit peptide (transA), is involved in Pchlide-dependent transport. Deletion of this motif from the COOH-terminal end of transA abolished both Pchlide binding and protein import. Incorporation of the TTSPG motif into normally non-Pchlide-responsive transit sequences conferred the pigment binding properties onto the engineered chimeric precursors but was insufficient to render protein import substrate dependent. An additional motif was identified in the NH2-terminal part of transA that was needed for binding of the precursor to the Pchlide-dependent translocon complex. Point mutations of the TTSPG motif identified glycine as the Pchlide binding site. By analogy to the major light-harvesting chlorophyll a/b binding protein of photosystem II, we propose that the peptidyl carbonyl oxygen of glycine may bind directly or via a water molecule to the central Mg atom of the pigment.

Cells contain distinct subcellular compartments, such as the nucleus, the endoplasmic reticulum (ER), the Golgi apparatus, peroxisomes, mitochondria, and, in plants, chloroplasts. Each of these compartments has a well-defined morphology and is surrounded by one or more limiting membranes. The various functions of the different compartments depend on an array of different proteins. Because the majority of the organellar proteins is encoded in nuclear DNA and synthesized by cytosolic ribosomes, efficient mechanisms have evolved to transport these proteins to their final destinations (Goerlich and Mattaj, 1996; Schatz and Dobberstein, 1996; Wickner and Schekman, 2005; Rapoport, 2007).

Topogenic signals have been identified in nucleus-encoded organelle proteins that dictate their proper compartmentalization (Blobel, 1980; von Heijne, 1990). Proteins destined to chloroplasts contain chloroplast transit peptides that are variable in length and amino acid composition (von Heijne et al., 1991). Chloroplast transit peptides are characterized by an uncharged NH2 terminus and a high content of hydroxylated amino acids (Ser and Thr). Furthermore, unlike typical mitochondrial transit sequences, chloroplast transit peptides are neither strongly basic nor amphiphilic (von Heijne et al., 1991). Chloroplast transit peptides guide the precursor to the protein translocon complex of the outer chloroplast envelope membrane, named TOC (Schnell et al., 1997).

TOC is a trimeric protein complex that mediates the recognition, binding, and translocation of cytosolic precursors across the outer chloroplast envelope membrane (Hirsch et al., 1994; Kessler et al., 1994; Perry and Keegstra, 1994; Schnell et al., 1994; Ma et al., 1996; Hinnah et al., 1997; Kouranov and Schnell, 1997; Bauer et al., 2000). Its constituents are TOC159, which establishes the main presequence receptor, TOC34, as...
regulatory GTPase, and TOC75 that operates as the translocation channel (for review, see Bedard and Jarvis, 2005; Hofmann and Theg, 2005; Kessler and Schnell, 2006). Recent work provided direct evidence for the existence of multiple, regulated protein import complexes in chloroplasts, which differ by an interchange of receptor components (Bauer et al., 2000). In addition to TOC159, three more TOC159-related genes were identified by the Arabidopsis (Arabidopsis thaliana) genome sequencing project (Bauer et al., 2000; for review, see Bauer et al., 2001). Their encoded products display distinctive precursor binding specificities (Ivanova et al., 2004; Kubis et al., 2004; Smith et al., 2004). For example, Smith et al. (2004) showed that TOC159 interacts with transit sequences of photosynthetic proteins but had little activity in binding assays with transit peptide fusions of nonphotosynthetic precursors, such as that for NADPH:protochlorophyllide (Pchlide) oxidoreductase A (pPORA), and dihydrofolate reductase (DHFR) of mouse. The latter finding is consistent with our previous results that pPORA does not bind TOC159 and TOC75 (Reinbothe et al., 2000) but interacts with several distinctive components forming a Pchlide-dependent translocon complex (PTC) during plastid import in vitro (Reinbothe et al., 2004a, 2004b). Among its constituents were an as yet unidentified 130-kD presequence receptor (PTC130), a 16-kD translocation pore (PTC16) related to the outer plastid envelope protein OEP16, and a 33-kD protein (PTC33) with similarities to TOC34 and TOC33 of Arabidopsis and pea (Pisum sativum; Reinbothe et al., 2004a, 2004b). In addition, the PTC complex was shown to contain a 52-kD protein named PTC52 with similarity to chlorophyllide a oxygenase. Both Pchlide b and PTC52 cofractionated with pPORA in membrane junction complexes formed between the outer and inner plastid envelopes during import (Reinbothe et al., 2004a). Time course analyses over import of pPORA provoked the hypothesis that nascent Pchlide b synthesized by virtue of PTC52 may bind to the transit peptide of pPORA, transA, and thereby could provide the trigger for the import step (Reinbothe et al., 2004a). As shown previously, pPORA’s translocation through the plastid envelope into the stroma is substrate dependent (Reinbothe et al., 1995, 1997, 2000), and transA contains a pigment binding site implicated in Pchlide-dependent import (Reinbothe et al., 1997).

In this work, an in vitro mutagenesis approach was taken to dissect the pigment binding site in transA and to study its role in vitro and in planta. We demonstrate that both unique features in transA as well as the PTC complex assure the proper targeting of pPORA to the plastid compartment.

RESULTS

Comparative Analysis of the Transit Peptides of pPORA and pPORB

pPORA and pPORB, the two Pchlide-reducing enzymes of etiolated barley (Hordeum vulgare) plants, are structurally related. Their overall amino acid sequence identity is 74.9% (Holtorf et al., 1995). In their mature parts, beginning at positions 68 (pPORA) and 82 (pPORB), respectively, their amino acid sequence identity is even higher and accounts to 81.5% (Holtorf et al., 1995). pPORA and pPORB are most divergent in their NH2-terminal transit peptides (transA and transB), and two major differences can be discovered (Fig. 1A). First, transA contains a short sequence motif, Thr-Thr-Ser-Pro-Gly (TTSPG), in its COOH-terminal end that is lacking in transB. Second, there are three insertions in the NH2-terminal half that distinguish transB from transA. We hypothesized that either difference could cause the transit peptides to operate in a different fashion, mediating differential receptor binding and substrate-dependent (pPORA) versus constitutive (pPORB) import (Reinbothe et al., 1997, 2004a, 2004b).

Identification of the Pchlide Binding Site in TransA

To dissect the Pchlide-responsive transit peptide of pPORA, a PCR-based in vitro mutagenesis approach was taken. Deletion derivatives were produced, in which transA was stepwise truncated from its COOH-terminal end and translationally fused to the DHFR (Fig. 1B). The reason for these constructions lay in the observation that a fraction of the full-length pPORA synthesized in wheat germ extracts was targeted to Pchlide-free chloroplasts (Aronsson et al., 2000; Dahlin et al., 2000; Philippar et al., 2007). A 14:3:3 protein recognition motif is present in the mature region of pPORA that governs Pchlide-independent default import (Schemenewitz et al., 2007). To avoid this complication, transA-DHFR fusions were made and first tested for Pchlide binding in vitro. Radiolabeled precursors were synthesized by coupled transcription/translation (Fig. 1C) and incubated in the dark with isolated Pchlide. To remove excess pigment not bound to the different precursors, the assays were subjected to gel filtration on Sephadex G15 (Reinbothe et al., 1995). Finally, protein-pigment complexes eluted with the flowthrough were extracted with acetone and pigments quantified by HPLC and fluorescence measurements (Reinbothe et al., 2003).

As shown in Figure 2A, the chimeric protein bearing the nontruncated transit peptide of the pPORA fused to the DHFR (transA-DHFR) was able to bind Pchlide, confirming previous results (Reinbothe et al., 1997). Deletion of amino acids 56 through 67, including the pentapeptide TTSPG, reduced the extent of Pchlide binding of the generated transA(Δ56–67)-DHFR protein to barely detectable levels (Fig. 2B). The same effect was observed when the transit sequence was truncated further, giving rise to transA(Δ43–67)-DHFR (Fig. 2C), transA(Δ30–67)-DHFR (Fig. 2D) and transA(Δ16–67)-DHFR (data not shown), respectively.

In Vitro Import of TransA-DHFR Deletion Derivatives

We next examined the import characteristics of the different truncated precursors. Chloroplasts were iso-
lated from 5-d-old light-grown barley seedlings and purified by differential centrifugation and density gradient centrifugation on Percoll. All steps of plastid isolation and manipulation were done in media containing Suc, which provides a greater stability than sorbitol for the recovery of functional PTC complex (C. Reinbothe, unpublished data). The final plastid pellet obtained was resuspended in import buffer containing Suc. Half of the plastid suspension was preincubated with 5-aminolevulinic acid (5-ALA) and Mg-ATP to induce intraplastidic Pchlide synthesis, whereas the other half was mock incubated with phosphate buffer (Reinbothe et al., 1995). Pchlide-containing and Pchlide-free chloroplasts were repurified on Percoll and depleted of endogenous energy sources by keeping the organelles in darkness on ice for 1 h (Theg et al., 1989; Reinbothe et al., 1995). Then the different 35S-labeled precursors were added and binding as well as import studied. To this end, the incubation temperature and nucleoside triphosphate concentrations were varied. As shown previously, binding of transA-DHFR to the plastids is initially reversible and occurs in an

Figure 1. Construction of deletion derivatives of transA-DHFR. A, Amino acid sequence alignment of the transit sequences of PORA (transA) and PORB (transB) of barley. Vertical bars indicate identical amino acid residues. B, Design of deletion derivatives of transA that lack the indicated COOH-terminal parts of the transit sequence, fused to a DHFR reporter protein of mouse. C, Production of 35S-transA-DHFR and its deletion derivatives by coupled in vitro transcription/translation in a wheat germ lysate and detection of the proteins by SDS-PAGE and autoradiography.

Figure 2. Protochlorophyllide binding to transA-DHFR derivatives in vitro. A to D, TransA-DHFR and its deletion derivatives were synthesized and pigment binding capability was determined by fluorescence spectroscopy at an excitation wavelength of 440 nm. The spectra show pigments extracted from transA-DHFR (A), transAΔ56–67-DHFR (B), transAΔ43–67-DHFR (C), and transAΔ30–67-DHFR (D), respectively. Pchlide has a fluorescence emission maximum at 631 nm in acetone. One microgram of transA-DHFR bound approximately 20 ng of Pchlide, which corresponds to 32.2 pmol of precursor and 32.6 pmol of pigment, respectively.
equilibrium reaction unless low, ≤0.1 mM Mg-ATP concentrations are present (Reinbothe et al., 2000, 2004b). Binding is strongly favored at 4°C. In the presence of 0.1 mM Mg-ATP and 0.1 mM Mg-GTP, the precursor inserts across the outer and inner envelope membranes and engages both components of the outer and inner PTC complexes (Reinbothe et al., 2004b). Figure 3B (a) confirms these previous observations and shows that in the presence of low, 0.1 mM concentrations of Mg-ATP transA-DHFR bound to the plastids in assays kept at 4°C. This is apparent from the decline in the amount of the precursor in the supernatant and its appearance in the plastid fraction, respectively, obtained after centrifugation of the assays. The envelope-bound precursor was chased into the plastids when the reaction temperature was raised from 4°C to 23°C. However, translocation required Pchlide and supplementing the assays with ≥2 mM Mg-ATP. Upon import, the precursor was processed to mature size (Fig. 3). Resistance against thermolysin proved that the mature DHFR was localized inside the plastids. In the absence of Pchlide, no import occurred (Fig. 3) and the plastid-bound precursor was released into the supernatant fraction or degraded by added thermolysin.

Figure 3 also indicates that removal of amino acids 56 to 67 in transA led to an almost complete loss of import of the resulting transA(Δ56–67)-DHFR into chloroplasts lacking or containing Pchlide produced by 5-ALA pretreatment. With transA(Δ43–67)-DHFR and with all of the other mutant precursors in which the transit peptide had been truncated further, similar results were obtained (Fig. 3B, b). Except for transA(Δ16–67)-DHFR, all tested precursors nevertheless were able to bind to the plastids. This is apparent from the disappearance of the precursors in the supernatants and their simultaneous appearance in the plastid fractions, respectively (Fig. 3B, a). The lack of binding of transA(Δ16–67)-DHFR unveiled the presence of a second regulatory motif in transA that is responsible for the faithful recognition of the respective envelope protein import machinery, i.e. the PTC complex. One can speculate that the receptor binding domain is located in region 17 to 30 of transA. Interestingly just in this region of their transit sequences transA and transB are most divergent, where transB has an eight-amino-acid insertion when compared with transA (see Fig. 1A).

Fine Mapping of the Pchlide Binding Site in TransA

Point mutations were engineered into transA to precisely determine which amino acid in the TTSPG motif might be involved in Pchlide binding and the regulation of protein import. Visual inspection and detailed computer-assisted sequence comparisons highlighted a distant relationship of the TTSPG motif to a chlorophyll binding site in the major light harvesting chlorophyll binding protein of higher plants, LHCII (Kühlbrandt et al., 1994; Liu et al., 2004). In this polypeptide, two Gly residues, either directly or via a water molecule, coordinate the central Mg atom of the tetrapyrole ring. These interactions occur in regions of the polypeptide chain where Pro residues disturb the formation of adjacent α-helices (Kühlbrandt et al., 1994; Liu et al., 2004). Because the TTSPG motif principally obeyed this criterion, point mutations were engineered that displaced either amino acid, or both, by Ala residues. The generated mutant precursors, transA(Gly→Ala)-DHFR, transA(Pro→Ala)-DHFR,
Figure 4. Effects of site-directed mutagenesis of the TTSPG motif in transA. A and B, The TTSPG motif was genetically modified to contain Gly-to-Ala or Pro-to-Ala substitutions as well as Gly-to-Ala plus Pro-to-Ala replacements. After in vitro transcription/translation, the pigment binding properties (A) and import characteristics into Pchlide-containing chloroplasts (B) of the engineered transA(Gly→Ala)-DHFR (solid red line), transA(Gly→Ala)/Pro→Ala)-DHFR (dotted blue line), transA(Pro→Ala)-DHFR (dashed blue line), and transA(Ala17→Ala, Pro→Ala)-DHFR (solid blue line). Pigment and precursor quantification confirmed that 1 μg of transA-DHFR (32.2 pmol) bound approximately 20 ng (32.6 pmol) of Pchlide. B, Precursor (P) and mature protein (m) levels of transA(Gly→Ala)-DHFR (G→A, lane 1), transA(Pro→Ala)-DHFR (P→A, lane 2), transA(Gly→Ala, Pro→Ala)-DHFR (G→A/P→A, lane 3) and the native, nonmutated transA-DHFR (native, lane 4) in the supernatant (Sup) and plastid (Plastids) fractions obtained after 15 min of import.

and transA(Gly→Ala, Pro→Ala)-DHFR subsequently were tested with respect to their pigment binding properties and import characteristics in vitro. Import reactions were carried out at 23°C in the presence of 2.5 mM Mg-ATP. Figure 4 illustrates that both transA(Gly→Ala)-DHFR and transA(Pro→Ala)-DHFR entirely lost their pigment binding and import capabilities. In fact, neither mutant precursor was able to bind Pchlide in vitro (Fig. 4A) or to be taken up by Pchlide-containing chloroplasts (Fig. 4B, lanes 1 and 2, respectively). Identical results were obtained for the transA(Gly→Ala,Pro→Ala)-DHFR double mutant (>Fig. 4, A and B, lane 3), underscoring that both Gly and Pro played important roles for establishing the Pchlide binding site in transA. Taking into account that all three mutant proteins were present in the supernatant fraction obtained after sedimentation of the plastids, we concluded that the precursors did not enter a productive import pathway. By contrast, the nonmutagenized transA-DHFR was readily imported into Pchlide-containing chloroplasts (Fig. 4B, lane 4), confirming the previously detected Pchlide requirement of import (Fig. 3A).

The TTSPG Motif Confers Pchlide Binding onto Other Model Precursors But Is Insufficient to Render Protein Import Substrate Dependent

To provide further evidence for a role of the TTSPG motif in Pchlide binding and import, we engineered a chimeric precursor in which the TTSPG was introduced into the linker region between the transit peptide of plastocyanin of Silene pratensis, transPC, and the DHFR (transPC-DHFR). By analogy, a construct was created in which the TTSPG motif was inserted between amino acids 68 and 69 of transB, giving rise to transB-TTSPG-DHFR. Pigment binding and import were determined as described (i.e. 23°C, 2.5 mM Mg-ATP) and are shown in Figure 5. Low but significant Pchlide binding was found for transPC-TTSPG-DHFR, but no difference in import was detectable for Pchlide-containing and Pchlide-free chloroplasts. Both plastid types imported and processed similar precursor levels. Indistinguishable results were obtained for an engineered transB-TTSPG-DHFR precursor that gained the pigment binding property in Pchlide binding tests but was taken up by either plastid type (see Supplemental Fig. S1). Together, these findings corroborated the view that both the TTSPG motif and the receptor binding domain (amino acids 17–30) were required for Pchlide-dependent import. Indeed when a plastocyanin double mutant was created containing both the receptor binding domain (amino acids 17–30) and the pigment binding domain (TTSPG), import became substrate dependent and occurred only with Pchlide-containing chloroplasts (Fig. 5D).

In Vivo Evidence for the Operation of the Substrate-Dependent, TransA-Mediated Import Pathway of pPORA

Transient expression assays were performed in Arabidopsis to validate the conclusions obtained in vitro. The various truncated versions of transA were fused to jellyfish GFP and transformed into leaf epidermis cells by ballistic bombardment. Leaf epidermis cells contain proplastids that can differentiate into other plastid forms. Plastids comprise a family of interconvertible forms (Gibbs, 1971; Kirk and Tilney-Basset, 1978) that import a variety of cytosolic precursor proteins in vitro.
and in vivo (Boyle et al., 1986; de Boer et al., 1988; Dahlin and Cline, 1991). To allow Pchlide synthesis, leaf tissues obtained after transformation were placed in darkness for variable periods and GFP and pigment fluorescences were monitored by confocal microscopy. Pilot experiments using peeled epidermal layers confirmed Pchlide accumulation to occur after darkening (see Supplemental Fig. S2).

Figure 6 (see also Supplemental Figs. S3 and S4) shows that transA-GFP was readily taken up into the plastids of leaf epidermis cells. That the stained particles indeed represent plastids was proven by the simultaneous expression of a fusion protein consisting of amino acids 1 to 66 of the full-length Arabidopsis small subunit precursor of Rubisco and the Discosoma coral DsRed protein (Fig. 6, B and C). When the various transA-GFP deletion derivatives were analyzed, strikingly different results were obtained. For transA(D56–67)-GFP, transA(D43–67)-GFP, transA(D30–67)-GFP, and transA(D16–67)-GFP, GFP fluorescence remained largely confined to the cytosol and no discrete, particulate localization of GFP indicative of plastid import of the respective precursors was found (Fig. 6, D–G; see also Supplemental Fig. S3). Identical results were obtained for GFP without a plastid signal sequence attached to it (Fig. 6I; Supplemental Fig. S4), whereas transB-GFP (Fig. 6H) and transPC-GFP (Supplemental Fig. S4) were readily imported.

**DISCUSSION**

Transit peptides are crucial elements in the targeting pathways of cytosolic precursors to their final destinations. They can operate as export or import signals (Schatz and Dobberstein, 1996; Wickner and Schekman, 2005; Rapoport, 2007). In either case, they sequentially interact with cytosolic targeting factors, receptors on their respective target membranes, and translocation channel components embedded into these membranes. The presequence initiates translocation. The driving force for the actual translocation across the membrane can be provided by the ribosome itself (cotranslational...
import of secretory proteins into the ER) or ATP-powered molecular chaperones on the trans-side of the target membrane (posttranslational transport into the ER, the mitochondrial matrix, and the chloroplast stroma; Schatz and Dobberstein, 1996; Wickner and Schekman, 2005; Rapoport, 2007). A TOC159-mediated, GTP-powered “push” of translocation of cytosolic precursors into chloroplasts has been proposed (Becker et al., 2004) but is highly disputed (Kessler and Schnell, 2004).

In this work, a different import mechanism is described. We demonstrated that import of transA-DHFR and transA-GFP requires Pchlide. A pigment binding site, TTSPG, was identified in transA that governed Pchlide-dependent import. Mutagenesis studies showed that replacements of Gly or Pro in this pentapeptide motif by Ala residues abolished pigment binding and import. By analogy to LHCII (Kühlbrandt et al., 1994; Liu et al., 2004), it is likely that a Gly-bound H2O molecule coordinated the central Mg atom of the tetrapyrrrole ring system, while the adjacent Pro residue disturbed the formation of adjacent α-helices (Kühlbrandt et al., 1994). Alternatively, the peptide bond carbonyl could directly bind the pigment. In LHCII, the peptide bond carbonyl at Tyr-24 binds chlorophyll b (Liu et al., 2004). Pchlide is probably more similar to chlorophyll b (and chlorophyll c) than to chlorophyll a and could coordinate directly with a ligand with a strong dipole such as the carbonyl group. In this sense, the pigment binding mode for transA is obviously different from that described for other chlorophyll- and heme-containing chromoproteins where often Cys, His, and/or Asp residues chelate the pigment (e.g. Adachi et al., 1993; Goodin and McRee, 1993; Dumont et al., 1994; Kühlbrandt et al., 1994).

The TTSPG is necessary for Pchlide binding in vitro but it was insufficient to render import substrate dependent. A supplementary motif was identified in transA that governed Pchlide-dependent import. Mutagenesis studies showed that replacements of Gly or Pro in this pentapeptide motif by Ala residues abolished pigment binding and import. By analogy to LHCII (Kühlbrandt et al., 1994; Liu et al., 2004), it is likely that a Gly-bound H2O molecule coordinated the central Mg atom of the tetrapyrrrole ring system, while the adjacent Pro residue disturbed the formation of adjacent α-helices (Kühlbrandt et al., 1994). Alternatively, the peptide bond carbonyl could directly bind the pigment. In LHCII, the peptide bond carbonyl at Tyr-24 binds chlorophyll b (Liu et al., 2004). Pchlide is probably more similar to chlorophyll b (and chlorophyll c) than to chlorophyll a and could coordinate directly with a ligand with a strong dipole such as the carbonyl group. In this sense, the pigment binding mode for transA is obviously different from that described for other chlorophyll- and heme-containing chromoproteins where often Cys, His, and/or Asp residues chelate the pigment (e.g. Adachi et al., 1993; Goodin and McRee, 1993; Dumont et al., 1994; Kühlbrandt et al., 1994).

The TTSPG is necessary for Pchlide binding in vitro but it was insufficient to render import substrate dependent. A supplementary motif was identified in the NH2-terminal part of transA that directed the precursor toward the PTC complex. The precursor then sequentially interacted with Ptc130, Ptc90, Ptc33, and Ptc16 and also approached Ptc52. From previous cross-linking data (Reinbothe et al., 2004a, 2004b; Schemenewitz et al., 2007) we conclude that the NH2-terminal part of transA is able to trigger opening of the Ptc16 translocation pore and to initiate translocation across the outer envelope membranes of chloroplasts. How this process may be regulated remains to be resolved.
It is tempting to hypothesize that import of pPORA may involve successive binding of Pchlide to the TTSPG motif in transA and the first of the four evolutionarily conserved Cys residues (Cys-106) in the mature PORA. The pigment binding Gly residue in the TTSPG motif and Cys-80 of the DHFR as well as Cys-106 of the mature PORA are located at equidistant positions of the polypeptide chain. Point mutations replacing either Cys residue by Thr and Ala residues, respectively, rendered the transA-DHFR and pPORA transport incompetent (Reinbothe et al., 2004b; C. Reinbothe, unpublished data), highlighting a stepwise mode of pigment-dependent import. The TTSPG motif is conserved in monocots, but apparently not in dicots. In Arabidopsis, where pPORA import has been demonstrated to be substrate dependent both in vitro (Reinbothe et al., 2000) and in planta (Kim and Apel, 2003), a Cys residue is present in the COOH-terminal part of transA that could fulfill the role of the TTSPG motif and operate as the pigment binding site during Pchlide-dependent import.

A quite different example of porphyrin-regulated import of an organellar protein has been reported by Lathrop and Timko (1993). The authors observed that import of mitochondrial 5-ALA synthase is inhibited by heme. A conserved Cys residue was identified in the presequence of 5-ALA synthase that bound the tetrapyrole pigment and blocked precursor uptake, presumably as part of a feedback mechanism limiting excess porphyrin synthesis in mitochondria (Lathrop and Timko, 1993). Deregluation of mitochondrial porphyrin metabolism in fact causes disease and cell death (e.g. May et al., 1990; Ajikawa et al., 2006). Last but not least, the import of the major light-harvesting protein of PSI, LHCII, into chloroplasts has been demonstrated to be dependent on chlorophyll(ide) b in vitro (Reinbothe et al., 2006). Although the evidence is still circumstantial, these results provoke a novel, common concept of pigment-regulated protein import into organelles.

**MATERIALS AND METHODS**

**DNA Constructs**

cDNA clones encoding transA-DHFR and transB-DHFR, derivatives in which the transit sequences of the pPORA (transA) and pPORB (transB) were fused to a cytosolic DHFR of mouse, have been described (Reinbothe et al., 1997). TransPC-DHFR consists of the chloroplast transit peptide of plastocyanin of *Silene pratensis* linked by a stretch of 10 amino acids (IQDSSRGSSG) to the DHFR (Hageman et al., 1990). For construction of deletion derivatives of transA and point mutants displaying amino acid substitutions in transA, fused to either the DHFR or jellyfish GFP, a PCR-based approach was used (Maniatis et al., 1982; Innis et al., 1990). Details on the primers used can be found as supplemental data (Supplemental Materials and Methods S1). The identity of the different constructs was confirmed by GAWCT Biotech.

**In Vitro Protein Synthesis and Pigment Binding Assay**

Radiolabeled precursors were synthesized by coupled in vitro transcription/translation of the recombinant clones specified in the text and purified as described previously (Reinbothe et al., 2004a). Equal amounts of the different precursors, as determined by counting their radioactivities and correcting the rates of incorporation for the different Met contents (Reinbothe et al., 1997), were supplemented with synthetic Pchlide a or Pchlide b (10 μM final concentrations each) or a pigment mixture prepared from 5-d-old etiolated barley (*Hordeum vulgare*) plants that contained equimolar amounts of either pigment (Reinbothe et al., 2003, 2004c). After incubation for 15 min in darkness, the assay mixtures were subjected to gel filtration on Sephadex G15 equilibrated in assay buffer (Reinbothe et al., 1995). Enzyme-pigment complexes eluted with the flowthrough were extracted with acetone and pigments identified and quantified by HPLC or fluorescence measurements in a Perkin Elmer LS50B spectrometer (Perkin Elmer; Reinbothe et al., 1995, 2003).

**Import Assay**

Protein import was studied as described previously, using postribosomal supernatants of in vitro translated precursors and isolated barley chloroplasts that had been prepared from 5-d-old light-grown plants. Plastid isolation and purification is described elsewhere and involved Suc-containing buffers (Reinbothe et al., 1995). All operations were done under a dim green safelight. For production of Pchlide, the plastids were preincubated in darkness with 5-ALA dissolved in 10 mM phosphate buffer, pH 8.0, or with phosphate buffer alone (Reinbothe et al., 1995). If not stated otherwise, plastids were repurified on Percoll after incubation. Import assays contained 37.5 μL of complete premix consisting of 25 μL of doubly concentrated, ATP- and GTP-free import buffer, 10 μL of urea-denatured, diluted (0.2 M final urea concentration), radiolabeled precursors, and 2.5 μL each of stock solutions of Mg-GTP and Mg-ATP, to give rise to the final nucleoside triphosphate concentrations specified in the text. If needed, bidistilled water was added to adjust the final reaction volume.

All import reactions were initiated by the addition of 10 μL of the repurified, diluted plastids (1 × 10⁷). After 15 min in darkness, the import mixtures were diluted with buffer containing 50 mM HEPES-KOH, pH 8.0, 350 mM Suc, 10 mM β-mercaptoethanol, and 50 μM each of the proteinase inhibitors leupeptin and pepstatin (Reinbothe et al., 1995). After centrifugation at 3,500 rpm in a Sorvall RC-5B centrifuge (DuPont de Nemours) at 4°C, using a HB4 rotor, nonimported precursors contained in the supernatant were precipitated with trichloroacetic acid and were processed for PAGE (Reinbothe et al., 1995). Plastids obtained in the sediment after centrifugation were repurified on Percoll cushions and treated with or without thermolysin as indicated (Cline et al., 1984). Plastid protein was extracted with trichloroacetic acid, depleting of chlorophyll by repeated extractions with acetone, ethanol, and ether, and resuspended in SDS sample buffer (Laemmli, 1970). Heat-denatured proteins were analyzed electrophoretically and detected by autoradiography (see below).

**Transient Expression of TransA-GFP Derivatives in Arabidopsis**

Transient expression of transA-GFP derivatives was performed in Arabidopsis (*Arabidopsis thaliana*) leaf epidermis cells. Ballistic bombardment was carried out according to Finer et al. (1992) using a pneumatic particle inflow gun. The conditions of bombardment were adjusted to helium pressure of 6.5 bar, 12-cm target distance, with a discrete grid at 7 cm, using 1 μM of gold microcarriers (Bio-Rad). After bombardment, the plantlets were kept under sterile conditions and further incubated on plates for variable time periods in darkness. Confocal laser scanning microscopy was carried out in LSM 510 Meta (Zeiss) with argon laser excitation at 488 nm. GFP and chlorophyll fluorescence were detected at emission wavelengths of 505 to 530 nm and 650 to 730 nm, respectively. Pchlide fluorescence was monitored between 600 and 650 nm. LSM 510 Meta software release 3.2 (Zeiss) and Adobe Photoshop 7 (Adobe Systems) were used for image acquisition and processing, respectively.

**Other Procedures**

Denaturing and non-denaturing PAGE were carried out as described (Reinbothe et al., 2004b).

**Supplemental Data**

The following materials are available in the online version of this article.

Supplemental Figure S1. Pigment binding and import characteristics of transB-TTSPG-DHFR.
ACKNOWLEDGMENTS

We are indebted to F. Buhr for critical reading of the manuscript and to L. Reinbothe for editorial work.

Received April 3, 2008; accepted April 15, 2008; published April 25, 2008.

LITERATURE CITED


Reinbothe et al.