Biol. Chem., Vol. 381, pp. 1263 – 1267, December 2000

Short Communication

NADPH:Protochlorophyllide Oxidoreductase Uses the General Import Route into Chloroplasts

Henrik Aronsson[1,*], Karen Sohrt[2], and Jürgen Soll[2]

[1] Department of Plant Physiology, Göteborg University, Box 461, S-40530 Göteborg, Sweden
[2] Botanisches Institut, Universität Kiel, D-24098 Kiel, Germany

* Corresponding author

Chloroplast differentiation in angiosperm plants depends on the light-dependent conversion of protochlorophyllide to chlorophyllide by NADPH:protochlorophyllide oxidoreductase (PORA; EC 1.6.99.1), a nuclearly encoded protein. The protein import of the precursor form of PORA into plastids was shown previously to strictly depend on the presence of its substrate protochlorophyllide. PORA seemed to follow a novel, posttranslationally regulated import route. Here we demonstrate that the precursor of PORA from barley is imported into isolated barley plastids independently of protochlorophyllide. PORA as well as PORB import is competed for by the precursor of the small subunit of Rubisco. The data demonstrate that the PORA precursor uses the general import pathway into plastids. Furthermore, *en route* into chloroplasts the pea POR precursor can be cross-linked to the protein import channel in the outer envelope Toc75 from pea.

Key words: Chloroplasts / NADPH:Protochlorophyllide oxidoreductase / *Pisum sativum* / Protein import / Toc75.

In angiosperm plants chloroplast development and differentiation is light regulated. Dark-grown etiolated leaf tissue contains etioplasts, which are highly specialized precursor organelles that differentiate light-dependent into chloroplasts (Virgin *et al*., 1963). Light exposure also results in the activation of transcription for many nuclear genes that encode chloroplast proteins (Silverthorne and Tobin, 1984). These proteins are synthesized in the cytosol in a precursor form, which contains a targeting signal as an N-terminal presequence. All precursors described to date seem to follow a general protein import pathway into the chloroplasts which involves a translocon at the outer envelope of the chloroplasts, the Toc complex (Schnell *et al*., 1994; Heins *et al*., 1998; Soll and Tien, 1998; Keegstra and Cline, 1999).

The light-dependent reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) is catalyzed by the NADPH:protochlorophyllide oxidoreductase (POR) (Griffiths, 1975). Three nuclear genes exist which encode three POR proteins: PORA, PORB and PORC (Armstrong *et al*., 1995; Holtorf *et al*., 1995; Oosawa *et al*., 2000). The PORA isoform accumulates in dark-grown plants in large amounts in the prolamellar bodies inside etioplasts and is down-regulated in the light (Apel, 1981). The PORB isoform is present in dark-grown plants but is not down-regulated in the light and can thus supply the necessary chlorophyll precursors during greening (Holtorf *et al*., 1995). The precursor form of PORA from barley has been reported to being imported into barley plastids only in the presence of its substrate Pchlide (Reinbothe *et al*., 1995a,b, 1997, 2000). Chloroplasts that normally do not contain significant levels of Pchlide imported pPORA only after the synthesis of Pchlide from added δ-aminolevulinic acid (Reinbothe *et al*., 1995a). Pchlide was suggested to interact with pPORA at the envelope membranes and was thought to enable the translocation of pPORA . However, pPORB was readily imported into chloroplasts in the absence of Pchlide. Furthermore, when the transit peptides were exchanged between PORA and PORB the import of PORB became substrate dependent, while PORA containing the PORB transit signal was imported also in the absence of Pchlide (Reinbothe *et al*., 1997). Together, the data suggested that import of pPORA could be regulated through a Pchlide responsive transit peptide and that pPORA did not use the general import pathway. In addition, it was indicated that the import of pPORA into plastids was not inhibited by competition with an excess of the precursor of the Rubisco small subunit (pSS), which uses the general import pathway (Reinbothe *et al*., 2000). However in the same communication it was also reported that the pPORA translation product was not import competent directly by itself after translation, but first had to be denatured by urea (Reinbothe *et al.*, 2000). It seems doubtful whether such an approach can faithfully describe a novel import route. In our study we use an import competent pPORA translation product and demonstrate that its import into barley chloroplasts is independent of Pchlide.

The study of protein import into isolated organelles requires an elaborate *in vitro* system which is delicate and susceptible to many experimental factors, *e. g.* organelle isolation and translation conditions (see below). Optimized experimental conditions were used here to assay the import of pPORA and pPORB into barley chloroplasts. The chloroplasts were isolated from green plants which

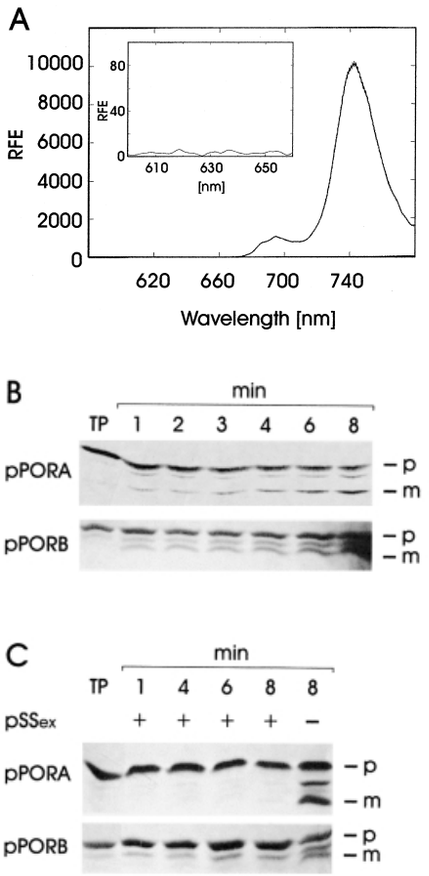

Fig. 1 The Barley PORA Precursor Protein Is Imported into Barley Chloroplasts**.**

Seedlings of barley (*Hordeum vulgare* cv. *Carina*) and pea *(Pisum sativum)* were grown for 8–10 days in a greenhouse before harvest. Plastids were isolated from leaves (Bölter *et al*., 1998). Crude plastids were further enriched by density gradient centrifugation using a 40/80% Percoll step gradient for pea and a linear 50% Percoll gradient for barley plastids. The Percoll step gradient was centrifuged at 7000 *g* for 4 min and the linear Percoll gradient at 7000 *g* for 8 min. The plastids were washed twice in wash medium (330 mM sorbitol, 50 mM Hepes/KOH, pH 7.6 and 3 mM $MgCl_2$). Plastids were recovered by centrifugation at 2000 *g* for 2 min. Chlorophyll content was determined according to Arnon (1949). (A) Fluorescence emission spectra were recorded at 77 K with the excitation wavelength at 440 nm according to Lindsten *et al*. (1988). The insert shows the same spectrum but recorded with higher sensitivity in the region of Pchlide fluorescence emission (compare Figure 2A). (B,C) Barley pPORA, pPORB, pea pPOR and pSS were synthesized by coupled *in vitro* transcription-translation in the presence of [^{35}S]-methionine/cysteine (May and Soll, 2000) for 60 min at 27–30 °C in a wheat germ lysate. Translation was stopped by cooling to 4 °C and a postribosomal supernatant was obtained by centrifugation at 100 000 *g* for 15 min. Chloroplasts were incubated with [^{35}S]-labeled wheat germ synthesized translation product (*TP*) of barley PORA and PORB precursor proteins for the times indicated at 25 °C essentially as described in Waegemann and Soll (1991). The import mixture consisted of radiolabeled precursor protein, ATP (final concentration 2 mM) and chloroplasts equivalent to 10 μg chlorophyll in a 100 μl import assay. The import reaction was run in the linear time range and terminated by the addition of icecold EDTA (final concentration 35 mM). Chloroplasts were recovered by centrifugation and washed once prior to analysis of the proteins by SDS-PAGE and fluorography (Waegemann and Soll, 1991). Overexpressed unlabeled pSS from inclusion bodies was used as a competitor (Waegemann and Soll, 1995). The protein was denatured in 8 M urea and added to the import assay. The final urea concentration did not exceeded 150 mM. p, m denote the position of the precursor and mature form, respectively. (B) Time course experiment of pPORA and pPORB import into barley chloroplasts. (C) Competition of pPORA and pPORB import into barley chloroplasts in the absence (–) and presence (+) of 10 μM heterologously expressed Rubisco small subunit precursor (pSS_{ex}).

had been in the light for 3 h of the normal night/day cycle. These chloroplasts did not contain detectable amounts of Pchlide, since only the typical chlorophyll fluorescence was measurable (Figure 1A). Both pPORA and pPORB were imported rapidly into isolated chloroplasts with similar efficiency. A typical experimental data set is shown out of more than 3 repeats (Figure 1B). We conclude that pPORA can translocate and becomes processed faithfully into chloroplasts in the absence of its substrate Pchlide. Furthermore, pPORA and pPORB seem to use components of the general import pathway. When an excess of the precursor form of the stroma localized Rubisco small subunit, pSS, was added, the import of pPORA and pPORB was completely abolished, even if longer import times were used (Figure 1C). Import of pPORA and pPORB was also dependent on ATP (not shown). Together the data indicated a cosubstrate-independent import of pPORA into chloroplasts, which could be competed for by a substrate of the general import pathway.

Although pPORA import seemed possible in the absence of Pchlide, it might be highly stimulated by the presence of its substrate. Therefore light-grown barley plants were cut at the base of the leaf and incubated overnight in the presence or absence of δ-aminolevulinic acid (ALA). This treatment resulted in a large accumulation of Pchlide inside chloroplasts as determined by the typical fluorescence emmission at 637 nm (Figure 2A). pPORA import was then tested into chloroplasts containing this large excess of endogenous Pchlide or into chloroplasts from mock treated plants. Both the rate as well as the yield of import were independent of the presence of endogenous Pchlide (Figure 2B). We conclude that pPORA import into chloroplasts is neither stimulated nor dependent on the presence of its substrate.

Our earlier results had indicated that the POR precursor could use components of the general import pathway (see above, Figure 1). Toc75 forms the protein translocation channel in the outer envelope import complex (Waegemann and Soll, 1991; Perry and Keegstra, 1994; Hinnah *et al*., 1997). We therefore tested whether POR precursor protein can be crosslinked to Toc75, which could indicate the direct involvement of translocon components. The Toc75 antiserum was raised against the pea protein (Hinnah *et al*., 1997). Preliminary experiments had shown that it was not able to immunoprecipitate barley Toc75; therefore the subsequent experiments were done with pea

pPOR and pea chloroplasts. Pea pPOR as well as barley pPORA were imported into isolated pea chloroplasts in a time- and ATP-dependent fashion (Figure 3A and not shown), demonstrating again that even in a heterologous system pPORA can be imported into chloroplast independent of its substrate. The import of pea pPOR as well as of pSS can also be competed for by the addition of overexpressed pSS (Figure 3B), indicating that also in this system POR uses the general import route. Crosslinking experiments were carried out under conditions which allow the preproteins to interact and insert into the translocon, but which do not allow complete translocation (*i.e.* 100 μM ATP, 2 °C). Only in the presence of crosslinker several radiolabeled high molecular weight products were formed from pea pPOR (Figure 3C, lanes 1 and 3). The Toc75 antiserum immunoprecipitated a crosslinked product of around 120 kDa for pea pPOR (Figure 3C, lane 4). In the

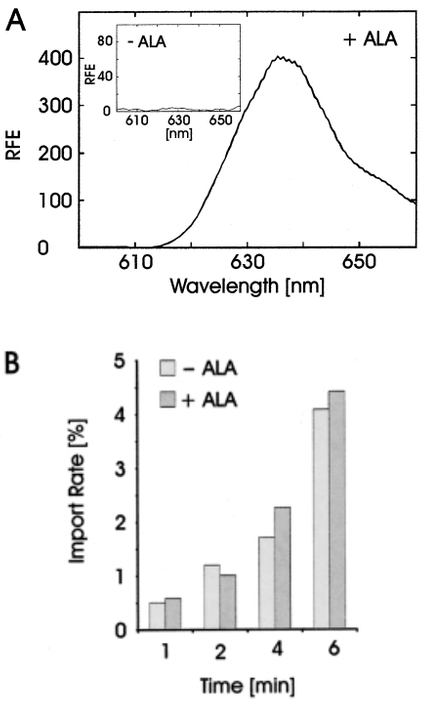

Fig. 2 The Import of pPORA Is Independent and Not Stimulated by the Presence of Pchlide.
8–10 days old greenhouse-grown barley plants were cut at the soil surface and immediately put into 0.1 M sucrose with or without 1 mg/ml of δ-aminolevulinic acid (ALA). The plants were incubated in darkness for 16 hours in a fume hood to stimulate transpiration and solute uptake. Fluorescence emission spectra were recorded as described in Figure 1. Chloroplasts were isolated in dim green safe light from treated or non-treated plants as described above. (A) The Pchlide content was determined by fluorescence spectroscopy. The inset shows the Pchlide content of mock treated leaves. Note the different scales of the relative fluorescence emission intensity (RFE). The spectra were normalized to equal fluorescence emission intensity of photosystem II at 743 nm (not shown). (B) Chloroplasts were isolated from leaves treated without (–) or with (+) δ-aminolevulinic acid and used for a standard import experiment in the presence of [^{35}S]-labeled pPORA. The import rate is given as percentage of the total pPORA translation product added to one import. The mean of two independent time course experiments is shown.

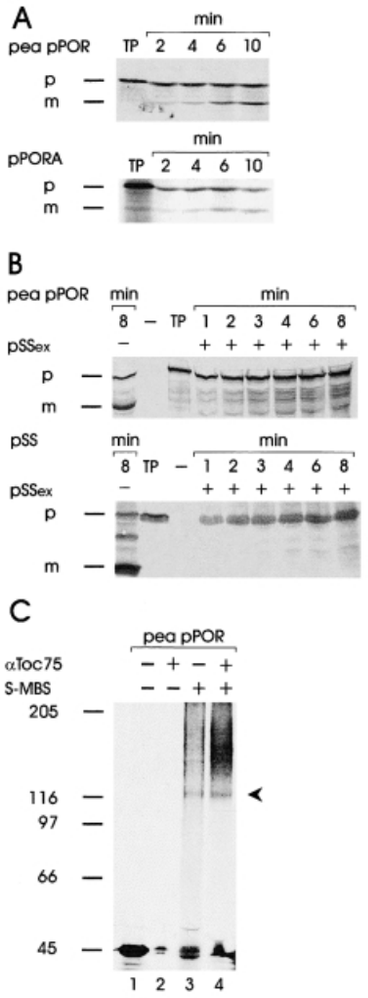

Fig. 3 POR Precursor Proteins Use the General Import Route Also in Pea.
(A) Purified pea chloroplasts import pea POR and barley pPORA precursor proteins in a time-dependent manner. (B) The import of pea pPOR as well as the import of pSS can be competed for by heterologously expressed pSS_{ex}. Competition of pea pPOR and pSS import in presence (+) of heterologous 10 μM pSS_{ex} was performed simultaneously in one import reaction in a time course assay. The very left lane shows the longest time point in the absence (–) of overexpressed pSS. TP denotes translation product, 10% of which was added to an import reaction. All other conditions were as in Figure 1. (C) Pea pPOR translation product was bound to intact pea chloroplasts equivalent to 60 μg chlorophyll in 150 μl binding buffer [330 mM sorbitol, 50 mM Hepes/KOH, pH 7.6, 3 mM $MgCl_2$, 100 μM ATP, 20 mM potassium gluconate, 10 mM methionine, 10 mM cysteine, 10 mM $NaHCO_3$, 2% (w/v) BSA] for 10 min on ice. The chloroplasts were recovered through a 40% Percoll cusion (Waegemann and Soll, 1991) and washed once in wash medium before crosslinking was performed with 2.5 mM sulfomaleimidobenzoyl-N-hydroxy-succinimide ester in 150 μl wash medium for 15 min on ice. The chloroplasts sedimented by centrifugation, were washed once in wash medium, solubilized in 2% (w/v) SDS and used for immunoprecipitation with antiserum against pea Toc75. A fluorogram is shown. Numbers on the left indicate molecular mass markers in kDa.

absence of crosslinker no labeled proteins were precipitated by Toc75 antiserum (Figure 3C, lane 2). The Toc75 preimmune serum did not recognize any labeled proteins (not shown). From these data we conclude that the precursor proteins of POR from different plants use the general import pathway.

The light-dependent conversion of Pchlide to Chlide in angiosperm plants is a sensory element in the differentiation of plastids. The POR enzyme, which catalyzes the conversion in barley, is encoded by two genes, one predominantly expressed in the dark, *PORA,* and one in the light, *PORB* (Holtorf *et al*., 1995). The possibility that the appearance of the PORA enzyme in plastids is regulated not only at the transcriptional level but also posttranscriptionally by making its import dependent on the presence of the substrate was intriguing and would allow for independent regulatory circuits in chloroplast differentiation (Reinbothe *et al*., 1995a, b, 1997, 2000). However, the data presented here and by Dahlin *et al*. (2000) clearly show that the POR expressed in the dark only, namely pPORA, can be imported into chloroplasts in the complete absence of its substrate. In our experiments care was taken to measure import in the linear range of translocation time and not as single time points as performed by Reinbothe *et al*. (2000). Furthermore, import of pPORA can be competed for by a standard preprotein like pSS. This result is again different from those reported by Reinbothe *et al*. (2000), where no competition could be observed between pPORA and pSS. Further evidence that the POR precursor takes the general import pathway comes from our crosslinking data (Figure 3 C) which demonstrate that pea pPOR can be crosslinked to the protein import channel Toc75.

What could be the reasons for these discrepancies? Protein import into plastids is prone to several *in vitro* experimental pitfalls (Waegemann and Soll, 1995). A major protein import receptor, Toc160, is extremely sensitive to proteolysis upon cell lysis (Bölter *et al*., 1998; Bauer *et al*., 2000; Chen *et al*., 2000). This very recent observation indicates that Toc160 degrades with a half life of a few minutes if not strict precautions are taken, *e. g.* rapid chloroplast isolation (about 15 min) at 2 °C, or inclusion of specific protease inhibitors. Prolonged isolation or incubation procedures at temperatures > 2 °C (as in Reinbothe *et al*., 2000) or in the absence of selective protease inhibitors result in the degradation of Toc160 and in the formation of proteolytic fragments of 86 kDa and 52 kDa. The preprotein translocation channel Toc75 has a precursor binding site (Kouranov and Schnell, 1997), which can mediate some protein import even in the absence of other receptor proteins. Denatured precursor proteins seem to directly interact with Toc75, bypassing the receptors Toc34 and Toc160 (Soll, unpublished). The import experiments of pPORA (Reinbothe *et al*., 2000) contained no less than 600 – 800 mM urea in addition to other salts and osmoactive compounds. In our studies 150 mM urea is not exceeded, because higher concentrations seem to influence chloroplast integrity and import competence. It is evident that the functional integrity of the Toc complex strongly influences the requirements for import *in vitro.*

The nature of a precursor protein after *in vitro* translation depends largely on the experimental conditions used. Synthesis in a wheat germ lysate, as also used for POR (Reinbothe *et al*., 1995a, 1997, 2000), results in at least four different preprotein populations. The preproteins can be found as monomers, various hetero-oligomers in association with cytosolic proteins or aggregated (May and Soll, 2000). The different precursor forms differ in import competence. The ratio between the precursor populations is determined by the translation conditions used (May and Soll, 2000); prolonged incubation times > 60 min, elevated temperatures > 30 °C or ATP > 1 mM lead to more monomeric and especially aggregated proteins, which require special conditions to regain their import competence.

It is difficult to pinpoint which experimental deviations described above actually caused the failure of barley pPORA to import into chloroplasts in the absence of Pchlide (Reinbothe *et al*., 1995a, b, 1997, 2000), but in these studies an urea denatured preprotein had to be used since the translated protein was not import competent (Reinbothe *et al*., 2000). The presence of large amounts of the substrate might somehow induce or stabilize an import competent state of the denatured protein *in vitro*. This is certainly unusual, but we doubt that the failure of a given protein to import under certain conditions is conclusive evidence for a new import pathway especially when an unfolded denatured preprotein is used. Our positive import results of barley pPORA into barley chloroplasts in the absence of substrate demonstrate, that Pchlide has no essential or regulatory role in the import of pPORA.

Acknowledgements

We thank T. May for his initial help in this project. The POR cDNA clones were kindly provided by Dr. M. P. Timko (pea POR) and Dr. K. Apel (barley PorA/B). This work was supported in parts by grants from the Deutsche Forschungsgemeinschaft, Fonds der Chemischen Industrie, the Human Frontier Science Program to J.S. and by the Adlerbertska Foundation, the Collianders Foundation and the Swedish Institute to H.A.

References

Apel, K. (1981). The protochlorophyllide holochrome of barley (*Hordeum vulgare* L.). Phytochrome-induced decrease of translatable mRNA coding for the NADPH:protochlorophyllide oxidoreductase. Eur. J. Biochem. *120*, 89 – 93.

Armstrong, G.A., Runge, S., Frick, G., Spreling, U., and Apel, K. (1995). Identification of NADPH: protochlorophyllide oxidoreductase A and B: a branched pathway for light-dependent chlorophyll biosynthesis in *Arabidopsis thaliana*. Plant Physiol. *108*, 1505 – 1517.

Arnon, D.J. (1949). Copper enzymes in isolated chloroplasts. Polyphenyloxidase in *Beta vulgaris*. Plant Physiol. *24*, 1 – 15.

Bauer, J., Chen, K., Hiltbunner, A., Wherli, E., Eugster, M., Schnell, D., and Kessler, F. (2000). The major protein import receptor of plastids is essential for chloroplast biogenesis. Nature *403*, 203–209.

Bölter, B., May, T., and Soll, J. (1998). A protein import receptor in pea chloroplasts, Toc86, is only a proteolytic fragment of a larger polypeptide. FEBS Lett. *441*, 59–62.

Chen, K., Chen, X., and Schnell, D. (2000). Initial binding of preproteins involving the Toc159 receptor can be bypassed during protein import into chloroplasts. Plant Physiol. *122*, 813–822.

Dahlin, C., Aronsson, H., Almkvist, J., and Sundqvist, C. (2000). Protochlorophyllide-independent import of two NADPH:Pchlide oxidoreductase proteins (PORA and PORB) from barley into isolated plastids. Physiol. Plant *109*, 298–303.

Griffiths, W.T. (1975). Characterization of the terminal stages of chlorophyll (ide) synthesis in etioplast membrane preparations. Biochem. J. *152*, 623–635.

Heins, L., Collinson, I., and Soll, J. (1998). The protein translocation apparatus of chloroplast envelopes. Trends Plant Sci. *3*, 56–61.

Hinnah, S.C., Hill, K., Wagner, R., Schlicher, T., and Soll, J. (1997). Reconstitution of a chloroplast protein import channel. EMBO J. *16*, 7351–7360.

Holtorf, H., Reinbothe, S., Reinbothe, C., Bereza, B., and Apel, K. (1995). Two routes of chlorophyllide synthesis that are differentially regulated by light in barley (*Hordeum vulgare* L.). Proc. Natl. Acad. Sci. USA *92*, 3254–3258.

Keegstra, K., and Cline, K. (1999). Protein import and routing systems of chloroplasts. Plant Cell *11*, 557–570.

Kouranov, A., and Schnell, D.J. (1997). Analysis of the interactions of preproteins with the import machinery over the course of protein import into chloroplasts. J. Cell. Biol. *139*, 1677–1685.

Lindsten, A., Ryberg, M., and Sundqvist, C. (1988). The polypeptide composition of highly purified prolammellar bodies and prothylakoids from wheat (*Triticum aestivum*) as revealed by silver staining. Physiol. Plant. *72*, 167–176.

May, T., and Soll, J. (2000). 14-3-3 proteins form a guidance complex with chloroplast precursor proteins in plants. Plant Cell *11*, 1–12.

Oosawa, N., Masuda, T., Awai, K., Fusada, N., Shimada, H., Ohta, H., and Takamiya, K.-H. (2000). Identification and light-induced expression of a novel gene of NADPH-protochlorophyllide oxidoreductase isoform in *Arabidopsis thaliana*. FEBS Lett. *474*, 133–136.

Perry, S.E., and Keegstra, K. (1994). Envelope membrane proteins that interact with chloroplastic precursor proteins. Plant Cell *6*, 93–105.

Reinbothe, S., Runge, S., Reinbothe, C., van Cleve, B., and Apel, K. (1995a). Substrate-dependent transport of the NADPH:protochlorophyllide oxidoreductase into isolated plastids. Plant Cell *7*, 161–172.

Reinbothe, S., Reinbothe, C., Runge, S., and Apel, K. (1995b). Enzymatic product formation impairs both the chloroplast receptor-binding function as well as translocation competence of the NADPH:protochlorophyllide oxidoreductase, a nuclear-encoded plastid precursor protein. J. Cell Biol. *129*, 299–308.

Reinbothe, C., Lebedev, N., Apel, K., and Reinbothe, S. (1997). Regulation of chloroplast protein import through a protochlorophyllide-responsive transit peptide. Proc. Natl. Acad. Sci. USA *94*, 8890–8894.

Reinbothe, S., Mache, R., and Reinbothe, C. (2000). A second, substrate dependent site of protein import into chloroplasts. Proc. Natl. Acad. Sci. USA *97,* 9795–9800.

Schnell, D.J., Kessler, F., and Blobel, G. (1994). Isolation of components of the chloroplast protein import machinery. Science *266*, 1007–1012.

Silverthorne, J., and Tobin, E.M. (1984). Demonstration of transcriptional regulation of specific genes by phytochrome action. Proc. Natl. Acad. Sci. USA *81*, 1112–1116.

Soll, J., and Tien, R. (1998). Protein translocation into and across the chloroplastic envelope membranes. Plant Mol. Biol. *38*, 191–207.

Virgin, H., Kahn, A., and Wettstein, D. (1963). The physiology of chlorophyll formation in relation to structural changes in chloroplasts. Photochem. Photobiol. *2*, 89–91.

Waegemann, K., and Soll, J. (1991). Characterization of the protein import apparatus in isolated outer envelopes of chloroplasts. Plant J. *1*, 149–158.

Waegemann, K., and Soll, J. (1995). Characterization and isolation of the chloroplast protein import machinery. Meth. Cell. Biol. *50*, 255–267.

Received August 31, 2000; accepted October 19, 2000