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Targeting of proteins to the thylakoid lumen by the bipartite transit peptide of the 33 kd oxygen-evolving protein

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Various chimeric precursors and deletions of the 33 kd oxygen-evolving protein (OEE1) were constructed to study the mechanism by which chloroplast proteins are imported and targeted to the thylakoid lumen. The native OEE1 precursor was imported into isolated chloroplasts, processed and localized in the thylakoid lumen. Replacement of the OEE1 transit peptide with the transit peptide of the small subunit of ribulose-1,5-bisphosphate carboxylase, a stromal protein, resulted in redirection of mature OEE1 into the stromal compartment of the chloroplast. Utilizing chimeric transit peptides and block deletions we demonstrated that the 85 residue OEE1 transit peptide contains separate signal domains for importing and targeting the thylakoid lumen. The importing domain, which mediates translocation across the two membranes of the chloroplast envelope, is present in the N-terminal 58 amino acids. The thylakoid lumen targeting domain, which mediates translocation across the thylakoid membrane, is located within the C-terminal 27 residues of the OEE1 transit peptide. Chimeric precursors were constructed and used in *in vitro* import experiments to demonstrate that the OEE1 transit peptide is capable of importing and targeting foreign proteins to the thylakoid lumen.

Key words: Arabidopsis thaliana/chimeric precursors/oxygen-evolving protein/protein targeting/thylakoid lumen

Introduction

Protein targeting for nuclear-encoded chloroplast proteins is complex, as polypeptides must be correctly sorted between both intracellular and intra-organellar destinations. Within chloroplasts there are six distinct compartments to which various proteins must target. These compartments are the three membrane systems (inner and outer envelope membrane and thylakoid membrane) and the three aqueous enclosures (thylakoid lumen, stromal space, and the intermembrane space between the outer and inner envelope). Polypeptides destined for the chloroplast must in many cases translocate across a combination of membranes and compartments before assembling at their functional sites. Thylakoid lumenal proteins such as plastocyanin or the subunits of the oxygen-evolving complex are presumed to transverse three distinct membranes to reach the lumen space. The signal directing chloroplast proteins to their appropriate compartment usually resides in the N-terminal region and is commonly referred to as the transit peptide. This amino-terminal transit peptide may contain sufficient information for both importing and intrachloroplast sorting (Smeekens and Weisbeek, 1988). Imported proteins are synthesized as precursors on cytoplasmic ribosomes and translocate across the chloroplast compartments via a post-translational mechanism. The transit peptides of these proteins may be processed at various steps of the translocation pathway. Multipartite signals involved in protein targeting have been demonstrated in other systems such as mitochondria (see reviews by Pfanner *et al.*, 1988; Verner and Schatz, 1988) and bacteria (von Heijne, 1983; Verner and Schatz, 1988). The signals and mechanism responsible for targeting proteins to and within the chloroplasts are less well understood.

Our knowledge about protein import into chloroplasts extends mostly from studies of the abundant stromal protein, the small subunit of ribulose-1,5-bisphosphate carboxylase (RBCS). The information necessary for mediating import and targeting to the stromal compartment, at least in the case of RBCS and ferredoxin, resides in the transit peptide (Schmidt and Mishkind *et al.*, 1986; Smeekens *et al.*, 1986). The transit peptides alone are capable of importing and targeting foreign proteins to the stroma (Van den Broeck *et al.*, 1985; Smeekens *et al.*, 1987). Import into other compartments of the chloroplast, such as the thylakoid membrane and the lumenal space, appears to be more complex. The transit peptide of the chlorophyll a/b (CAB) binding protein functions in import but not in targeting to the thylakoid membrane (Lamppa, 1988; Hand *et al.*, 1989). Replacement of the CAB transit peptide with the RBCS transit peptide does not abolish its import competence nor its integration into the thylakoid membrane, indicating that the signal for thylakoid targeting is present elsewhere in the mature body. In contrast, the transit peptide of plastocyanin has been shown to contain separate domains for import and targeting. Unlike the CAB protein, the transit peptide of plastocyanin plays a role in thylakoid lumen targeting (Smeekens *et al.*, 1986, 1987; Weisbeek *et al.*, 1987; de Boer *et al.*, 1988). Other parts of plastocyanin were also suggested to play a role in thylakoid lumenal targeting (de Boer *et al.*, 1988), since it was not possible to use the transit peptide alone to direct foreign passenger proteins to the lumen (Smeekens *et al.*, 1987).

In this paper, we present import studies of the transit peptide of a lumenal polypeptide, the 33 kd oxygen-evolving protein, which unlike the plastocyanin protein, is complexed with photosystem II. Utilizing chimeric transit peptides and block deletions of the transit peptide, we have revealed that the 33 kd transit peptide contains both a chloroplast importing and a thylakoid targeting domain. Furthermore, we have constructed chimeric precursor proteins to demonstrate that the 33 kd transit peptide is capable of importing and targeting foreign proteins to the thylakoid lumen.

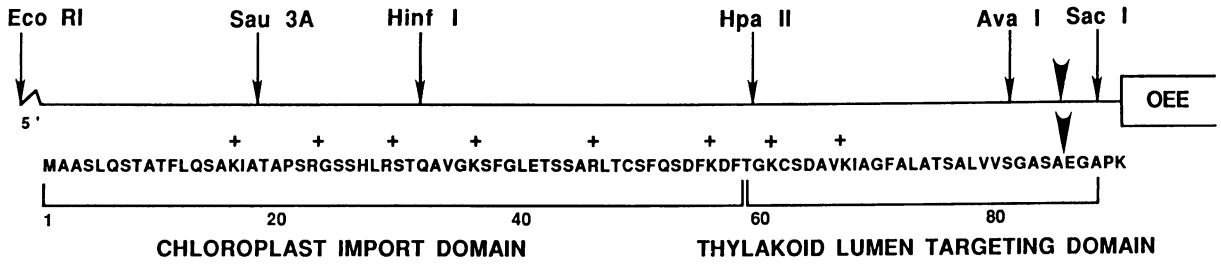

Fig. 1. The transit peptide of the 33 kd OEE1 protein. A map of restriction endonuclease sites employed in the making of the various gene constructs is shown at the top line. The amino acid sequence of the entire transit peptide together with the location of positively charged lysine and arginine residues is illustrated in the middle line. The regions in which the putative chloroplast importing and thylakoid lumen targeting domains are located are delineated by the brackets at the bottom. The processing site of OEE1 precursor is indicated by the large arrowhead. Sequences corresponding to the 5′ untranslated region and the mature body are marked 5′ and OEE, respectively.

Results

Import and localization of the 33 kd OEE1 precursor

The 33 kd OEE1 precursor protein was generated *in vitro* from an *Arabidopsis thaliana* cDNA clone. The *oee1* cDNA sequence comprises 35 bp of 5′ untranslated sequence, 996 bp of coding region, 110 bp of 3′ untranslated sequence and a 30 nucleotide poly(A) tail (Ko *et al.*, 1989). The cDNA fragment was inserted behind the SP6 and T7 promoter of the transcription plasmid pGEM4 as described in Materials and methods. These plasmids, designated pOEE1A and pOEE1B, were used as starting vectors for constructing chimeric gene fusions and served as templates for synthesis of *in vitro* transcripts of the OEE1 precursor. A detailed map of the OEE1 transit peptide depicting the restriction enzyme sites used in making the different gene fusions is shown in Figure 1. The amino acid sequence of the OEE1 transit peptide and the position of basic residues is also illustrated in Figure 1.

Precursors of OEE1 were made from the *in vitro* transcripts using the wheat germ extract translation system. The translation products, represented by OEE1A in Figure 2A, were used directly for assaying import into isolated intact pea chloroplasts. The predicted mol. wts from the nucleotide sequence were 35 and 27 kd for the precursor and the mature form, respectively. The migration of the proteins in a 12.5% SDS–polyacrylamide gel did not correspond to the calculated mol. wts. The relative mol. wts in SDS gel were estimated to be 41 and 33 kd for the precursor and the mature forms, respectively. This anomaly was also reported by other laboratories for the OEE1 protein (Oh-Oka *et al.*, 1986; Tyagi *et al.*, 1987; Watanabe *et al.*, 1987).

Radiolabeled OEE1 precursors were incubated with chloroplasts, after which intact plastids were re-isolated as described in Materials and methods. Thermolysin treatment of re-isolated intact chloroplasts was employed to differentiate bound and imported products. Bound products were susceptible to protease since they were exposed on the outside face of the chloroplast envelope. Imported proteins were located inside the chloroplasts and rendered protease resistant. The suborganellar location of the imported proteins as determined by hypotonically lysing the chloroplasts and subfractionating the components by differential centrifugation (Smeekens *et al.*, 1986). Plastids were subfractionated into stroma, envelopes and thylakoids. Since OEE1 is a thylakoid lumen protein, we used thermolysin treatment of isolated thylakoids to test whether the polypeptides were exposed on the surface (protease sensitive) or internalized into the membrane (protease resistant). As a control, an aliquot of the thylakoid fraction was sonicated briefly in the presence of thermolysin. The ability of thermolysin to completely digest proteins accessible on the stromal face of the thylakoids was evident in Coomassie Blue stained gels. Thermolysin reproducibly cleaved a portion of the CAB protein that was exposed, to yield a band of lower mol. wt. After the different treatments, the samples were analyzed by SDS–PAGE and fluorography.

Precursor OEE1 was imported into the chloroplast, processed and localized in the thylakoid lumen. The import data of OEE1A are presented in Figure 2B. The imported OEE1 protein was protease resistant and was processed to the mature form with a relative mol. wt of 33 kd. The mature OEE1 protein was correctly targeted to the thylakoid lumen rendering it resistant to thermolysin. Unprocessed precursors or intermediate forms were not detected. Deletion of the 35 bp 5′ untranslated DNA sequence of *oee1* had no effect on its *in vitro* expression or import competence of the OEE1 precursor. The product synthesized from pOEE1C was identical to the ones translated from the full-length clones pOEE1A and pOEE1B. The import characteristics were exactly the same as for the OEE1 precursor (OEE1A or OEE1B) (Figure 2A,B).

The transit peptide of the OEE1 precursor is 85 amino acids long (see Figure 1). Processing of the OEE1 precursor occurs between the residues alanine and glutamic acid (Ko *et al.*, 1989). Removal of the OEE1 transit peptide as in the construct OEE1D shown in Figure 2A, abolished the import competence of the translation product (results not shown). These results confirm that the transit peptide plays an essential role in the import of OEE1 into the chloroplast. The inability to import is most likely related to the lack of binding to the envelope by OEE1D. Import assays carried out in the presence of nigericin, an ionophore that inhibits import (Cline *et al.*, 1985), indicate that removal of the transit peptide abolished binding to the chloroplast envelope. Figure 2C, lane 2, shows this binding assay. Native OEE1 precursor (OEE1A) was bound to the chloroplast (Figure 2C, lane 3) whereas the mature OEE1 body (OEE1D) did not indicate that a role in import for the transit peptide includes a function in binding to the presumed receptor in the envelope.

Import of OEE1 protein directed by the RBCS transit peptide

We wished to determine if the mature 33 kd OEE1 protein could be targeted to the stromal compartment instead of the thylakoid lumen when its transit peptide was replaced by

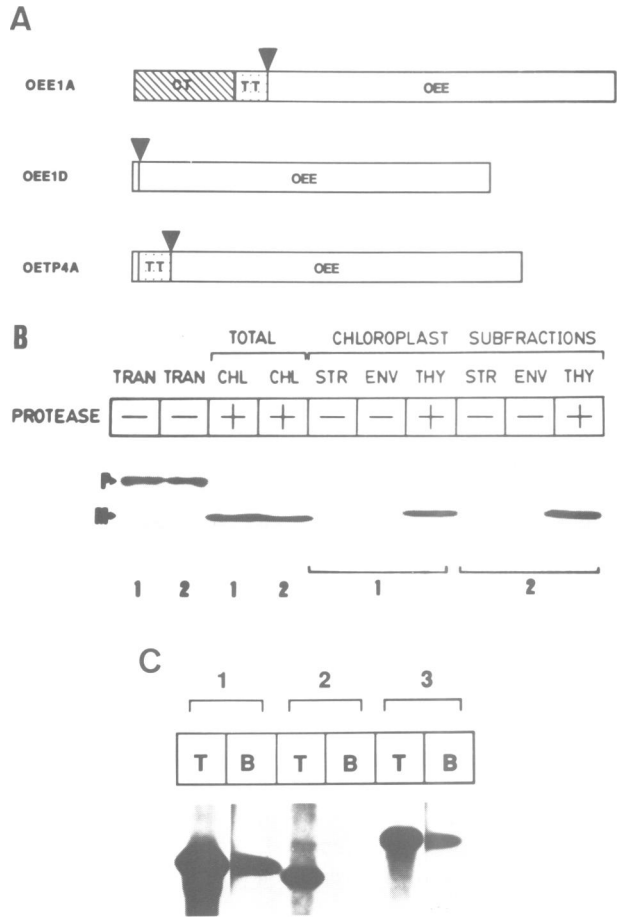

Fig. 2. Importing the 33 kd OEE1 precursor into chloroplasts. The *oee1* gene and various fusion constructs were subcloned into a transcription plasmid and used in *in vitro* import assays. Precursor proteins were generated by *in vitro* transcription and translation of cloned genes. The import assays were processed and analyzed by SDS−PAGE and fluorography. The resulting proteins are represented as bars in **panel A**. Construction schemes for these chimeric precursors are described in Materials and methods. The labels within the bars depict the origin of that segment: CT, putative chloroplast targeting domain of the transit peptide; TT, putative thylakoid lumen targeting domain of the transit peptide; OEE, mature body of the 33 kd oxygen-evolving protein. The solid triangle represents the cleavage/processing site. The first protein (labeled OEE1A) represents the native OEE1 precursor. The import data for OEE1A are shown in **panel B**. The application of sonication or protease-treatment is indicated in the squares. Translation profiles are shown in lanes marked TRAN; total chloroplast protein profiles are marked CHL; stromal, envelope and thylakoid subfractions are marked STR, ENV and THY, respectively. **Lanes 1** represent results of OEE1A and **lanes 2** represent import data of OEE1C. Suborganellar localization of the imported products was determined by subfractionation and thermolysin treatment. Binding assays were carried out as described in Materials and methods for all three constructs shown in panel A. **Lanes 1−3** in **panel C** represent binding results for OETP4A, OEE1D and OEE1A, respectively. The T and B inside the squares correspond to translation and binding samples, respectively. Import assay of OETP4A is discussed and shown in Figure 4C. The OEE1 precursor and mature form are indicated as P and M, respectively.

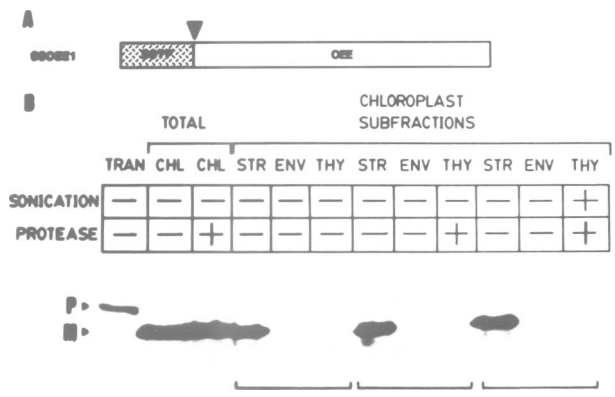

Fig. 3. Import of OEE1 directed by RBCS transit peptide. The 33 kd OEE1 transit peptide was replaced by the RBCS transit peptide, and assayed for import into chloroplasts. The polypeptide is represented in **panel A** (SSOEE1). The RBCS transit peptide is designated SSTP. The treatments and legends are explained in Figure 2. The import data are shown in **panel B**. Suborganellar localization of the imported products was determined by dividing an import reaction into thirds. The three aliquots are represented by the three sets of subfractions (STR, ENV and THY). The thylakoid fractions of each set were given different treatments as indicated in the squares. The STR and ENV subfractions are presented for each set to show the consistency of the untreated aliquots versus the changes observed in the treated thylakoid fractions. The chimeric precursor and the mature OEE1 form are indicated by P and M, respectively.

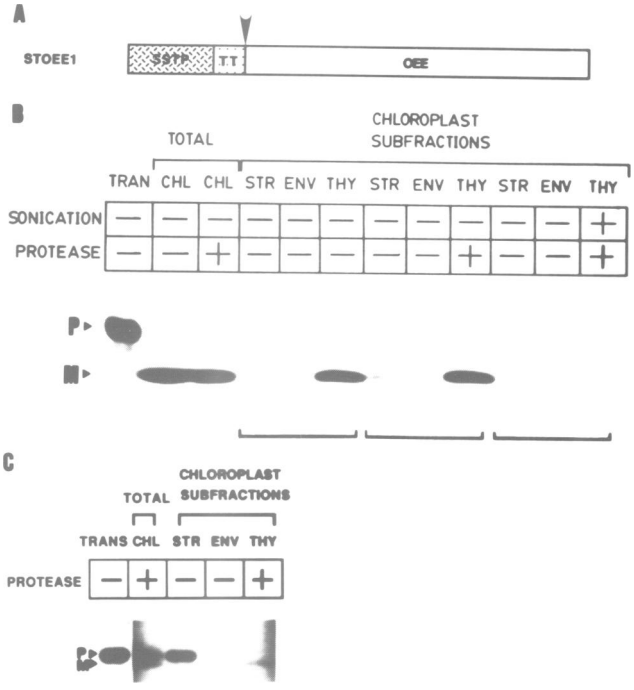

Fig. 4. Importing with the RBCS−OEE1 chimeric transit peptide. DNA sequences for the RBCS−OEE1 chimeric transit peptide were constructed as described in Materials and methods and assayed for import into chloroplasts. The polypeptide is represented in **panel A** as STOEE1. The import results are shown in **panel B**. All treatments and designations are described in the previous figures. In **panel C**, the import results of OETP4A, represented in Figure 2, panel A, are shown. The explanations for the treatments and legends are given in Figures 2 and 3.

the transit peptide of the stromal RBCS protein. A chimeric gene (pSSOEE1) was constructed as described in Materials and methods and the resulting protein (SSOEE1) is represented in Figure 3A. The DNA fragment for the mature body of OEE1 was fused in frame behind the RBCS transit peptide DNA sequence using the vector pSSTP. The processing site for the RBCS transit peptide (SSTP) remained intact and functional. The fusion created four additional amino acids from the polylinker region of the pGEM4 vector. The sequence of the four amino acids was Pro-Gly-Tyr-Arg. The SSOEE1 chimeric precursor was imported, processed and was resistant to subsequent thermolysin treatment.

The import data are shown in Figure 3B. The processed mature form had a relative mol. wt of 33 kd and was located in the stromal compartment as demonstrated by subfractionation experiments. The redirected OEE1 protein was correctly processed to the mature form and appeared to be stable in the stroma. Unprocessed or intermediate forms were not found in any of the subfractions. Protease-resistant radioactive protein bands were not detected in the thylakoid fractions.

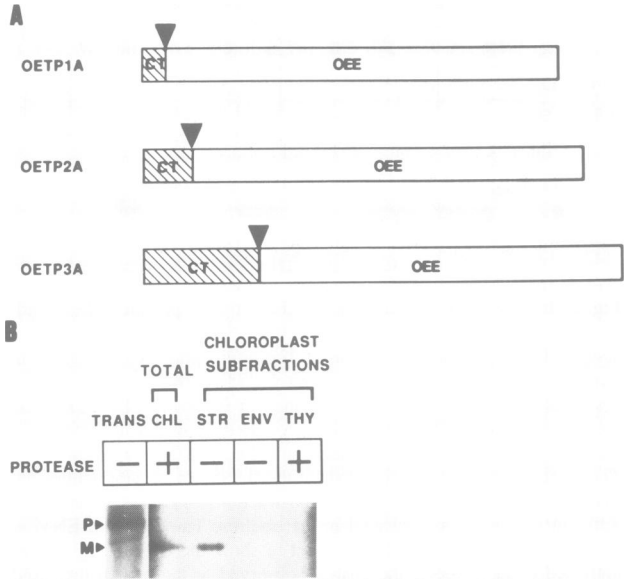

Fig. 5. Truncated OEE1 transit peptide import experiments. Deletions within the OEE1 transit peptide were prepared and assayed for import competence. The details of the construction of these deletions are described in Materials and methods. The polypeptides are illustrated in **panel A**. The polypeptides OETP1A and OETP2A were not imported into chloroplasts (data not shown). Only OETP3A was an import competent protein. The import data are shown in **panel B**.

Import directed by an RBCS – OEE1 chimeric transit peptide

The C-terminal region of the OEE1 transit peptide contains a series of residues which give rise to a prominent hydrophobic peak in a hydropathy profile of the OEE1 precursor (Hoffman *et al.*, 1988; Ko *et al.*, manuscript submitted). A region with similar characteristics has been hypothesized to provide the signal for the transfer of plastocyanin across the thylakoid membrane (Smeekens *et al.*, 1986; Weisbeek *et al.*, 1987). It was of interest to determine if insertion of this putative thylakoid lumenal targeting domain behind the RBCS transit peptide would reinstate the ability to target the OEE1 mature protein to the thylakoid lumen. A DNA sequence was constructed for an RBCS–OEE1 chimeric transit peptide which encoded the entire RBCS transit peptide fused to the C-terminal 27 amino acids of the OEE1 transit peptide. The details of the construction of this gene fusion (pSTOEE1) are described in Materials and methods and the resulting polypeptide (STOEE1) is illustrated in Figure 4A. The import results for the chimeric precursor are shown in Figure 4B. The STOEE1 precursor was imported, processed and localized in the thylakoid lumen. The fusion precursor was correctly

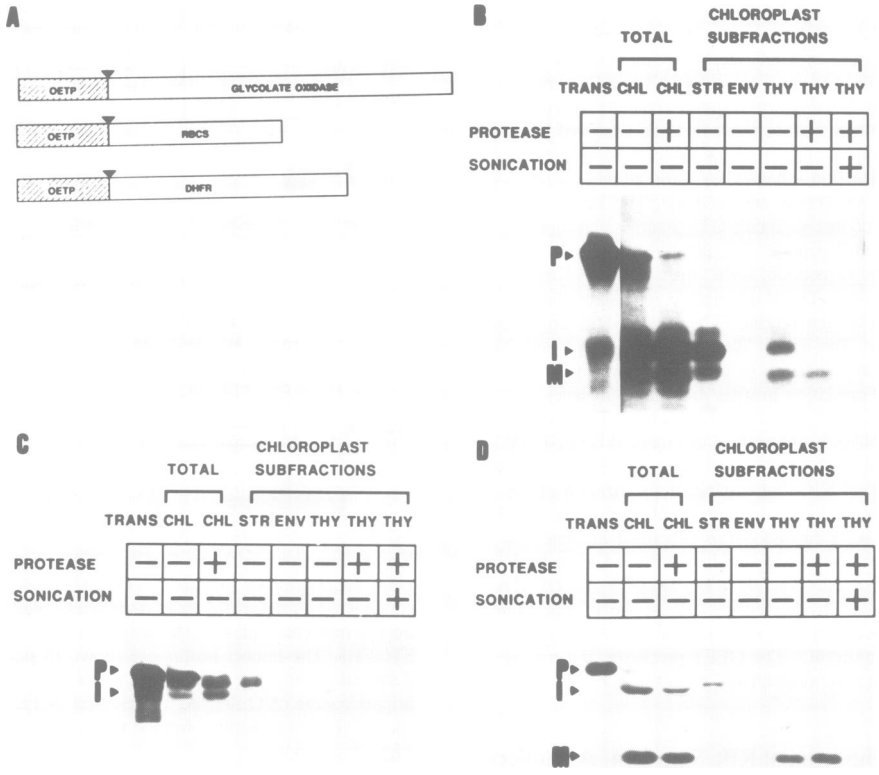

Fig. 6. Targeting foreign proteins to the thylakoid lumen. The DNA sequence for the OEE1 transit peptide was fused to the coding regions of glycolate oxidase (GO), RBCS and dihydrofolate reductase (DHFR). The corresponding polypeptides are represented in **panel A**. Details of the cloning strategies are described in Materials and methods. The chimeric proteins were assayed for import into chloroplasts and for suborganellar localization. The import results are shown for RBCS (**panel B**), GO (**panel C**) and DHFR (**panel D**). The different treatments and designations are discussed in previous figures. Intermediate forms are depicted by the letter I.

processed to the mature form with a relative mol. wt of 33 kd. The integrated product was resistant to thermolysin treatment. Unprocessed or intermediate forms were not detected even after prolonged exposure.

A second type of translation product (OETP4A) was prepared in which the C-terminal 27 residue domain of the OEE1 transit peptide (see Figure 1) was fused to the first seven amino acids of the RBCS transit peptide. The construction scheme for pOETP4A is described in Materials and methods and the resulting fusion protein is illustrated in Figure 2A. This chimeric precursor (OETP4A) was able to bind to isolated chloroplasts (Figure 2C, lane 1) and it was imported (Figure 4C) as indicated by resistance to subseqent thermolysin treatment. Subfractionation experiments established that ~60% of the imported OETP4A remained as the precursor form in the stromal compartment. A portion of the imported protein was processed to the mature form and localized in the thylakoids where it was resistant to protease degradation. The mature form represented ~40% of the imported products. Although for this fusion protein the integration of OEE1 into the thylakoids was far less efficient than OEE1A and STOEE1, the OETP4A transit peptide, which contained only the hydrophobic domain of the OEE1 transit peptide and the first seven amino acids of the RBCS transit peptide, possessed the capability to import OEE1 into chloroplasts and to target it to the thylakoid lumen.

Import of OEE1 protein directed by truncated transit peptides

In order to examine further the possible existence of distinct functional domains within the OEE1 transit peptide, DNA sequences for truncated transit peptides were prepared by using available restriction sites. These DNA fragments were fused, with the aid of linkers present in the multiple cloning site of pGEM4 or pBLUESCRIPT(KS), to the mature OEE1 DNA sequence. The construction schemes for pOETP1, pOETP2 and pOETP3 are described in Materials and methods. The three truncated transit peptides represent approximately successive thirds of the N-terminal putative chloroplast import domain which consists of a total of 58 amino acids (see Figure 1). These truncated transit peptides are illustrated in Figure 5A. The first construct, OETP1A, contains the first 17 amino acids of the OEE1 transit peptide. This truncated transit peptide did not import OEE1 in amounts that were detectable (results not shown). The addition of 14 more amino acids to give a truncated transit peptide of 31 residues still did not appreciably improve the import competence (OETP2A) (data not shown). Import capabilities were observed only with the longest truncated transit peptide, which contains the entire 58 residue segment (OETP3A). The 58 residue segment was capable of importing OEE1 into the chloroplast. The import data of OETP3A are shown in Figure 5B. The imported products were processed to the mature form and were resistant to thermolysin treatment. The final suborganellar location of the mature protein was in the stromal compartment. None of the imported protein was integrated in a protease-protected fashion into the thylakoid lumen membrane.

Import studies with foreign proteins fused to the OEE1 transit peptide

Although it has been possible to direct a number of different foreign (non-chloroplastic) polypeptides to the chloroplast stroma by simply adding a transit peptide to the N terminus of the protein (Van den Broeck *et al.*, 1985; Lubben and Keegstra, 1986; Smeekens *et al.*, 1987; Lubben *et al.*, 1988), the targeting of foreign proteins to the thylakoid lumen has been far more difficult and complex (Smeekens *et al.*, 1986,1987). The factor(s) determining the ability to target a foreign protein into the thylakoid lumen remains to be elucidated. Since the transportability of a foreign protein into the thylakoid lumen most likely involves yet to be identified features, two endogenous chloroplastic and two non-chloroplastic chimeric precursors were constructed using the OEE1 transit peptide and these precursors were assayed for import and suborganellar targeting. The gene sequences of these proteins were placed behind those for the OEE1 transit peptide using the vector pOETP (see Materials and methods). In-frame fusions were made by utilizing the appropriate restriction endonuclease sites available in the gene sequences and the multiple cloning site of pGEM4. The construction schemes for pOETPSS, pOETPGO and pOETPDHFR are described in Materials and methods and the resulting fusion proteins are illustrated in Figure 6A. The OEE1 transit peptide was shown to be capable of importing and targeting to the lumen the mature body of cytochrome *f*, an endogenous chloroplast DNA-encoded thylakoid lumen protein. The results of these experiments are reported separately (Ko *et al.*, manuscript in preparation).

The OEE1 transit peptide was shown to redirect a stromal protein (RBCS) to the thylakoid lumen. The import results for OETPSS are shown in Figure 6B. Replacement of the RBCS transit peptide with the OEE1 transit peptide resulted in the targeting of a significant portion of the mature RBCS to the thylakoid lumen. Most of the imported products were processed to a lower mol. wt intermediate form and rendered thermolysin resistant. This intermediate form was located in the stromal compartment and represented ~80–90% of the imported product. A portion of the processed intermediates was translocated across the thylakoid membrane. The integrated product was further processed to a lower mol. wt form corresponding to the relative size of the mature RBCS. The translocated protein was resistant to thermolysin treatment and was sensitive when sonicated in the presence of protease. This protease-resistant protein represented ~10% of the imported product. The final mature form appeared to be stable during the import and post import time period.

We also assayed the ability of the OEE1 transit peptide to mediate the import of two non-chloroplastic proteins. The two proteins were glycolate oxidase (GO), an enzyme of the photorespiratory carbon cycle present in plant peroxisomes and cytosolic dihydrofolate reductase (DHFR) from mouse. Both chimeric precursors (OETPGO and OETPDHFR) were imported into chloroplasts and processed to lower mol. wt forms (Figure 6C and D). Both imported precursors were protected from protease degradation. However, only DHFR was translocated to the thylakoid lumen and rendered resistant against subsequent exposure of isolated thylakoids to thermolysin. Imported glycolate oxidase remained in the stromal compartment as a form larger than the relative size of native GO but smaller than OETPGO precursor. A portion of the GO intermediate forms was associated with the thylakoids but it was sensitive to protease indicating that these molecules were exposed on the stromal face of the membrane. Protease resistant bands of GO were not detected

in the thylakoid subfraction even after prolonged exposure. In contrast, most of the DHFR was integrated and processed to a form similar in relative size to native DHFR. The mature form was located inside the thylakoid lumen and represented ~60–70% of the imported products. Approximately 30–40% of imported OETPDHFR remained in the stromal compartment as an intermediate form.

Discussion

In the results presented here we have described import experiments involving isolated chloroplasts and *in vitro* generated OEE1 polypeptides. The aim of these experiments was to characterize the nature of the OEE1 transit peptide and determine its role in targeting OEE1 to the thylakoid lumen. The OEE1 precursor, derived from *Arabidopsis thaliana*, was efficiently imported and processed by pea chloroplasts. The OEE1 protein was correctly translocated to the thylakoid lumen, as demonstrated by isolating thylakoids and showing that imported OEE1 protein was resistant to treatment with thermolysin but became sensitive subsequent to sonication. The translocation characteristics of OEE1 were demonstrated to be largely dependent on the nature of the OEE1 transit peptide. In the absence of the transit peptide, OEE1 was not imported, nor did it bind to isolated chloroplasts. Furthermore, the translocation of OEE1 could be altered by changing the transit peptide, and, by fusion to the OEE1 transit peptide, foreign proteins could be targeted to the thylakoid lumen.

The RBCS transit peptide redirects OEE1 protein to the stroma

Import of the OEE1 protein was redirected to the stromal compartment by exchanging the OEE1 transit peptide for that of the stromal RBCS transit peptide. The RBCS transit peptide in the fusion protein retained the processing site and the redirected fusion protein was correctly processed to the mature form and appeared to be stable in the stroma. The results indicate that sequences within the OEE1 transit peptide are primarily responsible for targeting to the lumen. If sequences within the mature OEE1 protein contribute to this targeting then these sequences do not appear to prevent redirection of the protein to alternative chloroplast compartments.

The OEE1 transit peptide contains separate domains for importing and thylakoid targeting

We tested whether the C-terminal hydrophobic domain within the OEE1 transit peptide acted as a targeting domain for the lumen by fusing this sequence to the RBCS transit peptide. The resulting transit peptide contained the entire RBCS transit peptide fused to the C-terminal 27 amino acids of the OEE1 transit peptide. This chimeric transit peptide functioned in targeting OEE1 to the thylakoid lumen in a manner essentially the same as the OEE1 transit peptide. Import was efficiently achieved, presumably via the RBCS transit peptide mediating translocation across both membranes of the chloroplast envelope. In contrast to import mediated by the RBCS transit peptide alone, which delivered OEE1 to the stroma, in this case the OEE1 protein was delivered exclusively to the thylakoid lumen. These experiments provide very good evidence that the C-terminal hydrophobic sequence of the OEE1 transit peptide contains a targeting domain for the thylakoid lumen.

A further experiment was performed to explore the characteristics of the C-terminal hydrophobic domain of the OEE1 transit peptide. In this experiment the first seven amino acids of the RBCS transit peptide were fused to the C-terminal 27 residue domain of the OEE1 transit peptide. Somewhat surprisingly, this fusion peptide directed import and translocation of OEE1 to the lumen, albeit at a level where only 40% of the imported products were integrated versus 100% observed in native OEE1 (OEE1A, Figure 2B) or STOEE1 (Figure 4) imports. The total length of this chimeric transit peptide is only 36 residues yet it contains information for both import into the chloroplast and targeting to the lumen. The exact contribution of the seven amino acids derived from the RBCS transit peptide to the properties of this fusion transit peptide is not known.

Having identified a C-terminal thylakoid lumen targeting domain within the OEE1 transit peptide, it seemed likely that the remaining positively charged N-terminal two thirds of the sequence functions to direct import via translocation across the two membranes of the chloroplast envelope. This was tested directly by determining the import characteristics of the OEE1 protein when it was fused to a truncated 58 amino acid transit peptide in which the C-terminal hydrophobic domain had been deleted. As expected, this protein was translocated to the stroma with no indication of targeting to the lumen. In this respect the truncated OEE1 transit peptide functioned in a manner similar to the RBCS transit peptide in terms of importing into the chloroplast and targeting to the stromal compartment.

We have succeeded in demonstrating that the 85 residue long OEE1 transit peptide contains separate signal domains for importing and for targeting to the thylakoid lumen. The importing domain is present in the N-terminal 58 amino acids and the thylakoid lumen targeting domain is located within the C-terminal 27 residues. From our studies it is quite clear that the C-terminal hydrophobic domain is both sufficient and necessary to direct translocation of OEE1 from the stroma to the lumen. We contrast this observation with the demonstration that integration of CAB proteins into the thylakoid is mediated not by the transit peptide but by sequences within the mature protein (Lamppa, 1988; Hand *et al.*, accompanying manuscript). Clearly, in some cases intra-organellar sorting of chloroplast proteins is determined largely, if not exclusively, by sequences residing within the transit peptide, whereas in other cases this is determined by sequences present within the processed protein.

Based on the experimental results presented here, the bipartite OEE1 transit peptide functions similarly to the transit peptide of the plastocyanin precursor (Smeekens *et al.*, 1986, 1988; Weisbeek *et al.*, 1987). The plastocyanin transit peptide also consists of two domains, one for mediating import into the chloroplast and a separate domain for translocation across the thylakoid membrane. The 'two domain' hypothesis proposed by Smeekens *et al.* (1986) suggests that pre-plastocyanin is transported to the thylakoid lumen in two steps. The import domain mediates translocation across the membranes of the chloroplast envelope. The precursors are processed probably in the stroma at the intermediate processing region by a processing protease located in the stroma. The hydrophobic thylakoid transfer

domain of the intermediate subsequently interacts with the thylakoid membrane and facilitates translocation across the membrane. The translocated intermediate is processed to the mature form by a thylakoid-based processing protease (Hageman *et al.*, 1986; Smeekens *et al.*, 1986, 1987; Weisbeek *et al.*, 1987; Smeekens and Weisbeek, 1988). The results presented here strongly suggest that OEE1 is transported to the lumen by the same two-step processing pathway as plastocyanin. The intermediate forms observed in the import of foreign proteins (Figure 6A–D) indicate that the OEE1 transit peptide is processed at an intermediate site by a stromal-based processing protease. Only translocated products are further processed to the mature form by a thylakoid-based processing protease. The processing of the OEE1 precursor by the two proteases was recently demonstrated by Watanabe *et al.* (1987). The exact nature and location of the intermediate processing site in the OEE1 transit peptide remains to be studied.

The OEE1 transit peptide directs import and targeting of foreign proteins to the thylakoid lumen

It has been difficult to engineer the translocation of foreign proteins to the thylakoid lumen (Smeekens *et al.*, 1986, 1987). In experiments reported in this manuscript we demonstrate that fusion of the OEE1 transit peptide will in certain cases import and target foreign proteins to the thylakoid lumen. The factors that determine the efficiency of targeting are not understood. Whereas the GO protein was not translocated to the lumen, some RBCS protein was, and most of the imported DHFR protein was located inside the thylakoid lumen. In the case of RBCS, assembly into holoenzyme may decrease the efficiency of translocation across the thylakoid membrane.

Additional features may exist within the mature region of certain proteins that may be involved in conjunction with domains in the transit peptide to effect the efficiency of translocation across the thylakoid membrane. Preliminary import data involving deletions from the C-terminal end of two proteins (cytochrome *f* and OEE1) indicate that these deletions abolish targeting to the thylakoid lumen. Both precursors were imported but were not integrated into the thylakoids in a way that rendered them thermolysin resistant. These results point to a possible involvement of C-terminal sequences in translocation across the thylakoid membrane. Whereas the primary translocation signals may reside at the N terminus, recent results indicate that an appropriately unfolded state of the protein may be necessary for efficient translocation (Della-Cioppa and Kishore, 1988; Pfanner *et al.*, 1988; Verner and Schatz, 1988). Conformational characteristics of precursor proteins are likely to be influenced by sequences in addition to those at the N terminus.

Materials and methods

Construction of chimeric genes

A cDNA clone of the nuclear-encoded 33 kd oxygen-evolving protein (OEE1) was previously isolated from an *A.thaliana* λgt10 library (Ko *et al.*, manuscript submitted). The 1200 base pairs (bp) cDNA insert contains sequence information for the complete OEE1 precursor protein. The cDNA insert was retrieved from recombinant phage clones by an *Eco*RI digestion and subcloned in both orientations relative to the transcription plasmid pGEM4 (Promega). These plasmids, designed pOEE1A and pOEE1B, were used to create gene fusions and to synthesize *in vitro* transcripts of native precursor OEE1. *In vitro* transcription was initiated from the SP6 promoter in pOEE1A and from the T7 promoter in pOEE1B.

Deletion of the 35 bp 5′ untranslated nucleotide sequence from *oee1* was achieved by subcloning a 1165 bp internal *Nco*I–*Xba*I fragment into the *Sma*I–*Xba*I sites of pGEM4. The *Nco*I–*Xba*I DNA fragment was recovered by restriction endonuclease digestion of pOEE1A. The *Nco*I end was converted into a blunt end by Klenow (Maniatis *et al.*, 1982). The resulting plasmid was named pOEE1C and *in vitro* transcription was initiated from the SP6 promoter.

A translatable gene construct of the OEE1 mature protein was made by fusing the OEE1 body to the first four amino acids of the RBCS transit peptide. The DNA sequence of the OEE1 mature protein was first subcloned as a 900 bp *Sac*I–*Eco*RI fragment into pGEM4. This plasmid was named pOEEMB. The multiple cloning sites present in pGEM4 enhanced the flexibility of making in-frame gene fusions. An internal 905 bp *Asp*518–*Eco*RI fragment was recovered from pOEEMB. Both ends were made blunt by Klenow before inserting into the *Eco*RV–*Xba*I sites of pSSTP. The *Xba*I site was converted to a blunt end. The vector pSSTP contains the DNA sequence of the RBCS transit peptide and pGEM4. The construction of this plasmid was reported recently (Ko and Cashmore, manuscript submitted). The final plasmid was called pOEE1D and *in vitro* transcription was initiated from the SP6 promoter. The amino acid sequence of the fusion point was -Met-Ala-Ser-Met-Met-Tyr-Arg-Ala-Pro-Lys-.

Replacement of the OEE1 transit peptide with the RBCS transit peptide was accomplished by inserting a 911 bp *Ava*I–*Eco*RI DNA fragment from pOEEMB into the *Bam*HI site of pSSTP. All restriction sites generated were converted to blunt ends by Klenow. The resulting plasmid was designated pSSOEE1 and was transcribed by SP6 polymerase. The amino acid sequence of the fusion point was -Met-Asp-Pro-Gly-Tyr-Arg-.

The OEE1 precursor with a RBCS–OEE1 chimeric transit peptide was made by joining the RBCS transit peptide to an N-terminal deletion of the native OEE1 protein. Unidirectional deletions from the N-terminal end of OEE1 were generated by exonuclease III/S1 digestion of pOEE1B according to Henikoff (1987). The starting point for exonuclease III digestion was the *Sal*I site of pOEE1B. The extent of the deletions was determined by 6% PAGE in 1 × Tris–borate buffer (Maniatis *et al.*, 1982). The appropriate size fractions were selected, digested with *Eco*RI, and separated by 0.7% low melt agarose gel electrophoresis in Tris–acetate buffer (Maniatis *et al.*, 1982). The DNA fragments were excised, extracted and then ligated into the *Eco*RV–*Eco*RI sites of pBLUESCRIPT (SK) (Stratagene). The gene deletion plasmids were transformed, characterized and isolated by standard methods (Maniatis *et al.*, 1982). One plasmid, designated pTTOEE1, contains the C-terminal 27 amino acids of the OEE1 transit peptide and the mature body. This DNA fragment was retrieved by digesting pTTOEE1 with *Cla*I and *Xba*I. The *Cla*I site was converted to a blunt end. The 990 bp *Cla*I–*Xba*I fragment was then inserted into the *Bam*HI–*Xba*I sites of pSSTP. The *Bam*HI site was also made a blunt end. The resulting construct was named pSTOEE1 and transcription was started from the SP6 promoter. The amino acid sequence of the fusion point was -Met-Asp-Pro-Ile-Ser-Leu-Ile-Thr-Gly-.

Truncated OEE1 transit peptides were generated by using the restriction sites available within the DNA sequence. A 300 bp *Eco*RI–*Sac*I fragment encoding the entire OEE1 transit peptide was first subcloned into pGEM4. This vector was designated pEOTP. The first 58 amino acids of the transit peptide were successively dissected into thirds. The lengths of the truncated transit peptides were 17, 31 and 58 residues. The DNA fragments that contained these deletions were the 84 bp *Eco*RI–*Sau*3A, the 126 bp *Eco*RI–*Hin*fI and the 211 bp *Eco*RI–*Hpa*II, respectively (see Figure 1). The *Sau*3A, *Hin*fI and *Hpa*II sites were converted into blunt ends by Klenow before inserting into the *Eco*RI–*Eco*RV sites of pBLUSCRIPT (SK). These vectors were designated pOETP1, pOETP2 and pOETP3, respectively. The mature OEE1 protein body was then rejoined to the truncated transit peptide by using various restriction fragments generated from pOEE1A, pOEE1B or pOEE1C.

A 925 bp *Ava*I fragment containing the mature OEE1 sequence was retrieved from pOEE1A, converted into blunt ends and inserted into the *Hin*cII site of pOETP1. The resulting plasmid was named pOETP1A. A 950 bp *Ava*I–*Hin*dIII fragment was generated from pOEE1C, converted to blunt ends and ligated into the *Hin*cII site of pOETP2. This plasmid was designated pOETP2A. The 925 bp *Ava*I fragment from pOEE1A was inserted into the *Hin*dII site of pOETP3 to yield pOETP3A. The *Hin*dIII site of pOETP3 was made blunt by Klenow before ligation. The amino acid sequences of the fusion points were -Ala-Lys-Ile-Lys-Leu-Ile-Asp-Thr-Val-Ser-Gly-; -Arg-Ser-Thr-Ile-Lys-Leu-Ile-Asp-Thr-Val-Ser-Gly-; and -Asp-Phe-Thr-Asp-Gln-Ala-Ser-Gly-, respectively.

The gene fusion plasmid pOETP4A was made by inserting a 980 bp *Hin*dIII–*Sma*I DNA fragment from pTTOEE1 into the *Cla*I–*Hin*dIII sites of pSSMET. The *Cla*I site was converted into a blunt end prior to ligation. The vector pSSMET was made by inserting the 45 bp *Eco*RI–*Eco*RV fragment of pSSTP, which encodes the first four amino acids of SSTP and its 5′ untranslated sequence, into the *Eco*RI–*Eco*RV sites of pBLUESCRIPT (SK). The amino acid sequence of the fusion point was -Met-Ala-Ser-Met-Ile-Ser-Ser-Leu-Ile-Thr-. Note that the fusion strategy recreated a DNA sequence that coded for the first seven amino acids of the RBCS transit peptide, therefore only -Leu-Ile- are newly created. All constructs made in pBLUSCRIPT (SK) were transcribed using T3 RNA polymerase. The processing or cleavage site of OEE1 was retained in all of the above gene constructs.

Foreign gene constructs were made by inserting the gene sequences behind the DNA sequence of the OEE1 transit peptide (OETP) using the vector pOETP. The mature *rbcS* gene sequence of pSS15 (Coruzzi *et al.*, 1983) was recovered as a 800 bp *Sph*I fragment and inserted into the *Sph*I site of pOETP. In order to achieve an in-frame fusion, pOETP was altered by joining the *Sal*I and *Sma*I sites together. The final plasmid was called pOETPSS. The amino acid sequence of the resulting fusion point was -Gly-Ala-Arg-Tyr-Pro-Arg-Pro-Ala-Gly-Met-Gln-Val-. The OETP-glycolate oxidase fusion was accomplished by inserting a 1500 bp *Sma*I fragment containing the coding region of glycolate oxidase into the *Sma*I site of pOETP. This plasmid was designated pOETPGO [the glycolate oxidase cDNA clone was a generous gift from M.Volokita and C.R.Somerville (Volokita and Somerville, 1987)]. The amino acid sequence of the fusion point was -Gly-Ala-Arg-Tyr-Pro-Gly-Ile-Thr-Asn-. Dihydrofolate reductase was fused to OETP by inserting a 1000 bp *Sac*I–*Bgl* II fragment containing DHFR coding sequences into the *Sac*I–*Bam*HI site of pOETP. The resulting plasmid was named pOETPDHFR. The amino acid sequence of this fusion point was -Gly-Ala-Arg-Ile-Pro-Ala-Ile-Met-. All foreign constructs were transcribed using SP6 polymerase. The processing or cleavage site of OEE1 was retained in all of the foreign gene fusions.

In vitro transcription and translation

Transcription plasmids were linearized at the appropriate restriction site 3′ to the gene construct according to the manufacturer's instructions. Linearized templates were repurified by extraction with phenol:chloroform:isoamyl alcohol (24:24:1) before *in vitro* transcription. The templates were transcribed *in vitro* using either SP6, T7 or T3 RNA polymerase (Melton *et al.*, 1984). Unmethylated cap analog (Pharmacia) was included in the transcription reactions. The transcripts were translated in a wheat germ system containing ^{35}S-radiolabeled methionine (New England Biolabs; Amersham) or TRANS-Label (ICN) which is a mixture of [^{35}S]methionine and cysteine. The wheat germ extract was prepared according to Erickson and Blobel (1983) except that the flotation step was omitted.

Chloroplast import and fractionation

Intact chloroplasts were isolated from pea seedlings (Progress no. 9) grown for 9–11 days in growth chambers set at 21°C under fluorescent lighting with 16:8 h light:dark photoperiod. Chloroplasts were prepared as described by Bartlett *et al.* (1982) or Cline *et al.* (1985). Purified plastids were resuspended in import buffer (1 × HS: 50 mM HEPES–KOH, pH 8.0; 0.33 M sorbitol) and kept on ice until use. The import assays were assembled in 0.3 ml volumes as described by Bartlett *et al.* (1982). The reactions contained an equivalent of 100 μg chlorophyll; ^{35}S-radiolabeled translation products were adjusted to 1 × HS, 10 mM methionine and import buffer to volume. The samples were incubated on a rotary shaker at room temperature under fluorescent lights for 30 min. Intact chloroplasts were re-isolated through 40% percoll cushions and analyzed directly or subjected to further protease treatment (using thermolysin) as described in Smeekens *et al.* (1986). Re-isolated, thermolysin-treated chloroplasts were sub-fractionated and the thylakoid fractions were divided into thirds and given different treatments according to the scheme described by Smeekens *et al.* (1986). The binding of precursors to the chloroplast envelope was assayed using the ionophore nigericin (Calbiochem). Binding assays were performed as described by Cline *et al.* (1985). Aliquots from *in vitro* wheat germ translations and import reactions were analyzed on a 12.5% SDS–polyacrylamide gel using the Laemmli buffer system (Laemmli, 1970). After electrophoresis, the gels were prepared for fluorography using ENHANCE (New England Nuclear) and exposed to Kodak X-AR or Fuji X-ray films. Quantitative analysis of the import data was carried out by scanning autoradiographic signals as described in Giuliano *et al.* (1988).

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References

Bartlett,S.G., Grossman,A.R. and Chua,N.-H. (1982) In Edelman,M., Hallick,R.B. and Chua,N.-H. (eds), *Methods in Chloroplast Molecular Biology*, Elsevier Biomedical Press, Amsterdam, pp. 1081–1091.

Cline,K., Werner-Washburne,M., Lubben,T. and Keegstra,K. (1985) *J. Biol. Chem.*, **260**, 3691–3696.

Coruzzi,G., Broglie,R., Cashmore,A. and Chua,N.-H. (1983) *J. Biol. Chem.*, **258**, 1399–1402.

de Boer,D., Creners,C., Teertstra,R., Smits,L., Hille,J., Smeekens,S. and Weisbeek,P. (1988) *EMBO J.*, **7**, 2631–2635.

Della-Cioppa,A. and Kishore,G.M. (1988) *EMBO J.*, **7**, 1299–1305.

Erickson,A.H. and Blobel,G. (1983) *Methods Enzymol.*, **96**, 38–50.

Giuliano,G., Pichersky,E., Malik,V.S., Timko,M.P., Scolnik,P.A. and Cashmore,A.R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7089–7093.

Hageman,J., Robinson,C., Smeekens,S. and Weisbeek,P. (1986) *Nature*, **324**, 567–569.

Hand,J.M., Szabo,L.J., Vasconcelos,A.C. and Cashmore,A.R. (1989) *EMBO J.*, **8**, 3195–3206.

Henikoff,S. (1987) *Gene*, **28**, 351–359.

Hoffman,N.E., Pichersky,E., Malik,V.S., Ko,K. and Cashmore,A.R. (1988) *Plant Mol. Biol.*, **10**, 435–445.

Ko,K., Granell,A., Bennett,J. and Cashmore,A.R. (1989) *Plant. Mol. Biol.*, in press.

Laemmli,U.K. (1970) *Nature*, **227**, 680–685.

Lamppa,G.K. (1988) *J. Biol. Chem.*, **263**, 14996–14999.

Lubben,T.H. and Keegstra,K. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 5502–5506.

Lubben,T.H., Theg,S.M. and Keegstra,K. (1988) *Photosynthesis Res.*, **17**, 173–194.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Melton,P.A., Krieg,P.A., Rebagliati,M.R., Maniatis,T., Zinn,K. and Green,M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.

Oh-Oka,H., Tanaka,S., Wada,K., Kuwabara,R. and Marata,N. (1986) *FEBS Lett.*, **197**, 63–66.

Pfanner,N., Hartl,F.-U. and Neupert,W. (1988) *Eur. J. Biochem.*, **175**, 205–212.

Schmidt,G.W. and Mishkind,M. (1986) *Annu. Rev. Biochem.*, **55**, 879–912.

Smeekens,S. and Weisbeek,P. (1988) *Photosynthesis Res.*, **16**, 177–186.

Smeekens,S., Bauerle,C., Hageman,J., Keegstra,K. and Weisbeek,P. (1986) *Cell*, **46**, 365–375.

Smeekens,S., van Steeg,H., Bauerle,C., Bettenbroeck,H., Keegstra,K. and Weisbeek,P. (1987) *Plant Mol. Biol.*, **9**, 377–388.

Tyagi,A., Hermans,J., Steppuhn,J., Jansson,Ch., Vater,F. and Hermann,R.G. (1987) *Mol. Gen. Genet.*, **207**, 288–293.

Van den Broeck,G., Timko,M.P., Kausch,A.P., Cashmore,A.R. and van Montagu,M. (1985) *Nature*, **313**, 358–363.

Van den Broeck,G., Van Houtven,A., Van Montagu,M. and Herrera-Estrella,L. (1988) *Plant Sci.*, **58**, 171–176.

Verner,K. and Schatz,G. (1988) *Science*, **241**, 1307–1313.

Volokita,M. and Somerville,C.R (1987) *J. Biol. Chem.*, **262**, 15825–15828.

von Heijne,G. (1983) *Eur. J. Biochem.*, **133**, 17–21.

Watanabe,A., Minami,E., Murase,M., Shinohara,K., Kuwabara,T. and Murata,N. (1987) In Biggins,J. (ed.), *Progress in Photosynthesis Research.* Nijhoff, The Hague, Vol. 4, pp. 629–636.

Weisbeek,P., Hageman,J., Keegstra,K., Robinson,C. and Smeekens,S. (1987) In Biggins,J. (ed.), *Progress in Photosynthesis Research.* Nijhoff, The Hague, Vol. 4, pp. 569–572.

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