Determinants for Cleavage of the Chlorophyll a/b Binding Protein Precursor: a Requirement for a Basic Residue That Is Not Universal for Chloroplast Imported Proteins

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Abstract. We demonstrate that the precursor of the major light-harvesting chlorophyll a/b binding protein (LHCP of Photosystem II), encoded by a Type I gene, contains distinct determinants for processing at two sites during in vitro import into the chloroplast. Using precursors from both pea and wheat, it is shown that primary site processing, and release of a ∼26-kD peptide, depends on an amino-proximal basic residue. Substitution of an arginine at position −4 resulted in an 80% reduction in processing, with the concomitant accumulation of a high molecular weight intermediate. Cleavage occurred normally when arginine was changed to lysine. The hypothesis that a basic residue is a general requirement for transit peptide removal was tested. We find that the precursors for the small

subunit of Rubisco and Rubisco activase do not require a basic residue within seven amino acids of the cleavage site for maturation. In the wheat LHCP precursor, determinants for efficient cleavage at a secondary site were identified carboxy to the primary site, beyond what is traditionally called the transit peptide, within the sequence ala-lys-ala-lys (residues 38–41). Introduction of this sequence into the pea precursor, which has the residues thr-thr-lys-lys in the corresponding position, converted it to a substrate with an efficiently recognized secondary site. Our results indicate that two different forms of LHCP can be produced with distinct NH_2-termini by selective cleavage of a single precursor polypeptide.

A N essential step in the pathway of import into the chloroplast of proteins thus far characterized is the proteolytic removal of the NH_2-terminal extension, the transit peptide. Chloroplasts (Robinson and Ellis, 1984; Abad et al., 1991), similar to mitochondria (Bohni et al., 1983; Hawlischek et al., 1988), appear to contain a general soluble processing enzyme that recognizes the large diversity of imported precursors which initially enter and traverse the stroma. The structural determinants for precursor processing have not been established, although deletions in the COOH-terminus of the transit peptide indicate that it plays an important role (Reiss et al., 1989; Ostrem et al., 1989; Smeekens et al., 1989). Transit peptides differ considerably even for the same protein between species, but sequence comparisons have revealed several potentially critical features for processing (for review see Keegstra, 1989; Gavel and von Heijne, 1990). First, most transit peptides contain a basic residue within seven amino acids of the cleavage site. Of 30 different transit peptides recently compared, 47% have a basic residue positioned between −1 and −4 relative to the processing site, and 80% have either an arginine or lysine between −1 and −7 (Gavel and von Heijne, 1990). For comparison, most precursors targeted to mitochondria contain a basic residue within two amino acids of the site cleaved, which may be essential for proteolysis (Hendrick et al.,

1989). Transit peptides also exhibit a propensity to form a β-sheet structure near their COOH-termini (Gavel and von Heijne, 1990; Cramer et al., 1990). Finally, in a subset of precursors (30%), the motif (val/ile)-X-(ala/cys)↓ala has been identified around the cleavage site, yet there are many exceptions (Gavel and von Heijne, 1990). All of these features have been proposed to in some way influence transit peptide removal, but thus far their importance has not been directly tested.

We have investigated the determinants for processing the precursor of the major light-harvesting chlorophyll a/b binding protein (LHCP[1] of Photosystem II encoded by a Type I gene), one of the most abundant polypeptides of the thylakoid membranes. When imported into wheat or pea chloroplasts in vitro, wheat preLHCP is processed at two sites, which we refer to as primary and secondary sites, giving rise to peptides of ∼26 and 25 kD (Lamppa and Abad, 1987; Clark et al., 1989, 1990). A similar result has been observed for preLHCP derived from a single gene from pea (Cline, 1988; Kohorn and Yokir, 1990), tomato (Pichersky et al., 1987), tobacco (Chaumont et al., 1990), and corn (Dietz and Bogorad, 1987). Although the relative abundance of

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[1] *Abbreviation used in this paper:* LHCP, light-harvesting chlorophyll a/b binding protein.

these two peptides varies depending on the origin of pre-LHCP, cleavage usually occurs preferentially at the primary site upon import, producing the 26-kD form. The primary site has been identified between residues 37 and 38 in the pea precursor (Mullet, 1983; Michel et al., 1990). In light of the prevalence of basic amino acids in the carboxy portion of transit peptides, we asked whether an amino-proximal basic residue is essential for cleavage at the primary site, and have found that this is indeed the case. However, when we tested the universality of this requirement by extending the analysis to the precursors of the small subunit of Rubisco (S) and Rubisco activase (RA), two stroma-localized proteins, we found that the loss of a basic residue in the region of interest had essentially no affect on precursor maturation during import.

Our previous studies suggested that there are distinct determinants for cleavage of preLHCP at the primary and secondary sites, and that these sites are independently recognized. Although both the 26- and 25-kD peptides are produced during import, only the 25-kD peptide is produced in an organelle-free assay enriched for the chloroplast soluble processing enzyme (Abad et al., 1989). Furthermore, a modified precursor with a four amino acid insertion at the transit peptide-mature protein junction gave rise to only the 26-kD peptide upon import, and this mutant was not cleaved in the organelle-free assay. Another mutant with three amino acids deleted at the primary processing site was still cleaved in both assays, producing only the 25-kD peptide (Clark et al., 1989). In this report we localize the determinants for secondary site processing to residues 5–7 amino acids carboxy to the primary site, within a domain previously thought to be at the NH_2-terminus of all mature LHCP molecules of Type I associated with PSII.

Materials and Methods

Plant Growth and Chloroplast Isolation

Peas (*Pisum sativum*, Laxton's Progress) were grown at 22°C under cool-white fluorescent lights on a 16-h light/8-h dark cycle, and harvested 7–8 d after germination. Plants were homogenized with a Polytron at 4°C in 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM Hepes-KOH, pH 7.5, 0.33 M sorbitol, 5 mM sodium ascorbate, 0.25% BSA at a ratio of 10 ml of buffer/g of tissue, fresh weight. Procedures described by Bartlett et al. (1982) were used to purify intact chloroplasts on Percoll gradients.

In Vitro Transcription, Translation, Import, and Organelle-free Processing

The T7 polymerase transcription (United States Biochemical Corp., Cleveland, OH) and the reticulocyte translation reactions (Bethesda Research Laboratories, Bethesda, MD) were carried out as recommended to synthesize ^{35}S-methionine-labeled precursor polypeptides. The import assays (350-μl vol), using radiolabeled precursors and pea chloroplasts, were carried out in 50 mM Hepes-KOH, pH 8.5, 0.33 M sorbitol, 8 mM methionine (HSM) as previously described (Lamppa and Abad, 1987), except that soluble fractions isolated after import were lyophilized instead of TCA precipitated. The organelle-free processing reactions were performed as described (Abad et al., 1989). Basically, 2 μl of radiolabeled precursor from a standard 30-μl reticulocyte translation reaction were incubated with a chloroplast soluble extract for 1 h at 27°C in the presence of 2 μg/ml chloramphenicol. All samples were solublized in loading buffer (0.125 M Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 2% SDS, 10% glycerol) and analyzed by SDS-PAGE on 12 or 15% acrylamide gels, followed by autoradiography. The relative abundance of different protein species was estimated by densitometric analysis.

Clone Construction

The coding sequences for wheat preLHCP (Lamppa et al., 1985) and for pea pre:LHCP (Cashmore, 1984) were inserted into the vector pIBI30 (International Biotech, Inc., New Haven, CT) to create the constructs pWhe: preLHCP and pPea:preLHCP, respectively. Both constructs were mutagenized (see In Vitro Mutagenesis) to create an SphI site at the sequences coding for the primary processing site (GCATGC, where the ATG codes for the methionine at the primary site; see Table I), producing pWhe:preLHCP[s] and pPea:preLHCP[s]. The construct pPea/whe:preLHCP was made by ligating an SphI-HindIII fragment from pWhe:preLHCP[s], containing the sequence coding for the wheat-mature region, with an SphI-HindIII fragment from pPea:preLHCP[s], containing the IBI30 vector and the sequence coding for the pea transit peptide. An SphI-StuI fragment from pPea:preLHCP[s], coding for the pea-mature region, was ligated to an SphI-StuI fragment from pWhe:preLHCP[s], containing the IBI30 vector and the sequence coding for the wheat transit peptide, to produce pWhe/pea:preLHCP. pPea:preS, coding for the small subunit of Rubisco from pea, was constructed as previously described (E9/E15; Abad et al., 1989); its sequence contained an SphI site at the coding sequence for the transit peptide-mature protein junction. pPreS/whe:LHCP was generated by ligating an SphI-PstI fragment from pWhe:preLHCP[s], coding for the wheat LHCP-mature region, with an SphI-PstI fragment from pPea:preS, containing the IBI31 vector and the sequence for the pea preS transit peptide. pPreS/pea:LHCP was constructed by ligating an SphI-PstI fragment from pPea:preLHCP [s], coding for the pea LHCP mature region, with an SphI-PstI fragment from pPea:preS, containing the IBI31 vector and the sequence for the pea preS transit peptide.
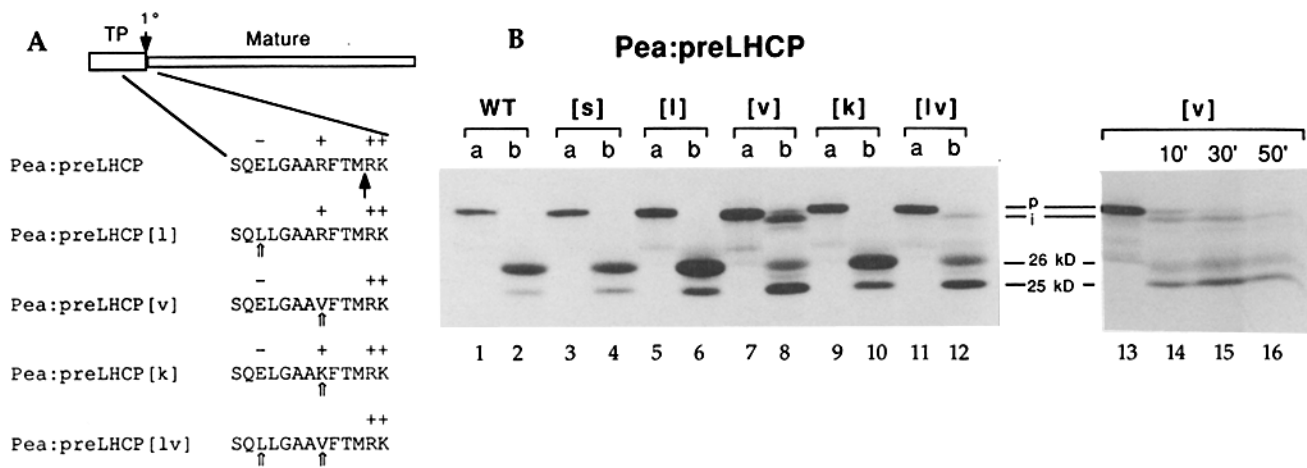
In Vitro Mutagenesis

pWhe:preLHCP, pPea:preLHCP, pPea:preS (see Clone Construction), and the construct coding for the Rubisco activase precursor from spinach (spn:preRA) (Werneke et al., 1988) were modified by oligonucleotide-directed mutagenesis. Mutations were generated using a protocol based on the method of Taylor et al. (1985), following the recommended procedure (Amersham Corp., Arlington Heights, IL). The construct coding for preRA[n] was identified by the presence of a novel HpaI recognition site (GTTAAC), which included the modified nucleotides coding for the substituted asparagine (AAC). All other mutated coding sequences were verified by sequencing, using the method of dideoxy chain termination (Sanger et al., 1977) with reagents supplied by United States Biochemical Corp.
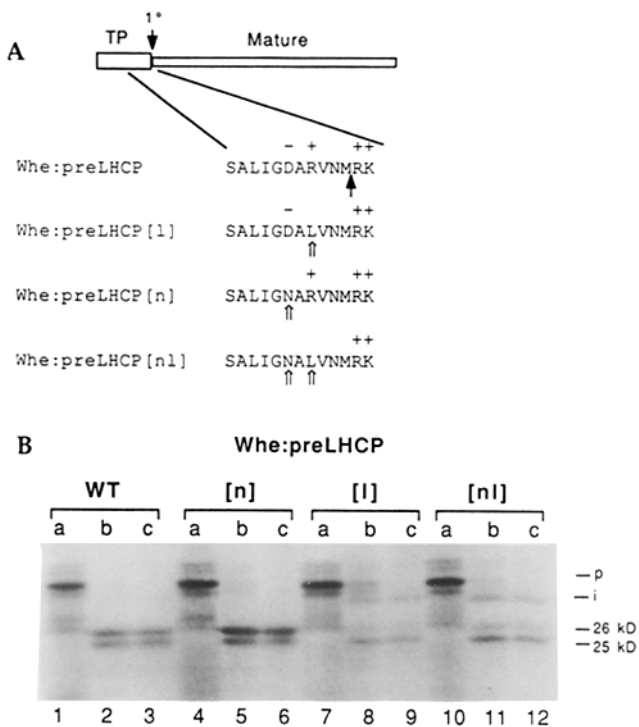
Results

PreLHCP Requires a Basic Residue for Primary Site Cleavage

During import into the chloroplast, pea:preLHCP is cleaved predominantly at its primary site giving rise to a ~26-kD peptide (Fig. 1 B, lane 2); typically 85% of the imported precursor is processed to this size (note the apparent M_r = 26,500, but for ease of discussion it is called 26 kD). Pea: preLHCP contains an arginine at position -4 and a glutamic acid at position -9 relative to the primary site. To investigate the importance of amino-proximal charged residues, especially the arginine, in the processing of preLHCP, these residues were replaced with uncharged amino acids (Fig. 1 A), and the modified precursors were used in an import reaction. Substitution of leucine for the glutamic acid had no effect on processing (Fig. 1 B, lane 6); both the 26- and 25-kD peptides were produced at wild-type levels. However, when valine was substituted for the arginine, creating pea: preLHCP[v], processing at the primary site was inhibited; only 20% of the imported protein was cleaved to yield the 26-kD peptide (Fig. 1 B, lane 8). Processing at the secondary site was not inhibited, and may have been slightly enhanced. There was a concomitant accumulation of a high molecular

A

TP 1°

Mature

Pea:preLHCP   SQELGAARFTMRK

Pea:preLHCP[l]   SQLLGAARFTMRK

Pea:preLHCP[v]   SQELGAAVFTMRK

Pea:preLHCP[k]   SQELGAAKFTMRK

Pea:preLHCP[lv]   SQLLGAAVFTMRK

B   Pea:preLHCP

Figure 1. Importance of a basic residue for primary site cleavage of pea:preLHCP during chloroplast import. (*A*) Schematic representation of the protein coded for by a pea genomic clone of preLHCP is shown (TP, transit peptide). Directly below are the amino acid sequences in single letter code of the primary processing site regions coded for by mutant constructs created by site-directed mutagenesis. The letters in brackets identify each mutant. Basic residues are indicated by a plus sign, acidic residues by a minus sign. Closed arrow indicates the location of primary site cleavage (1°; Mullet, 1983), and open arrows indicate modified amino acids. (*B*) The translation products for wild-type (*WT*) and mutant precursors (lanes *a*) described in *A* were incubated with chloroplasts for 30 min, and then the membrane fractions of thermolysin-treated organelles (lanes *b*) were analyzed by SDS-PAGE. Translation products of pea:preLHCP[v] (lane *13*) were incubated with chloroplasts. Aliquots were taken at 10 (lane *14*), 30 (lane *15*), and 50 min (lane *16*), treated with thermolysin, and membrane fractions were analyzed by SDS-PAGE. *p*, precursor; *i*, intermediate; 26 and 25 kD, mature peptides.

A

TP 1°

Mature

Whe:preLHCP   SALIGDARVNMRK

Whe:preLHCP[l]   SALIGDALVNMRK

Whe:preLHCP[n]   SALIGNARVNMRK

Whe:preLHCP[nl]   SALIGNALVNMRK

B   Whe:preLHCP

Figure 2. Importance of a basic residue for primary site cleavage of wheat:preLHCP during chloroplast import. (*A*) Schematic representation of the protein coded for by a wheat genomic clone of preLHCP is shown (*TP*, transit peptide). Directly below are the amino acid sequences in single letter code of the primary processing site regions coded for by mutant constructs created by site-directed mutagenesis. The letters in brackets identify each mutant. Basic residues are indicated by a plus sign, acidic residues by a minus sign. Closed arrow indicates the location of primary site cleav-

mass intermediate ($\sim$30 kD) that migrated slightly faster than the precursor, which also appeared at low levels. The 30-kD intermediate was imported into the chloroplasts as determined by its resistance to thermolysin treatment. Time course experiments examining the products of import after 10, 30, and 50 min, showed that the imported precursor disappeared more rapidly than the intermediate, which remained relatively stable (Fig. 1 *B*, lanes *14–16*). It is not clear whether this intermediate is on the normal pathway to LHCP maturation, or represents a novel incompletely processed form due to the inhibition of primary site processing.

The defective processing of pea:preLHCP[v] upon import indicated that the arginine was essential for efficient cleavage at the primary site. To determine whether this requirement was arginine-specific or could be satisfied with a different basic residue, lysine was substituted for arginine, creating the mutant pea:preLHCP[k]. This change had no effect on primary site processing upon import (Fig. 1 *B*, lane *10*), demonstrating that the requirement was for a basic amino acid. To maintain the net charge of the transit peptide (+2), a double mutant, pea:preLHCP[lv], was constructed in which leucine replaced glutamic acid and valine replaced arginine. Upon import, this mutant precursor also resulted in inhibition of processing at the primary site; only 40% of the imported protein was cleaved to the 26-kD form, and there was accumulation of the $\sim$30-kD intermediate (Fig. 1 *B*, lane *12*).

age (1°), and open arrows indicate modified amino acids. (*B*) The translation products for wild-type (*WT*) and mutant precursors (lanes *a*) described in *A* were incubated with chloroplasts for 30 min, and then the membrane fractions of either thermolysin-treated (lanes *c*) or nontreated (lanes *b*) organelles were analyzed by SDS-PAGE. *p*, precursor; *i*, intermediate; 26 and 25 kD, mature peptides.
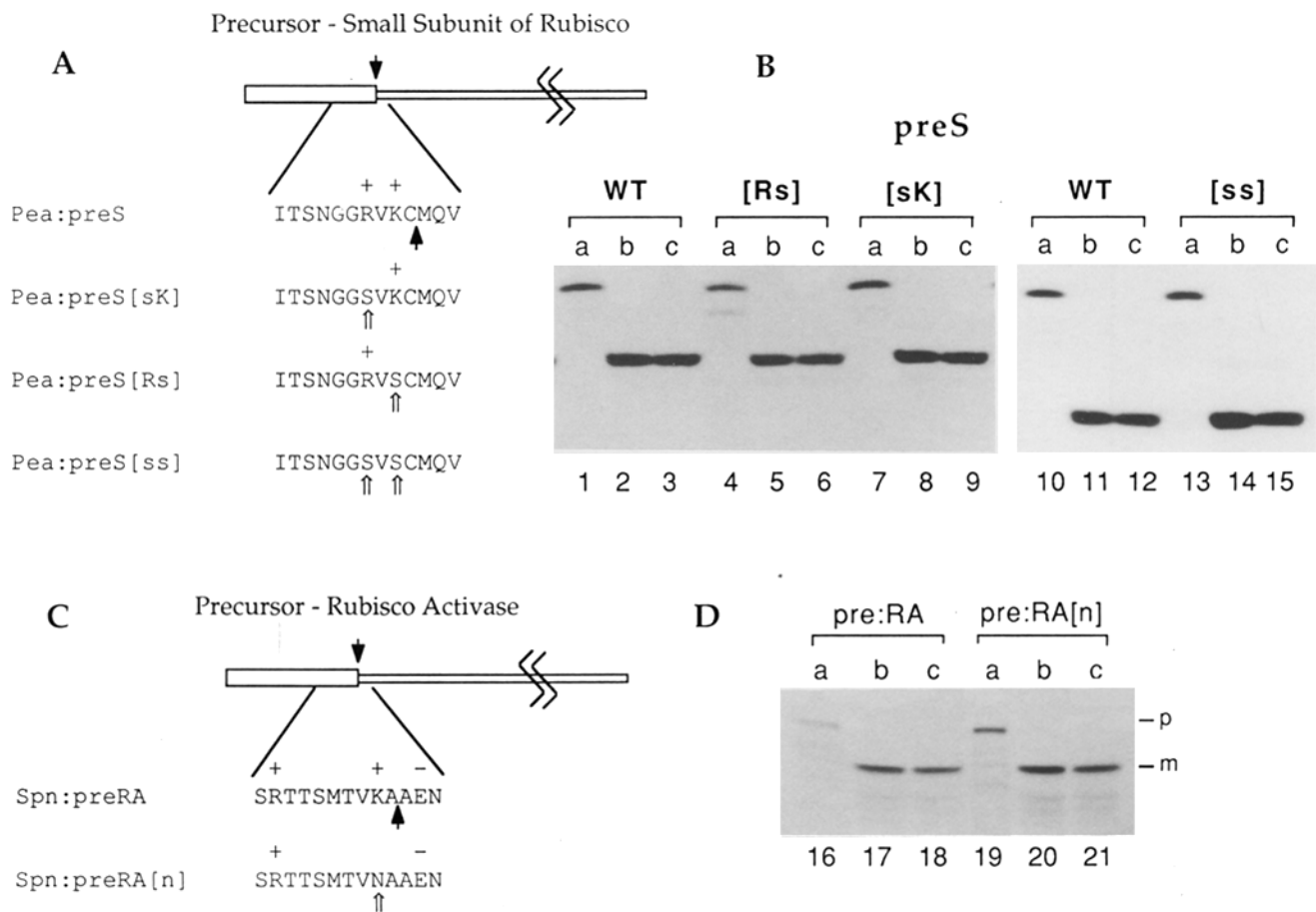
Figure 3. An amino-proximal basic residue is not required for maturation of pea:preS or spn:preRA. Schematic representation of the protein coded for by a pea clone of preS (*A*) and a spinach clone of preRA (*C*) are shown. Directly below are the amino acid sequences in single letter code of the primary processing site regions coded for by mutant constructs created by site-directed mutagenesis of the respective clone. The letters in brackets identify each mutant. Basic residues are indicated by a plus sign, acidic residues by a minus sign. Closed arrow indicates the location of cleavage, and open arrows indicate modified amino acids. (*B* and *D*) Import results of wild-type and mutant precursors (lanes *a*) described in *A* and *C*, respectively. The soluble fractions from chloroplasts either nontreated (lanes *b*) or treated with thermolysin before lysis (lanes *c*). *p*, precursor; *m*, mature.

An examination of the transit peptides of pea and wheat preLHCP reveals that the arginine positioned −4 to the primary site is one of four conserved residues between −1 and −10 (methionine at −1, arginine at −4, alanine at −5, glycine at −7). To determine whether whe:preLHCP primary site processing also required a basic residue at −4, mutant precursors were generated as described in Fig. 2 *A*. Similar to the results for pea:preLHCP, substitution of an uncharged residue, leucine, for the arginine at −4 resulted in an 80% reduction in the amount of 26-kD peptide produced upon import of whe:preLHCP[l] (Fig. 2 *B*, lane *9*). In addition, the accumulation of a high molecular mass intermediate (~29 kD) was again observed as a result of the leucine substitution preventing primary site cleavage. Substitution of asparagine for the aspartic acid positioned −6 to the primary site, producing whe:preLHCP[n], had no effect on the efficiency of processing during import, supporting the conclusion that the amino-proximal acidic residue is not essential for efficient cleavage at the primary site (Fig. 2 *B*, lane *6*). On the other hand, a mutant whe:preLHCP[nl], with the arginine and aspartic acid both changed, was not cleaved efficiently at the primary site, and produced the 29-kD intermediate (Fig. 2 *B*, lane *12*). Taken together, our analyses using substrates from two divergent plants, one a dicot and one a monocot, demonstrate the importance of an amino-proximal basic residue for primary site cleavage of preLHCP.

PreS and preRA Do Not Require a Basic Residue for Cleavage

To extend this analysis, two other precursors were mutagenized to determine if a basic residue near the cleavage site is a general requirement for processing of precursors localized to the chloroplast, or unique to preLHCP. Because preLHCP is an integral thylakoid membrane protein, its import pathway and intraorganellar location of processing might differ from those proteins localized to the stroma. In fact, it has been suggested that preLHCP inserts into the thylakoid membranes before transit peptide removal (Chitnis et al., 1988), although evidence exists to the contrary (Cline et al., 1989). If insertion occurs before processing, the integral membrane configuration of the precursor might place

the basic residue at −4 in a critical position for cleavage. Therefore, pea preS (pea:preS) and spinach preRA (spn:preRA) were selected for mutagenesis based on their localization to the stroma upon import. Mutations in pea:preS and spn:preRA were generated as shown in Fig. 3 (*A* and *C*). Pea:preS contains two basic residues in the region of interest, an arginine positioned −4 and a lysine positioned −2 to the processing site, respectively. When serines were substituted for these two residues either singly or together, no significant effect on processing during chloroplast import was observed (Fig. 3 *B*). Thus, it appears that these basic residues are not essential for pea:preS maturation. In addition, the arginine positioned −2 to the cleavage site of spn:preRA was replaced with asparagine. We observed no difference between processing of the wild-type and mutant precursors during import (Fig. 3 *D*).

Determinants for preLHCP Secondary Site Cleavage Reside within "Mature" LHCP

Both the wheat and pea precursors for LHCP give rise to two peptides upon import, but the relative amounts of the 26- and 25-kD mature forms consistently differ (Table I *A*). Pea:preLHCP was cleaved predominantly at the primary site to produce a 26/25-kD peptide ratio of ∼5:1, while whe:preLHCP was cleaved almost equally at both sites to produce a 26/25-kD peptide ratio of 1.5:1. Although these two precursors retain a high degree of sequence identity, and the mature proteins are 90% conserved, there are two regions of divergence which could influence processing at the two sites. First, while maintaining the general features necessary for transport, their transit peptides differ significantly, especially at the COOH-terminus, where six of ten residues positioned −1 to −10 from the primary cleavage site have diverged. Second, out of the first 10 residues following the primary site, six differences are also found (Table I *A*). A comparison of preLHCP from numerous higher plants has shown this to be the most variable region of the mature protein (Demmin et al., 1989). To investigate the relative importance of these two regions, hybrid precursors were constructed in which the pea and wheat transit peptides were exchanged. Specifically, we asked whether the determinants for selective cleavage characteristic of each substrate were located in the transit

Table I. Analysis of Cleavage at the Primary and Secondary Sites of preLHCP Using Hybrid Precursors and Secondary Site Mutants

Name	Structure/sequence	Import Results	

A

		26/25 kD	n=
Pea:preLHCP[s]	SQELGAARFSM-RKSATTKKVAS	4.9:1	9
Whe:preLHCP[s]	SALIGDARVSM-RKTAAKAKQVS	1.5:1	7
Whe/pea:preLHCP	SALIGDARVSM-RKSATTKKVAS	5.2:1	5
Pea/whe:preLHCP	SQELGAARFSM-RKTAAKAKQVS	1.6:1	5

B

		26/25 kD	n=
Pea:preLHCP[s]	RFSMRKSATTKKVASSGS	4.9:1	9
Pea:preLHCP[aka]	RFSMRKSAAKAKVASSGS	1.2:1	5
Whe:preLHCP[s]	RVSMRKTAAKAKQVSSSS	1.5:1	7
Whe:preLHCP[ttk]	RVSMRKTATTKKQVSSSS	*	5

The name of each precursor is indicated on the left. The structure of each precursor is shown in the middle along with the amino acid sequence in single letter code of the region of interest. The estimated ratio of 26 kD to 25 kD mature peptides observed after precursor import into pea chloroplasts is presented on the right (26/25 kD). The ratio was determined by densitometry of autoradiographs averaged over *n* experiments. The locations of primary (1°) and secondary (2°) sites are indicated by inverted triangles. (*A*) Analysis of import using the hybrid precursors. The dash (−) in each amino acid sequence indicates the location of the primary site and the location of the transit peptide-mature protein junction. (*B*) Analysis of import using the secondary site mutant precursors. Amino acid sequences covering the primary and secondary sites of each precursor are shown (see text). The modified residues are indicated with an arrow.
* Whe:preLHCP[ttk] produced no detectable 25-kD peptide, but 10% of the imported protein was a high molecular mass intermediate of ∼29 kD.

peptide, or carboxy to the primary site within the mature region of LHCP. The hybrid whe/pea:preLHCP contained the wheat transit peptide joined to the pea mature protein, and in pea/whe:preLHCP the pea transit peptide was joined to the wheat mature protein. In both cases the primary and secondary sites were maintained without the addition of extra residues (see Table I *A*). To accomplish this, an SphI site was
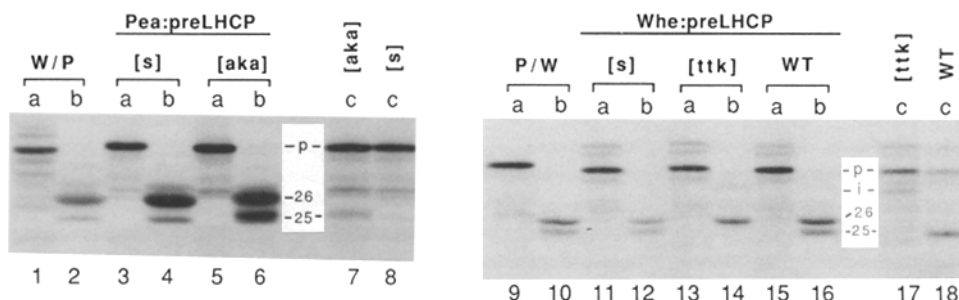

Figure 4. Import and processing of hybrid precursors and secondary site mutants. The translation products for wild-type (*WT*) and mutants precursors (lanes *a*) described in Table I were incubated with chloroplasts for 30 min, and then the membrane fractions of thermolysin-treated organelles (lanes *b*) were analyzed by SDS-PAGE. Translation products were also incubated with a chloroplast-soluble extract (lanes *c*). *W/P*, whe/pea: preLHCP; *P/W*, pea/whe:preLHCP; *p*, precursor; *i*, intermediate; 26 and 25 kD, mature peptides.

engineered into the wheat and pea coding sequence at the primary site, resulting in a serine substitution at −2 in the polypeptides pea:preLHCP[s] and whe:preLHCP[s]. This substitution had no effect on the ratio of 26 to 25 kD peptides produced upon import (Fig. 4, compare lanes 12 and 16; see also Fig. 1 B, lanes 2 and 4). The results of incubating the hybrid precursors with intact chloroplasts are shown in Fig. 4 and summarized in Table I A. The hybrid whe/pea:pre-LHCP, containing the pea mature region, produced a 26/25-kD peptide ratio of 5.2:1 (Fig. 4, lane 2), almost identical to wild-type pea precursor. The hybrid pea/whe:preLHCP, containing the wheat mature region, produced a 26/25-kD peptide ratio of 1.6:1 (Fig. 4, lane 10), characteristic of the wild-type wheat precursor.

In summary, the determinants for utilization of the secondary site reside carboxy to the primary site at the NH_2-terminus of "mature" LHCP. Because the secondary cleavage site is located in this region, between lys40 and ala41 in the wheat precursor (Abad et al., 1991), it seemed likely that the efficiency of secondary site recognition per se was governing the ratio of the 26- and 25-kD peptides produced upon import. That is, in contrast to whe:preLHCP, the pea precursor contained a poorly recognized secondary site. This was corroborated by incubation of the wheat and pea precursors with a chloroplast soluble extract in organelle-free processing reactions. This extract contains an endopeptidase, with the properties of the general stromal processing enzyme (Abad et al., 1989), that cleaves preLHCP only at the secondary site (Abad et al., 1991). Whe:preLHCP was processed efficiently in this reaction, whereas pea:preLHCP was processed to a very limited extent (Fig. 4, compare lanes 8 and 18).

The NH_2-terminal Domain of LHCP Contains an AKA Sequence Essential for Efficient Secondary Site Processing

We have recently established that the secondary processing site in the wheat precursor occurs between lys40 and ala41, within the sequence AK↓AK (Abad et al., 1991). This sequence is not found at the NH_2-terminus of pea:LHCP, which has a TTKK in the same position. We converted the three critical residues (underlined) in the pea substrate to AKA (pea:preLHCP[aka]), and conversely, changed the AKA to TTK in the wheat precursor (whe:preLHCP[ttk]) to investigate if this would be sufficient to alter secondary site cleavage (Table I B). In essence, the secondary cleavage sites were switched. When these precursors were incubated with intact chloroplasts, pea:preLHCP[aka] was processed efficiently at the secondary site to yield a 26/25-kD peptide ratio of 1.2:1 (Fig. 4, lane 6; see Table I B). In striking contrast, whe:preLHCP[ttk] showed a loss of secondary site processing; no 25-kD protein was detectable (Fig. 4, lane 14). Most of the imported whe:preLHCP[ttk] precursor was processed at the primary site to 26 kD, although ~10% of the imported protein was converted to a ~29-kD intermediate, similar to that observed for whe:preLHCP[l] (Fig. 2). To substantiate that it was secondary site cleavage that was being affected by these changes, each precursor was incubated in the organelle-free processing reaction (Fig. 4, lanes c). Pea:preLHCP[aka] was processed much more efficiently than pea:preLHCP[s] (Fig. 4, lanes 7 and 8), confirming that the TTK to AKA conversion produces an efficiently recognized secondary
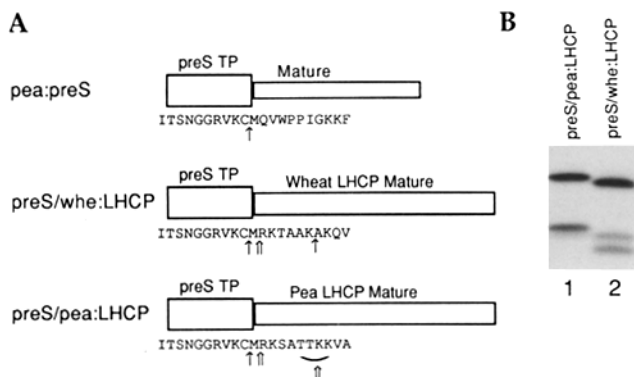
site. On the other hand, whe:preLHCP[ttk] was not processed to any detectable level, although it did give rise to a ~29-kD intermediate (Fig. 4, lane 17). We conclude that a limited number of residues confer specificity for secondary site cleavage.

AKA Motif Recognized Independently of the LHCP Transit Peptide

To determine if the LHCP transit peptide is required for cleavage at the AKA motif, hybrids were constructed with the wheat and pea LHCP "mature" regions placed carboxy to the transit peptide of pea:preS. This created the hybrids preS/whe:LHCP and preS/pea:LHCP, each potentially with three cleavable bonds: one between cysteine and methionine donated from preS, and the primary and secondary sites from preLHCP (see Fig. 5 A). In an organelle-free processing reaction, preS/pea:LHCP produced only a 26.5-kD peptide (Fig. 5 B, lane 1). Note again that pea LHCP migrates slightly slower than the wheat 26-kD protein. Because the LHCP transit peptide is absent and primary site cleavage does not occur in the organelle-free reaction, the 26.5-kD peptide is most likely the result of cleavage at the preS site (following cysteine), which is normally efficiently recognized in the organelle-free reaction (Abad et al., 1989). Significantly, the pea:LHCP secondary site was not recognized in this hybrid as indicated by the absence of the 25-kD peptide. On the other hand, the hybrid preS/whe:LHCP produced both the 26- and 25-kD peptides (Fig. 5 B, lane 2). The 26-kD peptide is probably due to cleavage at the preS site, and the 25-kD peptide from cleavage at the whe:LHCP secondary site. It appears that the AKA motif for secondary site processing can be recognized independently of the LHCP transit peptide.

Discussion

In this study we show that preLHCP contains two processing sites with distinct determinants for transit peptide removal. Efficient cleavage at the primary site, releasing a 26-kD peptide which is usually the predominant species upon import, depends on the presence of a basic residue at position −4. The same result was obtained for preLHCP from both wheat and pea, representatives of the monocots and dicots, respectively. Interestingly, an arginine or lysine in this position has been conserved in all preLHCP molecules (Type I of PSII) described to date (see Demmin et al., 1989), although this is in general a variable region of the transit peptide. We investigated whether the requirement for an amino-proximal basic amino acid is universal, because one is found within 7 amino aids of the cleavage site in the large majority (80%) of imported proteins, and it has been speculated that this may be an important feature for processing (Gavel and von Heijne, 1990). However, extending our analysis to the precursors of S and RA, localized to the stromal compartment, we found this is not the case. Modified precursors, with uncharged amino acids substituted for basic residues in the region of interest, were imported and processed as efficiently as the wild type polypeptides. Thus, despite their prevalence at the COOH-termini of many transit peptides, there is not a strict requirement for a basic residue in this domain for proteolytic processing upon precursor import into

A

pea:preS

preS TP | Mature

ITSNGGRVKCMQVWPP IGKKF

preS/whe:LHCP

preS TP | Wheat LHCP Mature

ITSNGGRVKCMRKTAAKAKQV

preS/pea:LHCP

preS TP | Pea LHCP Mature

ITSNGGRVKCMRKSATTKKVA

B

preS/pea:LHCP preS/whe:LHCP

1   2

Figure 5. Organelle-free processing of LHCP fused to the transit peptide of preS. (*A*) Schematic representations of pea preS and the hybrids preS/whe:LHCP and preS/pea:LHCP are shown (*TP*, transit peptide). Directly below each is the amino acid sequence in single letter code of the processing region. Closed arrows (↑) indicate peptide bonds predicted to be efficiently cleaved by a chloroplast soluble extract. Open arrows (⇑) indicate peptide bonds predicted to not be efficiently cleaved. (*B*) Translation products of hybrid precursors described in *A* were incubated with a chloroplast soluble extract and analyzed by SDS-PAGE.

the chloroplast. The fact that preLHCP requires a basic residue for processing at the primary site, suggests that the mechanism by which it is cleaved differs from that of preS and preRA.

PreLHCP also contains a secondary cleavage site, although it is not as efficiently recognized in some preLHCP substrates from different species. Wheat has an efficiently recognized site, and usually produces nearly equal amounts of the 26- and 25-kD peptides (Lamppa and Abad, 1987; Clark et al., 1989, 1990). On the other hand, the pea precursor used in the present study, and recently by others (Cline, 1988; Cline et al., 1989), is poorly recognized at the secondary site during import, and as a consequence, production of the 25-kD peptide has often been overlooked. Furthermore, pea:preLHCP does not cleave well in the organelle-free assay, which is specific for secondary site cleavage (Lamppa and Abad, 1987; Abad et al., 1989; Clark et al., 1989). NH₂-terminal sequence analysis of the 25-kD peptide released from wheat preLHCP synthesized in *E. coli* has located the secondary site between residues lys40 and ala41 (Abad et al., 1991), six amino acids carboxy to the primary processing site, within the sequence RKTAA**K**AK (underlined). These residues are not present in the pea precursor, instead one finds RKSATTKK in the same position. However, the sequence KAK is found (see underline below) in two-thirds of the precursors in the Type I class, which all share the sequence motif RKT(V/A)(T/A)KAK at the NH₂-terminus of "mature" LHCP. Hybrid precursors, in which the transit peptides of pea and wheat were exchanged, provided the first indication that the determinants for cleavage at the secondary site reside within this region, explaining why pea:preLHCP was such a poor substrate. This was confirmed by substituting the amino acids TTK with AKA in pea:preLHCP, and finding that the new precursor was now cleaved at the secondary site almost as efficiently as the wheat precursor, and it became cleavable in the organelle-free assay,

where only the secondary site is recognized. Conversely, changing the residues AKA in whe:preLHCP to TTK resulted in a complete loss of secondary site processing. Interestingly, in a recent study designed to investigate the migration of pea LHCP in the thylakoid membranes under different light regimes, it was observed that the conversion of the two threonines to alanine in a pea precursor produced a substrate more susceptible to a second processing event upon import (Kohorn and Yakir, 1990). In view of our own results, we interpret their data to indicate that secondary site recognition was enhanced by this change, analogous to the TTK to AKA conversion described herein. We suggest that precursors with the same sequence motif as whe:preLHCP following the primary cleavage site have the potential to be cleaved at two sites during import. It should be noted, however, that a mutant precursor with a four amino acid insertion between the transit peptide and mature protein abolished secondary site processing (Clark et al., 1989). Thus, the presence of this motif alone is not sufficient for cleavage but must be located within the correct structural context, i.e., conformation of the precursor.

An important question arises, is the same chloroplast enzyme responsible for proteolytic processing of preLHCP at both the primary and secondary sites? To date, we have not been able to reconstitute processing at the primary site in the organelle-free reaction, although our studies have not been exhaustive. However, a soluble enzyme with the properties of the general stromal processing endopeptidase (Robinson and Ellis, 1984) cleaves preLHCP at the secondary site: the enzyme is ~200-kD; it is sensitive to chelators, and functions optimally at basic pH at 26°C (Abad et al., 1989). In fact, a partially purified enzyme (7,000-fold enrichment based on recovered protein) also cleaves preS and preRA, as well as the precursors for plastocyanin, Hsp21, and acyl carrier protein (Abad et al., 1991). Therefore, either cleavage at the primary site of preLHCP does not occur because a key component, which normally facilitates its recognition in vivo, is missing in the organelle-free assay, or, alternatively, a different enzyme is involved with different requirements for detection.

In addition to there being distinct determinants for processing at the primary and secondary sites, our mutational analysis indicates that production of the 26- and 25-kD peptides can occur independently, i.e., the 26-kD peptide is not an obligatory intermediate that is subsequently cleaved to the 25-kD form. The 25-kD peptide is still produced at normal levels during import even when cleavage at the primary site is inhibited by amino acid substitution (see results for pre:preLHCP[v]; Fig. 1) or deletion (Clark et al., 1989). We observed no accumulation of the 26-kD peptide in the organelle-free assay when secondary site processing is blocked (Clark et al., 1989). We predict that preLHCP has, in effect, two transit peptides of different length, in wheat one ending at met34 and another at lys40. Selective cleavage at these two sites would produce two forms of LHCP with different NH₂-termini. Thus, as well as being encoded by a multigene family (see Demmin et al., 1989), selective cleavage of a single precursor contributes to the heterogeneity of LHCP which is found in vivo.

The physiological significance of secondary site cleavage is unknown at present, but it could have an important role in the organization of the light-harvesting complex. The NH₂-

terminal domain absent from the 25-kD peptide (RKTAAK in whe:LHCP) is rich in basic amino acids, and has been implicated in thylakoid stacking (Mullet, 1983). Its removal may influence how the 25-kD form of LHCP inserts into the membranes, and ultimately its final topology. Furthermore, the domain extends into the stroma and contains a threonine residue that has been shown to be a preferred site for phosphorylation (Mullet, 1983; Michel and Bennet, 1989). Hence, the 25-kD peptide may be phosphorylated at an alternate position, perhaps within the cluster of serines which are retained at the NH_2-terminus, or not at all. As an adaptive strategy in response to high intensity light, it has been proposed that phosphorylation of a subpopulation of LHCP triggers its migration away from PSII, decreasing the size of the peripheral light-harvesting complex (Anderson and Andersson, 1988). To explore the significance of secondary versus primary site cleavage, we are currently investigating when these sites are utilized during chloroplast development.

References

Abad, M. S., S. E. Clark, and G. K. Lamppa. 1989. Properties of a chloroplast enzyme that cleaves the chlorophyll a/b binding protein precursor. *Plant Physiol.* 90:117-124.

Abad, M. S., J. E. Oblong, and G. K. Lamppa. 1991. A soluble chloroplast enzyme cleaves preLHCP made in E. coli to a mature form lacking a basic N-terminal domain. *Plant Physiol.* 96:1221-1228.

Anderson, J. M., and B. Andersson. 1988. The dynamic photosynthetic membrane and regulation of solar energy conversion. *TIBS (Trends Biochem. Sci)* 13:351-355.

Bartlett, S., A. R. Grossman, and N.-H. Chua. 1982. In vitro synthesis and uptake of cytoplasmically synthesized chloroplast proteins. *In* Methods in Chloroplast Molecular Biology. M. Edelman, R. B. Hallick, and N.-H. Chua, editors. Elsevier Biomedical New York. 1081-1091.

Bohni, P. C., G. Daum, and G. Schatz. 1983. Import of proteins into mitochondria: partial purification of a matrix-located protease involved in cleavage of mitochondrial precursor polypeptides. *J. Biol. Chem.* 258:4937-4943.

Cashmore, A. R. 1984. Structure and expression of a pea nuclear gene encoding a chlorophyll a/b binding polypeptide. *Proc. Natl. Acad. Sci. USA.* 81:2960-2964.

Chaumont, F., V. O'Riordan, and M. Boutry. 1990. Protein transport into mitochondria is conserved between plant and yeast species. *J. Biol. Chem.* 265:16856-16862.

Chitnis, P. R., D. T. Morishige, R. Nechushtai, and P. J. Thornber. 1988. Assembly of the barley light-harvesting chlorophyll a/b proteins in barley etiochloroplasts involves processing of the precursors on thylakoids. *Plant Mol. Biol.* 11:95-107.

Clark, S. E., M. S. Abad, and G. K. Lamppa. 1989. Mutations at the transit peptide-mature protein junction separate two cleavage events during chloroplast import of the chlorophyll a/b-binding protein. *J. Biol. Chem.* 264:17544-17550.

Clark, S. E., J. E. Oblong, and G. K. Lamppa. 1990. Loss of efficient import and thylakoid insertion due to N- and C-terminal deletions in the light-harvesting chlorophyll a/b binding protein. *Plant Cell.* 2:173-184.

Cline, K. 1988. Membrane insertion, proteolytic processing, assembly into LHC II, and localization to appressed membranes occurs in chloroplast ly-sates. *Plant Physiol.* 86:1120-1126.

Cline, K., D. R. Fulsom, and P. V. Viitanen. 1989. An imported thylakoid protein accumulates in the stroma when insertion into thylakoids is inhibited. *J. Biol. Chem.* 264:14225-14232.

Cramer, W. A., J. W. Shiver, P. N. Furbacher, and K. Keegstra. 1990. Amphipathic β-sheet domains in chloroplast transit peptides. *Curr. Res. Photosyn.* 3:799-802.

Demmin, D. S., E. J. Stockinger, Y. C. Chang, and L. L. Walling. 1989. Phylogenetic relationships between the chlorophyll a/b binding protein (*CAB*) multigene family: an intra- and interspecies study. *J. Mol. Evol.* 29:266-279.

Dietz, K.-J., and L. Bogorad. 1987. Plastid development in *Pisum sativum* leaves during greening. II. Post-translational uptake by plastids as an indicator system to monitor changes in translatable mRNA for nuclear-encoded plastid polypeptides. *Plant Physiol.* 85:816-822.

Gavel, Y., and G. von Heijne. 1990. A conserved cleavage-site motif in chloroplast transit peptides. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 261:455-458.

Hawlitschek, G., H. Schneider, B. Schmidt, M. Tropschung, F.-U. Hartl, and W. Neupert. 1988. Mitochondrial protein import: identification of processing peptidase and of PEP, a processing enhancing protein. *Cell.* 53:795-806.

Hendrick, J. P., P. E. Hodges, and L. E. Rosenberg. 1989. Survey of amino-terminal proteolytic cleavage sites in mitochondrial precursor proteins: leader peptides cleaved by two matrix proteases share a three-amino acid motif. *Proc. Natl. Acad. Sci. USA.* 86:4056-4060.

Keegstra, K., L. J. Olsen, and S. M. Theg. 1989. Chloroplastic precursors and their transport across the envelope membranes. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40:471-501.

Kohorn, B. D., and D. Yakir. 1990. Movement of newly imported light-harvesting chlorophyll-binding protein from unstacked to stacked thylakoid membranes is not affected by light treatment or absence of amino-terminal threonines. *J. Biol. Chem.* 265:2118-2123.

Lamppa, G. K., and M. S. Abad. 1987. Processing of a wheat light-harvesting chlorophyll a/b protein precursor by a soluble enzyme from higher plant chloroplasts. *J. Cell Biol.* 105:2641-2648.

Lamppa, G. K., G. Morelli, and N.-H. Chua. 1985. Structure and developmental regulation of a wheat germ encoding the major chlorophyll *a/b* binding polypeptide. *Mol. Cell Biol.* 5:1370-1378.

Michel, H., and J. Bennett. 1989. Use of synthetic peptides to study the substrate specificity of a thylakoid protein kinase. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 254:165-170.

Michel, H. P., W. E. Buvinger, and J. Bennett. 1990. Redox control and sequence specificity of a thylakoid protein kinase. *Curr. Res. Photosyn.* 2:747-753.

Mullet, J. E. 1983. The amino acid sequence of the polypeptide segment which regulates membrane adhesion (grana stacking) in chloroplasts. *J. Biol. Chem.* 258:9941-9948.

Ostrem, J. A., R. T. Ramage, H. Bohnert, and C. Wasmann. 1989. Deletion of the carboxy-terminal portion of the transit peptide affects processing but not import or assembly of the small subunit of ribulose-1,5-bisphosphate carboxylase. *J. Biol. Chem.* 264:3662-3665.

Pichersky, E., N. E. Hoffman, V. S. Malik, R. Bernatzky, L. Szabo, and A. R. Cashmore. 1987. The tomato Cab-4 and Cab-5 genes encode a second type of CAB polypeptides localized in Photosystem II. *Plant Mol. Biol.* 9:109-120.

Reiss, B., C. C. Wasmann, J. Schell, and H. J. Bohnert. 1989. Effect of mutation on the binding and translocation functions of a chloroplast transit peptide. *Proc. Natl. Acad. Sci. USA.* 86:886-890.

Robinson, C., and R. J. Ellis. 1984. Transport of proteins into chloroplasts: partial purification of a chloroplast protease involved in the processing of imported precursor polypeptides. *Eur. J. Biochem.* 142:337-342.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463-5467.

Smeekens, S., D. Geerts, C. Bauerle, and P. Weisbeek. 1989. Essential function in chloroplast recognition of the ferrodoxin transit peptide processing region. *Mol. Gen. Genet.* 216:178-182.

Taylor, J. W., J. Ott, and F. Eckstein. 1985. The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. *Nucleic Acids Res.* 13:8765-8785.

Werneke, J. M., R. E. Zielinski, and W. L. Ogren. 1988. Structure and expression of spinach leaf cDNA encoding ribulosebisphosphate carboxy/oxygenase activase. *Proc. Natl. Acad. Sci. USA.* 85:787-791.