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Two nuclear mutations disrupt distinct pathways for targeting proteins to the chloroplast thylakoid

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Results of *in vitro* experiments have suggested the existence of at least three pathways by which nuclear-encoded proteins are targeted to the chloroplast thylakoid membrane. However, few components of the targeting machinery have been identified and the relationship between the three pathways is not clear. To investigate mechanisms underlying thylakoid protein targeting, we identified nuclear mutations in maize that cause targeting defects. We found two mutations, *tha1* and *hcf106*, that disrupt the localization of different sets of proteins to the thylakoid lumen. The *tha1* mutation interferes with the targeting of one chloroplast-encoded protein, cytochrome f, and three nuclear-encoded proteins, plastocyanin, the *psaF* gene product and the 33 kDa subunit of the oxygen-evolving complex. The *hcf106* mutation interferes with the targeting of the 16 and 23 kDa subunits of the oxygen-evolving complex. The *tha1* and *hcf106* phenotypes provide the first *in vivo* evidence supporting the existence of two distinct thylakoid-targeting pathways. Their phenotypes also provide evidence that one chloroplast-encoded protein, cytochrome f, engages the *'tha1'* pathway, indicating that nuclear- and chloroplast-encoded proteins can be targeted via common machinery.

Keywords: chloroplast/maize/organelle biogenesis/protein targeting/thylakoid

Introduction

Chloroplasts are structurally complex, consisting of three membrane and three aqueous compartments. The double envelope membrane encloses a soluble compartment, the stroma, which contains a variety of photosynthetic enzymes and the chloroplast gene expression machinery. The innermost membrane system, the thylakoid, lies within the stroma and encloses the thylakoid lumenal space. Photosynthetic electron transport is carried out by protein complexes located within the thylakoid membrane and the thylakoid lumen. The targeting of these polypeptides to the correct intrachloroplast location is critical to the functioning of the photosynthetic apparatus.

Whereas the integral thylakoid membrane proteins are encoded by either chloroplast or nuclear genes, all known lumenal proteins are encoded within the nucleus and synthesized on cytosolic ribosomes. Transport of nuclear-encoded proteins across the chloroplast envelope into the stroma is mediated by an N-terminal 'stromal targeting sequence' (reviewed in Keegstra *et al.*, 1989; de Boer and Weisbeek, 1991; Theg and Scott, 1993). Proteins destined for the thylakoid lumen are synthesized with a bipartite targeting sequence consisting of a stromal targeting sequence and an adjacent lumenal targeting sequence. Intrachloroplast targeting of lumenal proteins is believed to occur in two sequential steps (reviewed in Keegstra *et al.*, 1989; Berry-Lowe and Schmidt, 1991; Theg and Scott, 1993). Upon translocation across the envelope, the stromal targeting sequence is removed by a stromally localized processing enzyme. The resulting stromal intermediate is then translocated across the thylakoid membrane into the lumen, where a lumenal processing enzyme cleaves the lumenal targeting sequence to produce the mature protein (Hageman *et al.*, 1986; Kirwin *et al.*, 1987; Shackleton and Robinson, 1991). Although several integral thylakoid proteins are targeted to the membrane by a lumenal targeting sequence, most are directed for integration into the thylakoid by currently undefined signals within the mature proteins.

The results of *in vitro* experiments involving isolated chloroplasts and thylakoids have led to the description of at least three distinct targeting pathways that differ in their energetic requirements and substrate specificities (reviewed in Theg and Scott, 1993; Robinson and Klösgen, 1994). For example, import of the 33 kDa subunit of the oxygen-evolving complex (OEC33) and of plastocyanin (PC) into the thylakoid lumen is dependent upon ATP and is facilitated by a pH gradient across the membrane (Kirwin *et al.*, 1989; Cline *et al.*, 1992; Hulford *et al.*, 1994; Yuan and Cline, 1994). In contrast, the lumenal targeting of the 23 kDa and the 16 kDa subunits of the oxygen-evolving complex (OEC23 and OEC16) is absolutely dependent upon a pH gradient but is not facilitated by ATP (Mould and Robinson, 1991; Cline *et al.*, 1992; Hulford *et al.*, 1994). The integration of a nuclear-encoded protein, LHCP, into the thylakoid requires GTP (Hoffman and Franklin, 1994), and therefore involves a third pathway. The results of competition experiments support the assignment of these proteins to three distinct targeting pathways (Cline *et al.*, 1993).

Similarities between lumenal targeting sequences and the signal sequences that direct protein transport across the bacterial inner membrane suggest an underlying conservation in the transport mechanism. In *Escherichia coli*, protein transport across the inner membrane is mediated by a membrane-spanning translocation complex containing the *secY* and *secE* gene products (reviewed in Schatz and Beckwith, 1990; Wickner *et al.*, 1991; Pugsley, 1993). It has been proposed that proteins are delivered to this complex by two different pathways. One involves an interaction with the SecB chaperone, while the other involves a bacterial complex homologous to the eukaryotic

signal recognition particle (SRP) (reviewed in Schatz and Beckwith, 1990; Wickner *et al.*, 1991; Pugsley, 1993; Dobberstein, 1994; Wolin, 1994). Transport of proteins through the translocation complex is, in both cases, thought to be mediated by the *secA* gene product (Wolin, 1994). Recently, a chloroplast homologue of the bacterial SecA protein was identified and shown to be involved in the OEC33/PC transport pathway but not in the other two thylakoid-targeting pathways (Nakai *et al.*, 1994; Yuan *et al.*, 1994). A chloroplast homologue of an SRP protein, SRP54, has also been found (Franklin and Hoffman, 1993) and shown to function in the integration of LHCP into the thylakoid (Li *et al.*, 1995). The apparently unique energetics of the OEC23/OEC16 pathway suggest that it may be unlike previously defined transport systems. Aside from the SecA and SRP54 homologues, no other components of the thylakoid-targeting machineries have been identified.

To define the components of the thylakoid-targeting machinery, we are isolating transposon-induced mutations in maize nuclear genes that lead to targeting defects. Here, we describe the first such mutants to be recovered, *tha1* and *hcf106*. Our results indicate that the *tha1* mutation causes a defect in the targeting of OEC33 and PC but has no effect on the targeting of OEC23 and OEC16. *hcf106* has the complementary defect in that the targeting of OEC33 and PC is normal while that of OEC23 and OEC16 is defective. These results support the pathway specificity deduced from *in vitro* experiments. Analysis of *tha1* and *hcf106* mutants also revealed that the integration of cytochrome f (Cyt f), a chloroplast-encoded protein with a lumenal targeting sequence and a single membrane-spanning domain, is defective in *tha1* but not in *hcf106*. This suggests that the integration of Cyt f into the thylakoid involves the same pathway as the import of OEC33 and PC across the thylakoid membrane, and provides the first evidence that nuclear- and chloroplast-encoded proteins are targeted by a common machinery.

Results

The tha1 and hcf106 mutations cause similar losses of integral thylakoid membrane proteins

The *tha1* and *hcf106* mutations were recovered in screens for transposon-induced nuclear mutations in maize that cause the loss of components of the photosynthetic apparatus. Both mutations are recessive and result in pale green, non-photosynthetic seedlings that die after the expansion of three to four leaves, when endosperm stores are exhausted. Some aspects of the *hcf106* phenotype were described previously (Barkan *et al.*, 1986; Martienssen *et al.*, 1987).

The levels of representative subunits of the major chloroplast protein complexes were quantified by SDS–PAGE and immunoblot analysis (Figure 1). The profiles of leaf proteins visualized with Coomassie blue were not altered detectably in the mutants (Figure 1A). However, antibody probing revealed that both mutations cause decreased levels of the major thylakoid complexes—photosystem II (PSII), photosystem I (PSI), cytochrome f/b6 (Cyt f/b6), and ATPase. Figure 1B shows representative immunoblots on which subunits of each thylakoid complex were assayed. The levels of PSII-A and SubIV

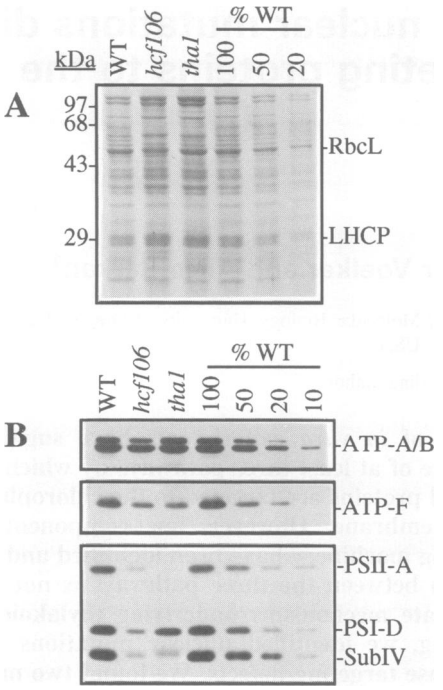

Fig. 1. SDS–PAGE and immunoblot analysis of total leaf proteins. Equal amounts of leaf proteins (15 μg) or the indicated dilutions of wild-type (WT) samples, were resolved by SDS–PAGE and (**A**) stained with Coomassie blue or (**B**) transferred to nitrocellulose and probed with the indicated antisera. Relative protein levels in mutant samples were quantified by visual comparison to the WT dilution series.

(components of the PSII and Cyt f/b6 complexes, respectively) were typically reduced to 10–20% of wild-type (WT) levels in both mutants. PSI-D (a subunit of the PSI complex) was typically reduced to 20% in *hcf106* seedlings but accumulated to 50% in *tha1* seedlings. Other subunits of these complexes (PSI-A/B, PSI-F, PSII-B, PSII-D, Cyt f, Rieske Fe-S, and cytochrome b6) were reduced to a similar extent (see below and data not shown). Accumulation of the thylakoid ATPase was less severely affected. The ATP-F protein, a subunit of the membrane-intrinsic portion of the thylakoid ATPase (CF_0), was reduced to 50% of normal levels in both mutants. However, components of the membrane-extrinsic portion of the ATPase (CF_1) accumulated normally (Figure 1B, ATP-A/B).

Extensive deficiencies such as these might reflect a primary defect in some global aspect of chloroplast gene expression. However, the normal accumulation of the chloroplast-encoded proteins RbcL, ATP-A and ATP-B (Figures 1A and B) and the failure to detect any aberrant chloroplast transcripts in either mutant (Barkan *et al.*, 1986 and data not shown) make this possibility unlikely. Likewise, the normal accumulation of the nuclear-encoded integral thylakoid protein LHCP (Figure 1A) eliminates the possibility that a global thylakoid membrane defect is the basis for these mutant phenotypes.

tha1 and hcf106 mutants accumulate increased levels of precursors to lumenal proteins

The pleiotropic losses of integral thylakoid proteins in the two mutants suggested that the mutations might disrupt some aspect of thylakoid protein targeting. To test for

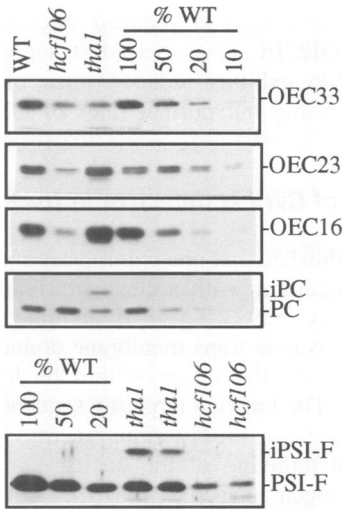

Fig. 2. Immunoblot analysis of leaf proteins. Blots identical to those shown in Figure 1 were probed with antisera specific for OEC33, OEC23, OEC16 or PC, as indicated. A similar blot containing proteins extracted from two individual *thal* or *hcf106* seedlings was probed with antiserum specific for PSI-F. Protein levels in mutant samples were quantified by visual comparison with the WT dilution series.

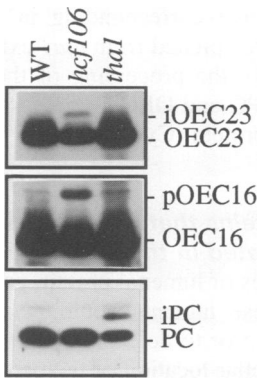

Fig. 3. Immunoblot analysis of chloroplast proteins. Chloroplasts were isolated from mutant or WT seedlings. Equal amounts of total chloroplast proteins (15 μg) were separated by SDS–PAGE and transferred to nitrocellulose. Blots were probed with affinity-purified antisera specific for the indicated proteins.

defects in the lumenal targeting machinery, lumenal proteins were quantified on immunoblots (Figure 2). While both mutations caused a 50% decrease in the level of OEC33, their effects on the accumulation of OEC23, OEC16 and PC differed. OEC23 and OEC16 accumulated normally in *thal* but were decreased to 20% of normal levels in *hcf106* plants. In contrast, mature PC was found at normal levels in *hcf106* but was reduced 2-fold in *thal*. Analysis of numerous *thal* individuals indicated that mature PC was more typically reduced to 10–20% of normal levels (data not shown).

Lumenal targeting sequences are processed by a protease that is localized in the thylakoid lumen (Hageman *et al.*, 1986; Kirwin *et al.*, 1987; Shackleton and Robinson, 1991). Defects in lumenal targeting might therefore be accompanied by increased levels of lumenal protein precursors. In fact, the PC-specific antibody detected a protein in *thal* that was ~3.5 kDa larger than mature PC (Figures 2 and 3). This higher molecular weight band is approximately the size expected of the stromal intermediate of PC (iPC) (Hageman *et al.*, 1986). This band was never detected in WT or *hcf106* samples. Likewise, antibody to PSI-F recognized a protein in *thal* that was ~4.5 kDa larger than mature PSI-F protein (Figure 2). This corresponds to the predicted size of the stromal intermediate (iPSI-F) (Scott *et al.*, 1994). Despite the fact that the level of mature PSI-F was reduced 10-fold in *hcf106*, no precursor was detected.

Longer exposures of blots containing total leaf proteins and probed with OEC23 or OEC16 antisera revealed the presence of putative precursor forms in *hcf106* but not in *thal* or WT seedlings (data not shown). These precursors were more clearly seen on blots containing chloroplast proteins rather than total leaf proteins (Figure 3). In *hcf106*, OEC23 antisera detected a protein 4 kDa larger than mature OEC23. This is the size predicted for the OEC23 stromal intermediate (iOEC23) (Bassham *et al.*, 1991). Antibody to OEC16 detected a protein 8 kDa larger than mature OEC16 in *hcf106* extracts but not in WT or *thal*. This protein is ~4 kDa larger than the expected size of the OEC16 stromal intermediate and is, instead, the size predicted for the full-length precursor (pOEC16) (Jansen *et al.*, 1987). No precursors to OEC33 were detected by immunoblot in either *thal* or *hcf106* (data not shown).

Lumenal targeting sequences are processed at reduced rates in hcf106 and thal

The increased accumulation of lumenal protein precursors suggested defects in either the translocation of these proteins across the thylakoid or in their subsequent processing by enzymes in the lumen. In either case, the rates of processing to the mature form would be reduced. To monitor the relative rates of processing, leaf proteins were pulse-labeled *in vivo* for 70 min and individual proteins were immunoprecipitated (Figure 4).

Although a small amount of iPC was occasionally detected in WT samples, the primary form detected in WT and *hcf106* plants was the mature protein (12 kDa) (Figure 4A). In contrast, little mature PC accumulated in *thal* during the 70 min pulse and higher molecular weight proteins of 14 kDa and 15.5 kDa were immunoprecipitated instead. The 15.5 kDa protein is the size predicted for the stromal intermediate (iPC) (Hageman *et al.*, 1986); the 14 kDa band may be the result of degradation of iPC, and/or may correspond to the second form of iPC described previously (Cline *et al.*, 1993).

Immunoprecipitation with antibody to OEC33 gave analogous results (Figure 4B). While a single protein the size of mature OEC33 was detected in *hcf106* and WT samples, the predominant protein detected in *thal* was a band 3 kDa larger than the mature protein, the predicted size of the OEC33 stromal intermediate (Bassham *et al.*, 1991). The failure to detect this precursor by immunoblot (Figure 2) may indicate that it is very unstable.

Analysis of OEC16 and OEC23 processing was complicated by the co-precipitation of an unrelated protein of 23 kDa, which obscured the signal for both mature OEC23 and pOEC16 (see asterisk in Figure 4C and D). Nonetheless, the results were consistent with those of the immunoblots. OEC23 antibody precipitated a radiolabeled protein of the size of iOEC23 from *hcf106* but not from WT and *thal* extracts (Figure 4C and E). OEC16 antibody

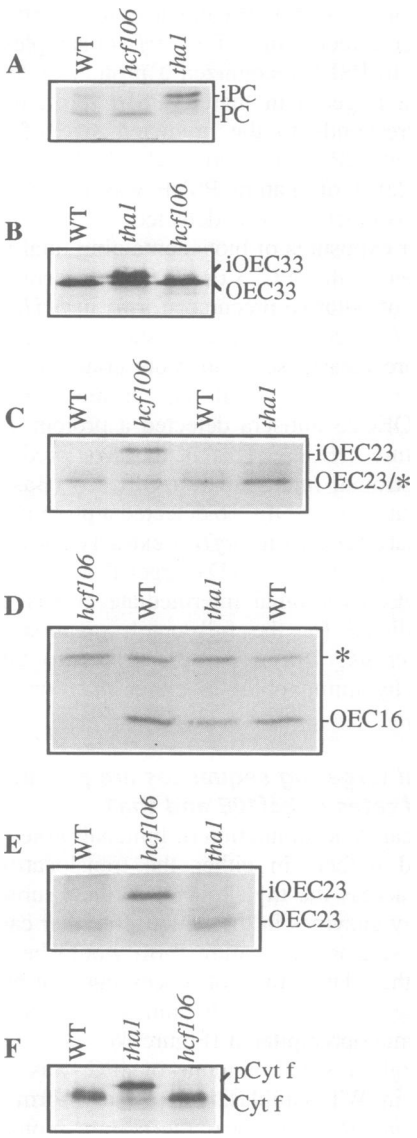

Fig. 4. Immunoprecipitation of proteins from *in vivo*-labeled leaf extracts. Seedling leaves were radiolabeled for 70 min by applying a mixture of [^{35}S]methionine and [^{3}H]leucine to small perforations. Specific proteins were immunoprecipitated from total leaf extracts, separated by SDS–PAGE, and detected by direct autoradiography (PC, OEC33 and Cyt f) or fluorography (OEC23 and OEC16). OEC23, OEC16 and Cyt f were immunoprecipitated using affinity-purified antisera. The band marked * corresponds to a radioactive protein that binds to *Staphylococcus* A cells and co-migrates with mature OEC23. The OEC16 precursor detected in *hcf106* migrates slightly faster than this band (very faint band in panel D and data not shown). Panel E shows the results of pre-clearing with *Staphylococcus* A cells before immunoprecipitation with anti-OEC23 antisera.

precipitated a radiolabeled protein of the size of mature OEC16 from WT and *tha1* extracts but not from *hcf106* extract (Figure 4D). When the contaminating 23 kDa protein was removed by pre-clearing the lysates with *Staphylococcus* A cells before antibody addition, a weak band corresponding in size to pOEC16 was observed only in the *hcf106* lane (data not shown) and a weak band corresponding in size to mature OEC23 was detected in WT and *tha1* lanes but was less intense in the *hcf106* lane (Figure 4E).

The results of these experiments indicate that OEC33 and PC are processed at reduced rates in *tha1* while OEC23 and OEC16 are processed at normal rates. Conversely, *hcf106* exhibits reduced rates of OEC23 and OEC16 processing but normal rates of OEC33 and PC processing.

Processing of Cyt f is defective in tha1 but not in hcf106

It is well established that one chloroplast-encoded protein, Cyt f, is synthesized with a cleavable lumenal targeting sequence (Gray, 1992). Cyt f is an integral membrane protein with a single trans-membrane domain and a large N-terminal domain that resides within the thylakoid lumen (Gray, 1992). The lumenal targeting sequence presumably functions in targeting the N-terminus across the membrane. Although the targeting of the nuclear-encoded lumenal proteins has been analyzed *in vitro*, similar studies of Cyt f have not been reported.

To determine whether the *tha1* or *hcf106* gene might function in the targeting of Cyt f, its rate of processing was examined in mutant seedlings (Figure 4F). The majority of the radiolabeled Cyt f detected after a 70-min pulse was fully processed in WT and *hcf106* seedlings. However, a single polypeptide 4 kDa larger than the mature protein and corresponding in size to the Cyt f precursor was precipitated from *tha1* extract. These results indicate that, like the processing of the nuclear-encoded proteins PSI-F, PC and OEC33, processing of the chloroplast-encoded protein Cyt f is slow in *tha1* but not in *hcf106*.

Precursor proteins that accumulate in tha1 and hcf106 are located in the stroma

The reduced rates of lumenal protein processing that were observed in these mutants could be due to defects in lumenal targeting or to defects in a processing peptidase. The intra-organellar location of the precursor proteins can be used to distinguish between these possibilities. Whereas a defect in a processing peptidase would likely result in increased accumulation of precursors in the lumen, defective translocation into the lumen would result in their increased accumulation within the stroma. To localize the precursor proteins, mutant chloroplasts were isolated and fractionated into stroma and intact thylakoids (containing lumenal proteins). Proteins of interest were then detected on immunoblots.

The results of fractionations of *hcf106* chloroplasts are shown in Figure 5. Because the accumulation of pOEC16 rather than iOEC16 in *hcf106* suggested that this precursor might be located outside of the chloroplast, a preliminary experiment was performed to test this possibility. Proteins enclosed by the chloroplast envelope will be protected from exogenous proteases. Therefore, intact chloroplasts were treated with protease in the presence or absence of Triton X-100 (Figure 5A). Addition of protease in the absence of detergent did not affect the integrity of the OEC16 precursor (Figure 5A, lane 5). However, in the presence of detergent both the precursor and mature forms were rapidly degraded (Figure 5A, lanes 3 and 6). These data indicate that pOEC16 is located inside of *hcf106* chloroplasts and suggest that this protein is not processed by the stromal peptidase before its translocation across the thylakoid.

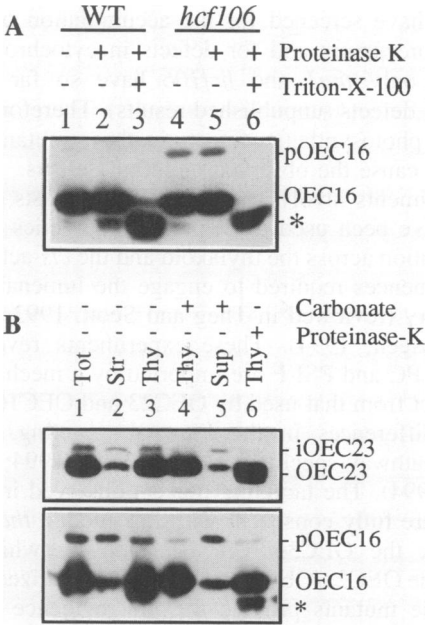

Fig. 5. Localization of precursors to OEC16 and OEC23 in fractionated *hcf106* chloroplasts. (**A**) Localization of pOEC16 inside *hcf106* chloroplasts. Intact WT and *hcf106* chloroplasts were treated with proteinase-K in the absence (lanes 2 and 5) or presence of Triton X-100 (lanes 3 and 6). Samples were fractionated by SDS–PAGE and analyzed on an immunoblot. Each lane contains proteins isolated from the same number of chloroplasts as determined by chlorophyll concentration. The band marked * is a proteolytic fragment of OEC16. (**B**) Intrachloroplast location of pOEC16 and iOEC23. Chloroplasts isolated from *hcf106* seedlings were lysed in hypotonic buffer under conditions that ruptured the envelope but maintained the integrity of the thylakoid. Total chloroplast lysate (Tot) was centrifuged to separate intact thylakoids (Thy) from stroma (Str). To remove peripheral proteins, thylakoids were washed with 200 mM sodium carbonate (lanes 4–5) or 2 M urea (not shown) and centrifuged to separate washed thylakoids from peripheral thylakoid proteins (Sup). Proteins in these supernatants were concentrated before electrophoresis by acetone precipitation. Alternatively, unwashed thylakoids were treated with proteinase-K (lane 6). Samples were fractionated by SDS–PAGE and analyzed on immunoblots. Each lane contains proteins isolated from the same number of chloroplasts. The band marked * is thought to be a proteolytic fragment of OEC16.

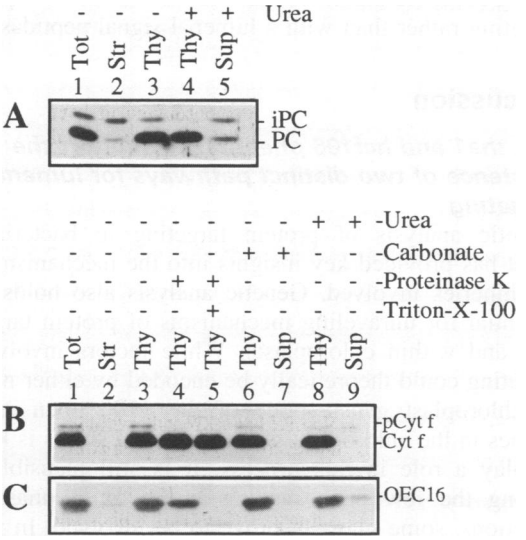

Fig. 6. Localization of precursors to PC and Cyt f in fractionated *tha1* chloroplasts. Chloroplasts were isolated from *tha1* seedlings and treated as described in Figure 5. Lane 5 in (B) and (C) represents a control experiment in which thylakoids were solubilized with 0.5% Triton X-100 before protease treatment.

Next, the intrachloroplast locations of pOEC16 and iOEC23 were determined (Figure 5B). Nearly all of the mature OEC16 and OEC23 protein was located in the thylakoid fraction (Figure 5B, lanes 1–3), indicating that the thylakoids were recovered largely intact. The iOEC23 and pOEC16 proteins, in contrast, were fairly equally distributed between the stromal and thylakoid fractions (Figure 5B, lanes 1–3). However, when the thylakoid pellets were washed with 200 mM sodium carbonate to remove peripherally associated proteins, these precursors were largely stripped from the thylakoids (Figure 5B, lanes 4–5). These results demonstrate that the OEC16 and OEC23 precursors in *hcf106* chloroplasts are located outside of the thylakoid.

The stromal location of the precursors was verified by protease treatment of the thylakoid fraction. Proteins enclosed by the thylakoid membrane are protected from exogenous proteases. Although mature OEC23 and OEC16 were insensitive to protease, the precursor forms were rapidly degraded (Figure 5B, lane 6). In control experiments (not shown), protease treatment following solubilization of the thylakoids with Triton X-100 resulted in the complete degradation of the mature forms of OEC23 and OEC16. Therefore, their resistance to proteolysis in the absence of detergent was due to their localization within the thylakoid lumen.

Figure 6 shows the results of analogous experiments to localize iPC and pCyt f in *tha1* chloroplasts. Approximately half of the iPC protein was located within the stromal fraction (Figure 6A, lanes 1–3). Nearly all of the membrane-associated iPC was removed by washing with urea (Figure 6A, lanes 4–5) or carbonate (data not shown), indicating that this protein was located outside of the thylakoid. Nearly all of the pCyt f was associated with unwashed thylakoid pellets (Figure 6B, lanes 1–3). The pCyt f protein was tightly associated with the thylakoids since it was not removed by washing the thylakoids with sodium carbonate and was only partially removed by washing with urea (Figure 6B, lanes 6–9). However, pCyt f displayed much higher sensitivity to proteolysis than the mature form (Figure 6B, lanes 3–5). Since mature Cyt f proved to be resistant to proteolysis even after detergent solubilization of the membrane (Figure 6B, lane 5), the intactness of the protease-treated thylakoids was confirmed by monitoring the lumenal protein OEC16 (Figure 6C, lanes 4–5). As expected, OEC16 was resistant to proteolysis in the intact thylakoids (Figure 6C, lane 4) but sensitive to proteolysis following Triton X-100 treatment (Figure 6C, lane 5). Cyt f, when fully integrated into the thylakoid, has only 15 C-terminal residues exposed on the stromal face (Gray, 1992). Therefore, the protease sensitivity and susceptibility to urea extraction displayed by the precursor suggests that it is not fully integrated into the membrane.

Taken together, these results indicate that the precursors that accumulate in *hcf106* and *tha1* chloroplasts are located outside rather than inside of the thylakoid, providing

strong evidence that these mutations interfere with protein targeting rather than with a lumenal signal peptidase.

Discussion

The tha1 and hcf106 phenotypes support the existence of two distinct pathways for lumenal targeting

Genetic analysis of protein targeting in bacteria and yeast has provided key insights into the mechanisms and machineries involved. Genetic analysis also holds great potential for unraveling mechanisms of protein targeting into and within chloroplasts. While factors involved in targeting could theoretically be encoded by either nuclear or chloroplast genes, none of the ~100 open reading frames in the chloroplast genome of land plants is known to play a role in this process. It is still plausible that among the few open reading frames with unassigned functions, some may yet prove to be involved. In fact, a mutation at an unknown site in the chloroplast genome of *Oenothera hookeri* leads to the over-accumulation of precursors to Cyt f, OEC23 and OEC16 (Johnson *et al.*, 1991); this mutation may define a chloroplast gene that functions in the targeting or processing of these proteins.

Since most of the components of the transport apparatus are likely to be encoded within the nucleus, we have focused on identifying nuclear mutations that interfere with the targeting of thylakoid proteins. Transposon-induced non-photosynthetic maize mutants were screened for protein deficiencies suggestive of targeting defects. Two mutants, *tha1* and *hcf106*, were recovered in this manner. Our results indicate that both the *tha1* and *hcf106* genes function in thylakoid protein targeting, but that each acts on a different set of proteins. The *tha1* gene functions in the targeting of OEC33, PC, PSI-F and Cyt f, while the *hcf106* gene functions in the targeting of OEC23 and OEC16 (see Figure 7). These conclusions are supported by two sets of observations. First, the rate of processing of each affected protein is reduced in the mutants. This is consistent with a decrease in their rate of localization to the thylakoid lumen, the site of the signal peptidase. Second, the precursor forms examined were localized on the external face of the thylakoid or in the stroma. This supports the view that the mutations affect protein translocation rather than the signal peptidase itself. While it is possible that the stromal location of the accumulated precursors could be a secondary consequence of a defect in the signal peptidase, this seems unlikely for two reasons. First, mutations in the *E.coli* leader peptidase do not affect translocation across the bacterial inner membrane (Dalby and Wickner, 1985). Second, mutations in the signal peptidase cleavage sites of secreted proteins in *E.coli* (Plückthun and Pfitzinger, 1988) and lumenal proteins in chloroplasts (Shackleton and Robinson, 1991) result in the accumulation of unprocessed proteins in the periplasm and lumen respectively. Therefore, processing of these proteins is not required for their translocation across the membrane.

Since transport of proteins across the thylakoid requires energy in the form of a pH gradient and/or ATP, targeting defects could be a pleiotropic effect of many mutations that interfere with photosynthetic electron transport. However, of the large number of non-photosynthetic mutants that we have screened for the accumulation of lumenal protein precursors and for defects in cytochrome f processing, only *tha1* and *hcf106* have so far exhibited targeting defects (unpublished results). Therefore, we feel that the photosynthetic defects in these mutants are not likely to cause the observed targeting defects.

Experiments involving isolated chloroplasts and thylakoids have been used to define the energetics of protein translocation across the thylakoid and the *cis*-acting amino acid sequences required to engage the lumenal targeting machinery (reviewed in Theg and Scott, 1993; Robinson and Klösgen, 1994). These experiments revealed that OEC33, PC and PSI-F are imported by a mechanism that is distinct from that used by OEC23 and OEC16, and that subtle differences in the lumenal targeting sequences dictate pathway specificity (Henry *et al.*, 1994; Robinson *et al.*, 1994). The targeting defects observed in *tha1* and *hcf106* are fully consistent with this model: *tha1* appears to block the OEC33/PC/PSI-F pathway while *hcf106* blocks the OEC23/OEC16 pathway (summarized in Figure 7). These mutants provide the first evidence that these distinct targeting pathways function *in vivo* and are genetically separable.

The OEC33/PC pathway has been proposed to be related to the bacterial SecA/Y/E translocation pathway. Strong support for this hypothesis comes from the recent identification of a chloroplast homologue of the SecA protein, CPSecA, which facilitates OEC33 and PC translocation *in vitro* (Nakai *et al.*, 1994; Yuan *et al.*, 1994). The *tha1* gene may encode this chloroplast SecA homologue or some other component that functions specifically in the OEC33/PC targeting pathway.

No components of the OEC23/OEC16 translocation apparatus have been identified. The apparent lack of an ATP hydrolysis step in the translocation of proteins by this pathway is unusual and suggests that this may represent a novel mechanism for protein translocation. The *hcf106* gene product may be a component of the OEC23/OEC16 targeting apparatus. Its localization to the thylakoid membrane is consistent with this idea (R.Martienssen, personal communication). However, it remains possible that the *hcf106* mutation indirectly affects protein translocation by destroying the trans-thylakoidal pH gradient. This might differentially block the OEC23/OEC16 pathway due its strict requirement for a pH gradient across the thylakoid.

Targeting of cytochrome f, a chloroplast-encoded protein with a lumenal targeting sequence

Cytochrome f is the only chloroplast-encoded protein known to be synthesized with a lumenal targeting sequence. The pathway by which it is targeted to the thylakoid membrane has not been reported. Our observation that Cyt f integration is defective in *tha1* but not in *hcf106* mutants provides strong evidence that this chloroplast-encoded protein engages at least one targeting component in common with the nuclear-encoded proteins OEC33, PC and PSI-F. This is consistent with the observation that Cyt f, when expressed in *E.coli*, inserts into the inner membrane in a *secA*-dependent manner (Rothstein *et al.*, 1985).

Chlamydomonas reinhardtii mutants have been generated with alterations in the Cyt f lumenal targeting sequence (Smith and Kohorn, 1994). The most severe

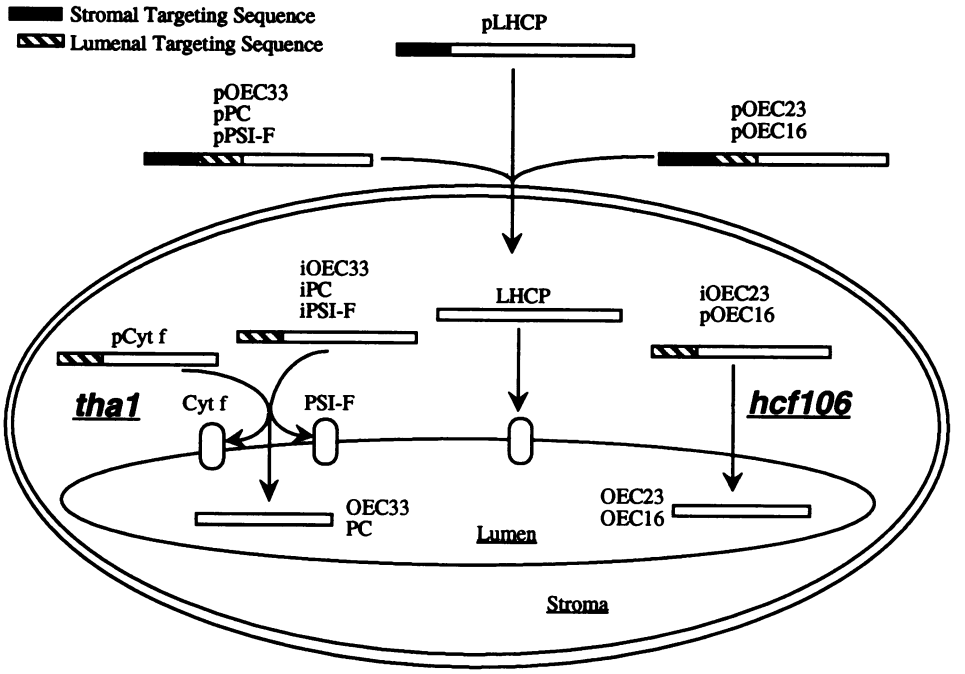

Fig. 7. Model for the roles of the *tha1* and *hcf106* gene products in thylakoid protein import. The nuclear-encoded proteins pLHCP, pOEC33, pOEC23, pOEC16, pPC and pPSI-F are synthesized in the cytosol and are targeted to the chloroplast by an N-terminal stromal targeting sequence. With the possible exception of pOEC16, the stromal targeting sequences are removed after transport into the stroma. The increased accumulation of stromally located pOEC16 in *hcf106* suggests that in maize, this protein is not processed in the stroma before crossing the thylakoid membrane. Transport of OEC33, OEC23, OEC16, PC and PSI-F across the thylakoid is directed by lumenal targeting sequences. PSI-F is thought to remain firmly anchored in the thylakoid by virtue of a single hydrophobic membrane spanning domain (Scott *et al.*, 1994). After transport across the membrane the lumenal targeting sequences are removed by a lumenal peptidase. *In vitro* experiments further suggested that OEC23 and OEC16 are targeted by a pathway distinct from that used by OEC33, PC and PSI-F. LHCP is targeted to the thylakoid by amino acid sequences internal to the mature protein; it has been proposed that LHCP is targeted by a pathway distinct from those involved in targeting the lumenal proteins. The data presented in this paper support the existence of multiple thylakoid targeting pathways: *tha1* functions specifically in the OEC33/PC/PSI-F pathway, while *hcf106* functions specifically in the OEC23/OEC16 pathway. The mutant phenotypes suggest that neither gene functions in the integration of LHCP. Furthermore, the defect in Cyt f targeting observed in *tha1* mutants provides evidence that this chloroplast-encoded protein is targeted via the same machinery as used by the nuclear-encoded proteins OEC33, PC and PSI-F.

allele, A15E, is similar to *tha1* in that the integration of Cyt f into the thylakoid is defective. However, the phenotypes of A15E and *tha1* also display significant differences. For example, A15E, unlike *tha1*, causes a reduction in the level of LHCP. While A15E and *tha1* both block the integration of Cyt f, phenotypic differences could be due to blockage at different points in the targeting pathway.

Other roles for the 'lumenal' targeting machinery?

The *tha1* and *hcf106* mutants exhibit similar and rather extensive losses of thylakoid proteins: in both mutants the levels of PSI, PSII and Cyt f/b6 are reduced 3- to 10-fold, the level of CF_0 is reduced 2-fold, and LHCP and CF_1 accumulate normally. An understanding of the basis for these protein deficiencies may reveal previously unsuspected roles for the 'lumenal' targeting machinery in the biogenesis of the thylakoid. Although thylakoid protein complexes can accumulate independently of one another, closely-associated subunits of the same complex are typically destabilized as a consequence of any mutational defect that interferes with their normal assembly (reviewed in Barkan *et al.*, 1995). Therefore, the loss of the PSI, PSII, Cyt f/b6 and CF_0 complexes in *tha1* and *hcf106* mutants could result from the defective targeting of just one subunit of each complex or from the defective targeting of non-structural proteins that play essential roles in complex biogenesis. The thylakoid protein complexes are largely composed of integral membrane proteins whose targeting mechanisms are unknown. While each complex does contain at least one subunit with a lumenal targeting sequence, defects in the targeting of only these subunits is not sufficient to explain all aspects of the mutant phenotypes. For example, it is useful to consider the basis for the loss of the Cyt f/b6 complex in the two mutants. The only subunit of the Cyt f/b6 complex known to be synthesized with a lumenal targeting sequence is Cyt f. The observed defect in Cyt f targeting is sufficient to explain the loss of the Cyt f/b6 complex in *tha1*. However, the loss of Cyt f/b6 proteins in *hcf106* must reflect a previously unknown role for the OEC23/OEC16 pathway since Cyt f appears to be targeted normally in this mutant. It has been proposed that the Rieske Fe-S protein is targeted via an N-terminal sequence that resembles an uncleaved lumenal targeting sequence (Breyton *et al.*, 1994; de Vitry, 1994; Madueño *et al.*, 1994). The loss of the Cyt f/b6 complex in *hcf106* mutants may then be due to the mistargeting of the Rieske Fe-S protein. It is also possible that this defect could be due to the mistargeting of a novel subunit, of one or more of the integral membrane subunits, or of some other factor involved in Cyt f/b6 biogenesis.

The loss of PSII in *tha1* could be due solely to the defect in OEC33 targeting, as suggested by the fact that

mutations in the *C.reinhardtii* OEC33 gene lead to a 10-fold reduction in the level of the PSII core proteins (Mayfield *et al.*, 1987a). Since defects in OEC23 and OEC16 have not been associated with a loss of the PSII core (Mayfield *et al.*, 1987b), defective targeting of these proteins in *hcf106* does not seem likely to be the cause of its PSII deficiency. However, PSII-T, a nuclear-encoded subunit of PSII (Ikeuchi, 1992), is targeted by the same pathway as OEC23 and OEC17 (Henry *et al.*, 1994). It is possible that a defect in the targeting of this protein results in the loss of PSII in *hcf106*.

At least two subunits of the PSI complex, PSI-F and PSI-N, are synthesized with lumenal targeting sequences. *In vitro* experiments have shown that PSI-N is imported by the OEC23/OEC16 pathway (Mant *et al.*, 1994; Nielsen *et al.*, 1994) while PSI-F is imported by the OEC33/PC pathway (Karnauchov *et al.*, 1994; Mant *et al.*, 1994). The defect we observed in PSI-F targeting may be sufficient to explain the loss of PSI in *tha1*. The targeting of PSI-N is likely to be defective in *hcf106* and this may also be sufficient to lead to the loss of the PSI core complex.

The accumulation of ATP-F, a component of the CF_0 complex, is reduced 2-fold in both *tha1* and *hcf106* mutants; other CF_0 subunits were not assayed due to lack of suitable antibodies. However, the reduction in ATP-F suggests that both mutations affect the integration of at least one CF_0 component. Although one nuclear-encoded subunit of the complex, ATP-G, is synthesized with an N-terminal sequence resembling a lumenal targeting sequence, it has been suggested that this protein is targeted by a pathway distinct from both the OEC33/PC and the OEC23/OEC16 pathways (Michl *et al.*, 1994). Analysis of the targeting of this subunit in *tha1* and *hcf106* mutants may shed light on this issue.

The major LHCP proteins accumulate normally in *tha1* and *hcf106* seedlings, suggesting that these are targeted by pathways distinct from those used by the lumenal proteins. This conclusion is consistent with the recent elucidation of an 'SRP-like' pathway for LHCP integration and the fact that LHCP does not compete with the lumenal proteins *in vitro* for access to their targeting machineries (Cline *et al.*, 1993; Li *et al.*, 1995).

The current body of data indicates that proteins are targeted to and across the thylakoid via multiple pathways. It remains unclear, however, whether these pathways are entirely independent or share common components. It is possible, for example, that these pathways reflect the presence of a variety of receptors that deliver different proteins to a common translocation complex. Such a mechanism appears to operate in *E.coli* where proteins destined for secretion may be delivered to the secY/E/A translocase either by SecB or by an SRP-like complex (reviewed in Dobberstein, 1994; Wolin, 1994). Clarification of the inter-relatedness of the targeting pathways within chloroplasts will require elucidation of the components of the transport machinery. Cloning of genes identified by mutations such as *tha1* and *hcf106* and characterization of their gene products will be important for this purpose. The *hcf106* gene has already been cloned (Martienssen *et al.*, 1989) and the characterization of its gene product is well under way (R.Martienssen, personal communication). Transposon-facilitated cloning of the *tha1* gene is currently in progress.

Materials and methods

Plant growth

Both *tha1* and *hcf106* were recovered from *Mutator* lines propagated by S.Hake, M.Freeling and co-workers (University of California, Berkeley). Mutant *tha1* seedlings often exhibited small dark-green sectors on their slightly pale-green leaves, confirming that the mutation was caused by transposon insertion. Unless otherwise noted, seedlings used for experiments were grown for 9–13 days in 16 h of light (400 $\mu E/s/m^2$) at 27°C and 8 h of dark at 25°C. Previously we observed that PSII levels are decreased when cytochrome f/b6-specific mutants are grown in high intensity light (manuscript in preparation). However, the growth conditions used here minimized this effect.

SDS–PAGE and immunoblot analysis

Proteins were extracted from seedling leaf tissue, fractionated by SDS–PAGE (13% acrylamide, no urea), transferred to nitrocellulose, and immunodetected as described previously (Barkan, 1993). Antigen/antibody–horseradish peroxidase (HRP) complexes were detected using the Renaissance chemiluminescence system (New England Nuclear) or as follows (based upon advice from G.Schuster). Blots were rinsed after incubation with the secondary antibody and incubated in 6 ml of 100 mM Tris–HCl, pH 8.5, containing 3 µl 2% hydrogen peroxide, 15 µl 90 mM *p*-coumaric acid (dissolved in DMSO) and 30 µl 250 mM Luminol (Fluka Chemicals).

Pulse-labeling experiments

Seedlings used for pulse-labeling experiments were germinated and grown under the conditions described above until the coleoptile emerged (3–4 days). They were then transferred to continuous darkness until the third leaf emerged (8–9 days after planting) and moved back into the light for 24–36 h before the labeling experiment. Mutant seedlings were then identified by their decreased chlorophyll content and/or their increased chlorophyll fluorescence when illuminated with a hand-held UV lamp (Miles, 1980). All labeling experiments were performed at the same time of day.

Forty to fifty tiny perforations were made in a 1 cm band in the mid-section of the second leaf by piercing with a hypodermic needle against a piece of cardboard placed against the back side of the leaf. Immediately thereafter, 20 µl of labeling mix [8 µl [^{3}H]leucine (140 Ci/mmol, 1 mCi/ml), 8 µl [^{35}S]methionine (1140 Ci/mmol, 8 mCi/ml), 3 µl 20 mM sodium phosphate (pH 7.0) and 1 µl 10% bromophenol-blue (to visually monitor uptake)] was applied to the perforations. The plants were then incubated in an illuminated fume hood for 70 min. During this time ~20 µl additional labeling mix was applied, as needed, to keep the perforations moist. After the pulse, the 2 cm of tissue surrounding the perforations was removed and total proteins were extracted and solubilized as described previously (Barkan *et al.*, 1994). Protein samples were either used immediately for immunoprecipitation or were frozen at –80°C.

Immunoprecipitations were performed as described previously (Barkan *et al.*, 1994) with the following modifications. Immunoprecipitations of nuclear-encoded proteins contained 500 000 d.p.m.. Immunoprecipitations of Cyt f contained 300 000 d.p.m.. The antibody binding reactions were performed in the following buffer: 0.15 M NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 20 mM Tris–HCl, pH 7.5, 2% non-fat dry milk, 10 mM EDTA, 2 mM PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 2 µg/ml pepstatin. 15 µl of crude antiserum or 5 µl of affinity-purified antibody was added to each reaction. The processing rate of each protein was analyzed in multiple independent labeling experiments, always with similar results: Cyt f was immunoprecipitated from seven *tha1* and three *hcf106* extracts; PC was immunoprecipitated from four *tha1* and two *hcf106* extracts; OEC33 was immunoprecipitated from four *tha1* and four *hcf106* extracts; OEC23 and OEC16 were precipitated from two *tha1* and two *hcf106* extracts. The lysates used in Figure 4E were pre-cleared before antibody addition as follows: a portion of washed *Staphylococcus* A cells equivalent to that used for the final binding of IgG was incubated with each lysate at 4°C for 1 h. The cells were then pelleted and the immunoprecipitations carried out with the cleared supernatants as described above.

Chloroplast isolation and fractionation

Seedlings used for chloroplast isolation were grown under day/night cycles (as described above) until the third leaf was expanded. The leaves from 12 seedlings (6–9 g) were homogenized briefly in 120 ml of ice-cold GM buffer (330 mM sorbitol, 50 mM HEPES–KOH, pH 8, 2%

BSA, 2 mM EDTA, 1 mM $MgCl_2$) in a VirTis homogenizer (VirTis Equipment Co.) fitted with razor blades (Gillette). The slurry was filtered through cheesecloth and the chloroplasts were pelleted by centrifugation at 4000 *g* for 1 min in a swinging bucket rotor. After gently resuspending the pellet in 4 ml GM, the material was layered onto a 40%/80% Percoll step gradient (made up in GM buffer). The gradient was centrifuged at 6000 *g* for 5 min and the lower (intact chloroplast) band was recovered. The chloroplasts were washed by resuspending in 5 vol SH buffer (330 mM sorbitol, 50 mM HEPES–KOH, pH 8) and pelleted by centrifugation at 4000 *g* for 1 min. To lyse the chloroplasts, pellets were resuspended in HM buffer (10 mM HEPES–KOH, pH 8, 2 mM $MgCl_2$) to 0.7 mg chlorophyll/ml. After 10 min on ice, the chloroplast lysate was aliquoted and fractionated or protease-treated as described below.

Fractionation. Chloroplast lysate was centrifuged at 10 000 *g* for 3 min and the supernatant (stroma) was removed. The pellet (thylakoid) was resuspended to the starting volume with HM buffer. For salt-washed thylakoids, pellets were resuspended to their starting volume in 2 M urea or 200 mM sodium carbonate, incubated for 5 min on ice, and centrifuged for 3 min at 10 000 *g*. Proteins were precipitated from the supernatant by the addition of 3 vol of ice-cold acetone, incubation on ice for 5 min and centrifugation at 15 000 *g* for 15 min. The washed thylakoid pellet was rinsed with HM buffer, pelleted as above, and resuspended to the starting volume with HM.

Protease treatment. Chloroplast lysate was incubated in the presence of proteinase-K (0.1 mg proteinase-K/mg chlorophyll) on ice for 40 min. Reactions were stopped by the addition of PMSF to 6 mM and EDTA to 12 mM. In control experiments designed to test protease sensitivity in the absence of intact thylakoids, Triton X-100 was added to 0.5%. Protease treatments involving intact chloroplasts were performed using these same conditions.

Antisera

Antisera against maize Cyt f, Cyt b6, Rieske, and Subunit 4 were described previously (Barkan *et al.*, 1986). Antisera against maize ATP-F, PC, and OEC33, sorghum OEC23 and OEC16 and barley PSI-D were obtained following injection of rabbits with fusion proteins generated with the pMAL expression system (New England Biolabs). The ATP-F fusion construct included only the 5′ exon of ATP-F, while the other fusion constructs included essentially the entire coding sequences. The ATP-F, OEC33, OEC23 and OEC16 antisera were affinity purified by chromatography using Sepharose–cyanogen bromide beads (Sigma) coupled with the purified fusion proteins against which the antibodies were raised.

The PSI-F antiserum was generously provided by R.Malkin, CF_1 antiserum by S.Merchant, D1 antiserum by G.Schuster and D2 antiserum by W.Vermaas. The early stages of this work involved OEC23/OEC16 antiserum provided by A.Staehelin, OEC23 antiserum provided by K.Cline, plastocyanin and PSI-D antisera provided by R.Malkin and plastocyanin antiserum provided by K.Burkey.

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