Transposon-Disruption of a Maize Nuclear Gene, *tha1*, Encoding a Chloroplast SecA Homologue: *In Vivo* Role of cp-SecA in Thylakoid Protein Targeting

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Manuscript received August 16, 1996
Accepted for publication October 14, 1996

ABSTRACT

A nuclear mutant of maize, *tha1*, which exhibited defects in the translocation of proteins across the thylakoid membrane, was described previously. A transposon insertion at the *tha1* locus facilitated the cloning of portions of the *tha1* gene. Strong sequence similarity with *secA* genes from bacteria, pea and spinach indicates that *tha1* encodes a SecA homologue (cp-SecA). The *tha1-ref* allele is either null or nearly so, in that *tha1* mRNA is undetectable in mutant leaves and cp-SecA accumulation is reduced ≥40-fold. These results, in conjunction with the mutant phenotype described previously, demonstrate that cp-SecA functions *in vivo* to facilitate the translocation of OEC33, PSI-F and plastocyanin but does not function in the translocation of OEC23 and OEC16. Our results confirm predictions for cp-SecA function made from the results of *in vitro* experiments and establish several new functions for cp-SecA, including roles in the targeting of a chloroplast-encoded protein, cytochrome *f*, and in protein targeting in the etioplast, a nonphotosynthetic plastid type. Our finding that the accumulation of properly targeted plastocyanin and cytochrome *f* in *tha1-ref* thylakoid membranes is reduced only a few-fold despite the near or complete absence of cp-SecA suggests that cp-SecA facilitates but is not essential *in vivo* for their translocation across the membrane.

MITOCHONDRIA and chloroplasts are highly compartmentalized organelles believed to be evolved from ancient endosymbiotic bacteria. Of the proteins localized to these organelles, only a minority are synthesized on organellar ribosomes. The majority are synthesized in the cytosol and subsequently targeted to the organelle. Regardless of their origin, all organellar proteins must ultimately be sorted to the correct intraorganellar compartment.

A "conservative sorting" hypothesis has been proposed whereby nuclear-encoded proteins are initially targeted to the mitochondrial matrix or chloroplast stroma (derived from the cytoplasm of the ancestral endosymbiont) and then "secreted" across internal organellar membranes via mechanisms evolved from the endosymbiont's secretory system (HARTL *et al.* 1987; SMEEKENS *et al.* 1990). This notion was first suggested when structural and functional similarities were noted between bacterial signal peptides and the domains that target proteins to the thylakoid lumen or mitochondrial intermembrane space. Although a conservative sorting mechanism for targeting to the mitochondrial intermembrane space remains controversial (GLICK *et al.* 1992), there is increasing evidence that such a mechanism is involved in the sorting of certain proteins to the thylakoid lumen. *In vitro* experiments involving isolated chloroplasts and thylakoid membranes demonstrated that sodium azide, a potent inhibitor of the bacterial *secA* gene product, inhibits the translocation of a subset of proteins across pea thylakoid membranes (HENRY *et al.* 1994; KNOTT and ROBINSON 1994). In addition, homologues of bacterial *secA* and *secY* have been found on the plastid genomes of several algae (SCARAMUZZI *et al.* 1992; FLACHMANN *et al.* 1993; REITH and MUNHOLLAND 1993; VALENTIN 1993), and the nuclear genomes of higher plants encode chloroplast-localized SecA (cp-SecA) and SecY homologues (NAKAI *et al.* 1994; BERGHOEFER *et al.* 1995; LAIDLER *et al.* 1995; NOHARA *et al.* 1995). Finally, cp-SecA can function *in vitro* to facilitate the translocation of several proteins across the thylakoid membrane (YUAN *et al.* 1994; NOHARA *et al.* 1995).

Intrachloroplast protein sorting has been studied primarily with *in vitro* targeting experiments in which radiolabeled protein precursors are incubated with isolated chloroplasts or isolated thylakoid membranes. These experiments have provided evidence for at least three pathways by which proteins can translocate into or across the thylakoid membrane. Nuclear-encoded proteins destined for the thylakoid lumen are synthesized with cleavable lumenal targeting sequences that resemble bacterial signal peptides (reviewed by ROBINSON and KLÖESGEN 1994; CLINE and HENRY 1996). A subset of these engages a "*sec*"-like pathway, which requires ATP and is facilitated by cp-SecA *in vitro*. Others are translocated across the membrane by a mechanism that does not require ATP nor any soluble factors but that is strictly dependent upon a *trans*-thylakoidal

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ΔpH. A third pathway is engaged by the nuclear-encoded protein LHCP, whose integration into the thylakoid requires GTP but does not involve a cleavable targeting sequence. LHCP integration is facilitated *in vitro* by 54CP, a homologue of the signal recognition particle protein SRP54 (HOFFMAN and FRANKLIN 1994; LI *et al.* 1995).

These *in vitro* experiments have revealed the mechanistic complexity of thylakoid membrane assembly. However, to fully appreciate the nature of these targeting pathways and their interrelationships *in vivo,* it is essential to study the consequences of genetic disruption of thylakoid membrane targeting. We described two maize mutants, *hcf106* and *tha1,* with defects in the localization of different sets of proteins to the thylakoid lumen (VOELKER and BARKAN 1995). The *tha1* reference allele described previously (*tha1-ref*) interferes with targeting of nuclear-encoded proteins thought to engage the *sec*-like pathway and also with the targeting of the chloroplast-encoded protein cytochrome *f.* In contrast, the *hcf106* mutation disrupts the targeting of proteins that engage the ΔpH-dependent pathway *in vitro.* These phenotypes provided evidence that the ΔpH-dependent and *sec*-like pathways are genetically separable *in vivo.*

Somatic instability of the *hcf106* and *tha1-ref* mutations indicated that both were the result of transposable element insertions. The *hcf106* gene was cloned previously by virtue of its association with a *Mutator* (*Mu*) transposon (MARTIENSSEN *et al.* 1989). Here, we describe the *Mu*-facilitated cloning of the *tha1* gene and report that the *tha1* gene encodes a homologue of the bacterial and plant SecA proteins. The accumulation of cp-SecA is exceedingly low in leaves of *tha1-ref* plants. These results, in conjunction with the specific targeting defects associated with the *tha1-ref* mutation, confirm and extend the roles for cp-SecA that were postulated based upon the results of *in vitro* experiments. It is interesting, however, that even those lumenal proteins whose translocation is affected in *tha1-ref* mutants do accumulate within the thylakoid lumen to substantial levels. This observation suggests the possibility that cp-SecA, while a facilitator of translocation across the thylakoid, is not absolutely required for the translocation of any protein yet examined.

MATERIALS AND METHODS

Plant material: The *tha1-ref* mutation was recovered from a *Mu*-active maize line propagated in the laboratories of S. HAKE (USDA Plant Gene Expression Center, Albany, CA) and M. FREELING (University of California, Berkeley). Numerous *tha1-ref/+* plants were propagated in parallel for several generations by crossing with inbred lines. Heterozygous plants were then self-pollinated to recover homozygous mutant seedlings. Mutant seedlings used for DNA and RNA extraction were identified initially by virtue of their pale green pigmentation. The protein composition of a leaf tip harvested from each potential *tha1-ref* seedling was analyzed on Western blots. In each case, we observed the characteristic "fingerprint" of the *tha1-ref* mutation [*i.e.,* the loss of the core subunits of the electron transport complexes and the over-accumulation of the stromal precursor to plastocyanin (VOELKER and BARKAN 1995)], which confirmed that these were homozygous *tha1-ref* seedlings.

Etiolated *tha1-ref* leaves were obtained by germinating and growing the F_1 progeny of a *tha1-ref/+* plant in the absence of light. After 9 days of growth at 26°, plants were numbered under a green safelight and individual etiolated leaf tips were harvested and immediately frozen. The plants were then transferred to continuous light for 24 hr and *tha1-ref* plants were identified by virtue of their reduced pigmentation and increased chlorophyll fluorescence when viewed with a handheld ultraviolet light (MILES 1982). Leaf tips of several greened *tha1-ref* and wild-type seedlings were harvested.

The *tha1* locus was mapped at the University of Missouri-Columbia Maize RFLP Laboratory to chromosome *3* in Bin 3.04.

Isolation and analysis of plant DNA: DNA was extracted from the above-ground portion of single 2-wk-old seedlings. Plants were ground in liquid nitrogen to a fine powder and thawed in a lysis buffer consisting of 7 M urea, 0.25 M NaCl, 50 mM Tris-HCl (pH 8.0), 1% N-lauryl-sarcosine, 20 mM EDTA, 0.25% β-mercoptoethanol. After a 5-min incubation at 37°, samples were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with an equal volume of isopropanol. Samples were resuspended in TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA] containing RNAse A (20 μg/ml), extracted once with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol. After the addition of an equal volume of a solution containing 1.6 M NaCl, 13% polyethylene glycol 8000, samples were incubated at room temperature for 30 min and the DNA pelleted by centrifugation at 12,000 × *g* for 15 min. Pellets were rinsed with 70% ethanol and the DNA was resuspended in TE.

For Southern analysis, 4 μg of DNA was digested overnight with 15 units of restriction enzyme. DNA was fractionated by electrophoresis (1.5 V/cm, 20 hr) in 0.8% agarose gels prepared with 1× TAE [40 mM Tris-Acetate, 1 mM EDTA (pH 8)] and 0.2 μg/ml ethidium bromide. DNA in the gel was depurinated, denatured, neutralized, and transferred to Magnacharge nylon membrane (MSI) as described (SAMBROOK *et al.* 1989). DNA was fixed to the membrane by exposure to UV light (1200 μJ/cm^2) in a Fisher UV Crosslinker.

Membranes were prehybridized in hybridization buffer [5× SSC (SAMBROOK *et al.* 1989), 0.1% *N*-laurylsarcosine, 0.02% SDS, 1% casein, 200 μg/ml salmon DNA] for 1–2 hr. DNA probes, prepared by polymerase chain reaction amplification of plasmid inserts in the presence of digoxigenin-labeled dUTP (dUTP:dTTP = 1:20), were denatured by incubation in boiling water for 5 min and added to the hybridization solution. Hybridization proceeded for 16–24 hr. Filters were washed six times for 15 min each in 0.5× SSC, 0.1% SDS. Standard prehybridization, hybridization, and washes were at 65° (Figure 1). Low stringency hybridization and washes were at 54° (Figure 5). Bound probe was detected with the GENIUS chemiluminescence system (Boehringer Mannheim).

Genomic cloning: DNA (30 μg) from a homozygous *tha1-ref* seedling was digested with *Eco*RI and fractionated in an agarose gel, as described above. A gel slice containing DNA fragments of 2000–3000 bp was excised. DNA was extracted from the gel with QIAEX beads, according to the manufacturer's instructions (Qiagen). The DNA was ligated into Blue Script SK+ plasmid (Stratagene) that had been digested with *Eco*RI, and electroporated into XL1-Blue MRF' cells (Stratagene). Colony lifts (SAMBROOK *et al.* 1989) were probed by hybridization with a radiolabeled *Mu1* probe.

Reverse transcription-PCR amplification of a partial tha1 cDNA: A degenerate primer, 5'-(G/A/C) GC(G/C) GT(G/C)CC(G/C) GTCAT (G/A/T/C)CC-3', was designed against the highly conserved SecA peptide sequence gly-met-thr-gly-thr-ala and was used to prime reverse transcription from poly A+ leaf RNA isolated from B73 inbred maize seedlings. Each reaction (20 μl) contained 1 ng of poly A+ RNA, 100 pmol of primer, 20 units RNAsin ribonuclease inhibitor, 15 units AMV reverse transcriptase, 1 mM each dNTP, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 50 ng/μl actinomycin-D. Reactions were incubated at 21° for 10 min and then for 1 hr at 42°. The template RNA was degraded by the addition of 4 μl 1 M NaOH/ 0.1 mM EDTA and incubation at 60° for 1.5 hr. Nucleic acids were precipitated by adding 2.5 μl 3 M NaAcetate, pH 5.2, and 75 μl ethanol, and recovered by centrifugation. The resulting pellets were rinsed with 70% ethanol, dried, and resuspended in 20 μl water.

PCR amplification using the cDNA as a template was performed by using a *tha1* gene specific primer, 5'-AGCCCGAGGTCT CCGCGC-3' (see Figure 3), and a degenerate primer, 5'-(G/C) CC(G/C)GT(G/A) AA(T/C) TC(G/A)TC(G/A/C) AC(G/T/A) AT-3', designed according to the highly conserved SecA motif ile-val-asp-glu-phe-thr-gly. Based upon the sequence of pea cp-SecA, we expected that this primer pair would amplify a cDNA fragment of ~900 bp. Each 100 μl reaction contained 10 μl of the reverse transcription product, 100 pmol of each primer, 0.2 mM each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.05% Tween-20. The reactions were incubated at 95° for 5 min after which 5 units Taq DNA polymerase were added and allowed to amplify for five cycles of 95°/30 sec, 37°/30 sec, and 72°/90 sec and 25 cycles of 95°/30 sec, 45°/30 sec, and 72°/90 sec. A second round of amplification was performed using 5 μl of the initial PCR products and the same buffer conditions used above. However, the reactions contained only 10 pmol of each primer and the following "touchdown" temperature profile was used for amplification: 20 cycles of 94°/30 sec, 56°/30 sec (−0.5°/cycle), and 72°/60 sec and 20 cycles of 94°/30 sec, 45°/30 sec, and 72°/60 sec. The 906-bp DNA fragment was gel-purified and subcloned into a Bluescript SK+ plasmid.

Multiple sequence alignments: SecA homologues from a variety of organisms (GenBank accession numbers: *Pisum sativum* (common pea), X82404, *Synechococcus PCC7942,* X74592, *Escherichia coli,* M20791, *Spinacia oleracea* (spinach) Z49124) were compared with the *tha1* protein sequence deduced from the partial genomic and cDNA clones (GenBank accession numbers U71124 and U71123, respectively). Alignments were calculated using the GCG program package (Wisconsin Genetics Computer Group). Default alignment parameters were chosen. Homologies were determined using the GES index scale for 85% homology.

PCR amplification of genomic DNA: Amplification reactions (100 μl) were performed containing 20–50 ng of maize genomic DNA, 20 pmol of each primer (primer-A: 5'-ACCGTGGC GCGTATC AAC-3', primer-B: CTGCAATC TGATACGAGAGT-3', Mu-primer: 5'-CGCCTCCATTT CGTCGAATCC-3'), 0.2 mM of each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 0.01% gelatin, 0.05% Tween-20. After a 5-min incubation at 95°, 5 units of Taq DNA polymerase were added and allowed to amplify for 30 cycles as follows: 94°/30 sec, 56°/30 sec, and 72°/45 sec. 10 μl of each reaction was analyzed by gel electrophoresis and DNA was visualized with ethidium bromide.

Isolation and analysis of mRNA: Total and poly A+ leaf RNA were purified and analyzed in Northern and RNAse-protection experiments as described previously (MARTIENSSEN *et al.* 1989; BARKAN 1993; BARKAN *et al.* 1994). The clone used to probe for actin mRNA was obtained from WIM VAN HEECKEREN (University of Oregon).

Isolation and analysis of protein: Leaf or chloroplast proteins were extracted and analyzed on Western blots as described previously (VOELKER and BARKAN 1995). Antisera raised against pea cp-SecA and LHCP were generously provided by T. ENDO (Nagoya University) and BILL TAYLOR (CSIRO, Canberra), respectively. Antisera specific for OEC23, cytochrome *f,* and plastocyanin were described previously (VOELKER and BARKAN 1995).

RESULTS

Molecular cloning of the *tha1* mutation: The *tha1-ref* mutation arose in a maize line with active *Mu* transposons. The frequent appearance of small phenotypically normal sectors on the pale green mutant leaves supported the notion that the mutation resulted from a *Mu* insertion. To allow the segregation of *tha1* from unlinked *Mu* insertions, numerous heterozygous plants were outcrossed to inbred lines. Resulting heterozygous plants were again outcrossed. After several rounds of this crossing scheme, DNA was prepared from individual mutant plants representing distantly related branches of the pedigree. DNA was also prepared from homozygous wild-type plants closely related to the mutant plants used in this analysis.

Mu insertions were visualized by Southern hybridization with probes corresponding to members of the *Mu* family (CHANDLER and HARDEMAN 1992). Probes for *Mu3, Mu8,* and *MuDR* failed to detect any insertions in common to all mutant individuals. However, a *Mu1* probe revealed a 2.4-kb *Eco*RI fragment in all mutant individuals that was absent in all homozygous wild-type individuals (representative results are shown in Figure 1A). Genomic DNA comigrating with this fragment was gel-purified and ligated into a plasmid, and bacterial transformants were screened by hybridization with a *Mu1* probe. The cloned 2.4-kb fragment contained the 1.4-kb *Mu1* element flanked by 333 bp and 719 bp of genomic DNA (Figure 1B). The genomic Southern blots used for the linkage analysis were reprobed with each of these flanking DNA segments (Figure 1C and data not shown). Both flanking probes detected just a single band in all *tha1* samples, of 2.4 kb. This band was absent in all homozygous wild-type samples. The wild-type plants were polymorphic with regard to the cloned locus (compare lane 1 with lanes 6 and 12), the size of the hybridizing fragments correlating with the line from which the wild-type allele arose. These results indicate that the cloned fragment is genetically linked to the *tha1* locus.

If the cloned genomic fragment contains the *Mu* insertion responsible for the *tha1-ref* phenotype, one would expect that excision of the *Mu1* element from these sequences would result in phenotypic reversion. To test this prediction, the structures of the corresponding genomic sequences in sectors of revertant tissue were analyzed. DNA was extracted from revertant sec-

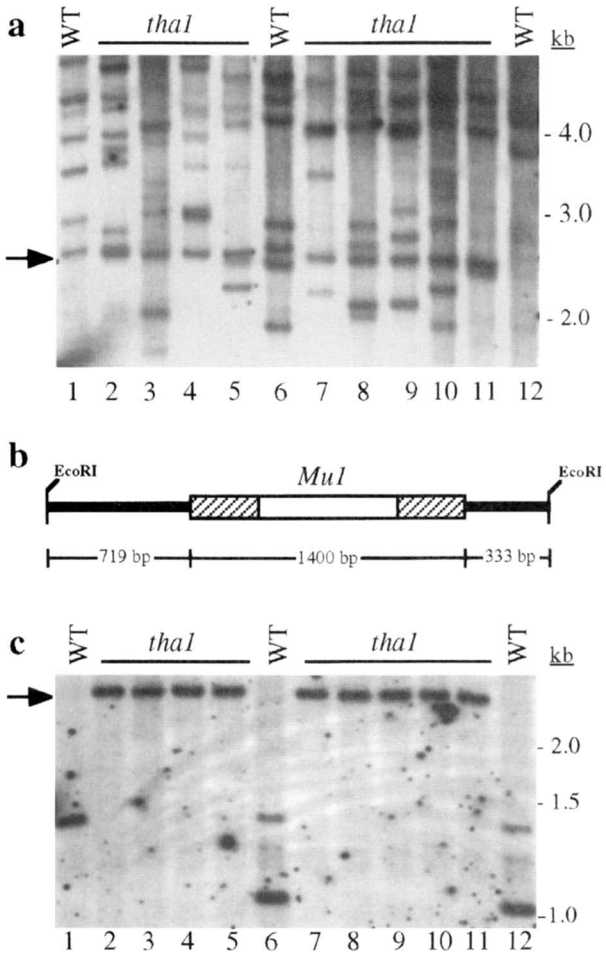

FIGURE 1.—Genomic Southern blots demonstrating genetic linkage between a *Mu1* insertion and *tha1*. Lanes contain *Eco*RI-digested DNA from homozygous wild-type (WT) or *tha1-ref* leaves. The mutant samples were selected from diverse arms of the *tha1* pedigree. Each WT sample was closely related to one of the adjacent mutant samples. The samples shown represent half of the samples that were analyzed; all samples gave analogous results. (A) A *Mu1* probe detected a 2.4-kb band (indicated by the arrow) that was present in all *tha1* samples and absent in all WT samples. A band of nearly the same size is present in the WT sample in lane 1; however, this band is actually slightly larger than the band present in all mutants (note the doublet in lane 2). (B) Map of the cloned 2.4-kb *Eco*RI fragment containing the *Mu1* insertion that is linked to *tha1*. (C) The blot shown in panel (A) was stripped and reprobed with the 719-bp genomic fragment flanking the cloned *Mu1* (see panel B). As expected, this probe detected the 2.4-kb fragment corresponding to the clone in mutant samples but not in WT samples. Bands of different sizes were present in WT samples containing the *Tha1* allele from the A188 (lanes 6 and 12) and B73 (lane 1) maize lines. The wild-type progenitor of *tha1* was not available for analysis.

tors and from flanking mutant tissue. The DNA structure surrounding the insertion site was analyzed with PCR, using primer pairs designed to selectively amplify either the mutant or wild-type allele (Figure 2A). Revertant sectors are most likely to be heterozygous and should therefore give rise to the products expected from both alleles.

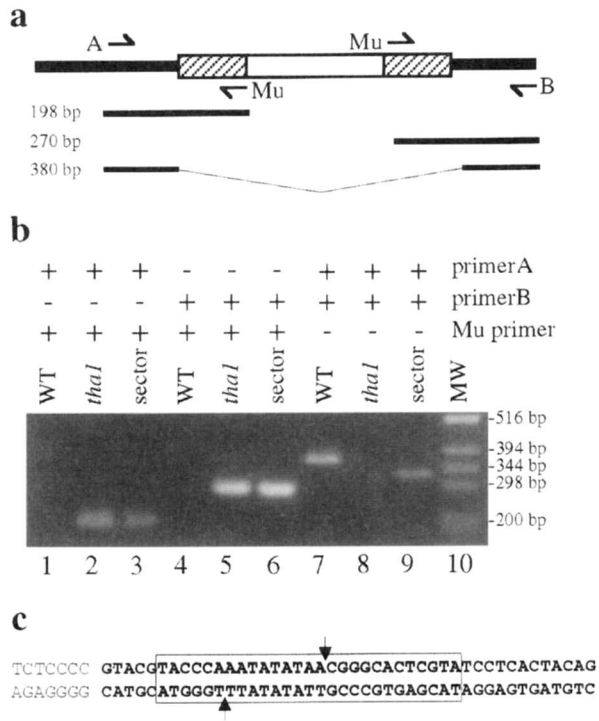

FIGURE 2.—*Mu1* excision from the cloned genomic region correlates with phenotypic reversion. (A) Primers used in the analysis of the cloned sequences in revertant sectors. Three primers were used, designated A, B, and Mu on the diagram. Amplification of the mutant allele with the Mu primer and primer A would result in a 198-bp fragment. Amplification of the mutant allele with the Mu primer and primer B would result in a 270-bp fragment. Amplification of the wild-type progenitor allele with primers A and B would result in a 380-bp fragment. Primers A and B are not expected to amplify the mutant allele at all because of the inability of the PCR to amplify through intact *Mu* elements. (B) PCR analysis of wild-type, mutant, and revertant DNA. DNA was extracted from a small revertant sector ("sector") and from mutant tissue on the same leaf (*tha1*). Wild-type (WT) DNA was extracted from a homozygous wild-type sibling of the sectored seedling. The primer pairs used in each reaction are indicated above the gel. (C) Nucleotide sequence of the cloned *Mu1* insertion site. Intron sequences are shown in bold. These were identified by comparison of the *tha1* cDNA and genomic sequences (see results below). The staggered *Mu1* insertion site, deduced by the sequence of the 9-bp target site duplication in the cloned mutant allele, is indicated by arrows. Nucleotides deleted in the revertant sector analyzed above are indicated by the box.

Control experiments were performed to establish that the PCR faithfully amplified the predicted DNA fragments from material of known genotype. As expected, DNA from homozygous wild-type tissue (WT) gave no amplification products in reactions involving the *Mu* primer in conjunction with either of the gene-specific primers A or B (Figure 2B, lanes 1 and 4). Primers A and B together, however, gave rise to an amplification product of 380 bp, which corresponds in size to that expected for amplification of the wild-type progenitor of the cloned sequences (Figure 2B, lane 7). Amplification of homozygous *tha1-ref* DNA with the *Mu* primer in conjunction with primers A or B resulted

in DNA fragments whose sizes were consistent with those predicted (198 and 270 bp, respectively). No amplification was observed from the homozygous *tha1-ref* template when the gene-specific primers A and B were paired. This was expected since the polymerase chain reaction fails to amplify across intact *Mu* elements (A. B. and M. WALKER, unpublished observations).

DNA extracted from a revertant sector that flanked the mutant tissue used for the *tha1* control reactions was analyzed in the same way. Primers A and B paired with the *Mu* primer amplified the two fragments predicted for the mutant allele (Figure 2B, lanes 3 and 6), as expected if just one of the two alleles had reverted. Primer A paired with primer B amplified a fragment of ~340 bp (Figure 2B, lane 9). This amplification provided evidence that *Mu1* had excised from one of the mutant alleles in the cell that gave rise to the revertant sector. The revertant allele, however, was ~40 bp smaller than the progenitor wild-type allele in the region between primers A and B. These results suggest that imprecise excision of *Mu1* caused a small deletion of flanking genomic sequences and that this excision nonetheless resulted in reversion to a wild-type phenotype.

A second revertant sector, excised from a different plant, was analyzed in the same way and with similar results (data not shown). Once again, the *Mu1* insertion was homozygous in the phenotypically mutant tissue and was heterozygous in the revertant tissue. Excision of *Mu1* was again accompanied by a small deletion of flanking sequences, although the size of this deletion differed slightly between the two revertant samples. The amplification products derived from both excision events were cloned and their DNA sequences were determined. In both cases, the deletions (27 and 38 bp) extended into what proved to be intron sequences (Figure 2C and results below) and did not disrupt sequences essential for splicing at the 5′ splice junction (LUEHRSEN *et al.* 1994). The *tha1* gene would likely be expressed fairly normally in these derivative alleles since it is unlikely that these small deletions of intronic sequences would interfere with splicing. Taken together, these results indicate that excision of *Mu1* from genomic sequences corresponding to the clone correlates with reversion to a wild-type phenotype, providing strong evidence that the cloned sequences represent the *tha1* gene.

The *tha1* gene encodes a SecA homologue: DNA sequence analysis revealed a 627-bp open reading frame in the 719-bp cloned genomic region flanking the *Mu1* insertion (Figure 1B). This region has the potential to encode the amino acids shown upstream of the vertical arrow in Figure 3. The deduced amino acid sequence shows similarity to the amino-terminus of the pea (*P. sativum*) and spinach (*S. oleracea*) chloroplast-localized SecA homologue, cp-SecA (Figure 3) (NAKAI *et al.* 1994; BERGHOEFER *et al.* 1995). The similarity between the open reading frame in the *tha1* genomic clone and sequences encoding pea and spinach cp-SecA ended 19 bp 5′ to the *Mu1* insertion and did not continue on the other side of the *Mu* element, suggesting that *Mu1* had inserted near the 5′-end of an intron (data not shown; GenBank accession No. U71124).

To determine definitively whether the cloned fragment derives from a *secA* homologue, reverse transcription followed by PCR amplification was used to obtain downstream mRNA sequence. Published sequences of *secA* homologues in other organisms were used to design two degenerate primers that were expected to anneal with highly conserved mRNA regions ~1000 and 900 nucleotides downstream of those encoded by the cloned genomic sequences. The more downstream primer was used to prime a reverse transcription reaction on a template of wild-type leaf mRNA. The nested primer was then used in a polymerase chain reaction, in conjunction with a "*tha1*-specific" primer complementary to the genomic clone in a region that is less conserved in known *secA* genes (see horizontal arrow Figure 3). This yielded a 906-bp cDNA fragment (data not shown).

Nucleotide sequence analysis indicated that this cDNA contained a continuous open reading frame with the capacity to encode a protein with extensive similarity to SecA homologues in many organisms (Figure 3). The nucleotide sequence of the cDNA clone in the region adjacent to the "*tha1*-specific" primer was identical for 126 bases to that of the genomic clone, diverging in sequence 19 bp upstream of the *Mu1* insertion (data not shown; GenBank accession No. U71123). This site of sequence divergence between the genomic and cDNA clones corresponds precisely with a 5′-splice site consensus sequence (shown in Figure 2C) (LUEHRSEN *et al.* 1994) and with the point of divergence between pea cp-SecA and the deduced product of the *tha1* genomic clone (as described above). We concluded, therefore, that the amplified cDNA does correspond to the genomic clone, that *Mu1* had inserted within an intron 19 bp from the 5′-splice junction, and that *tha1* encodes a SecA homologue.

Both the pea and spinach cp-SecA proteins are predicted to be synthesized with a 60–80 amino acid N-terminal extension when compared to bacterial SecA homologues (BERGHOEFER *et al.* 1995; NOHARA *et al.* 1995) (Figure 3). These N-terminal sequences facilitate protein targeting across the chloroplast outer envelope. We did not directly determine the start codon for the *tha1* gene product. However, translation initiated at an AUG 420 nt upstream of the *Mu1* insertion site would result in an N-terminal extension comparable in length to those predicted for the pea and spinach cp-SecA preproteins (see * in Figure 3). The predicted sequence of the N-terminus of a protein starting at this site exhibits properties characteristic of stromal targeting sequences (reviewed by CLINE and HENRY 1996): it is rich in hy-

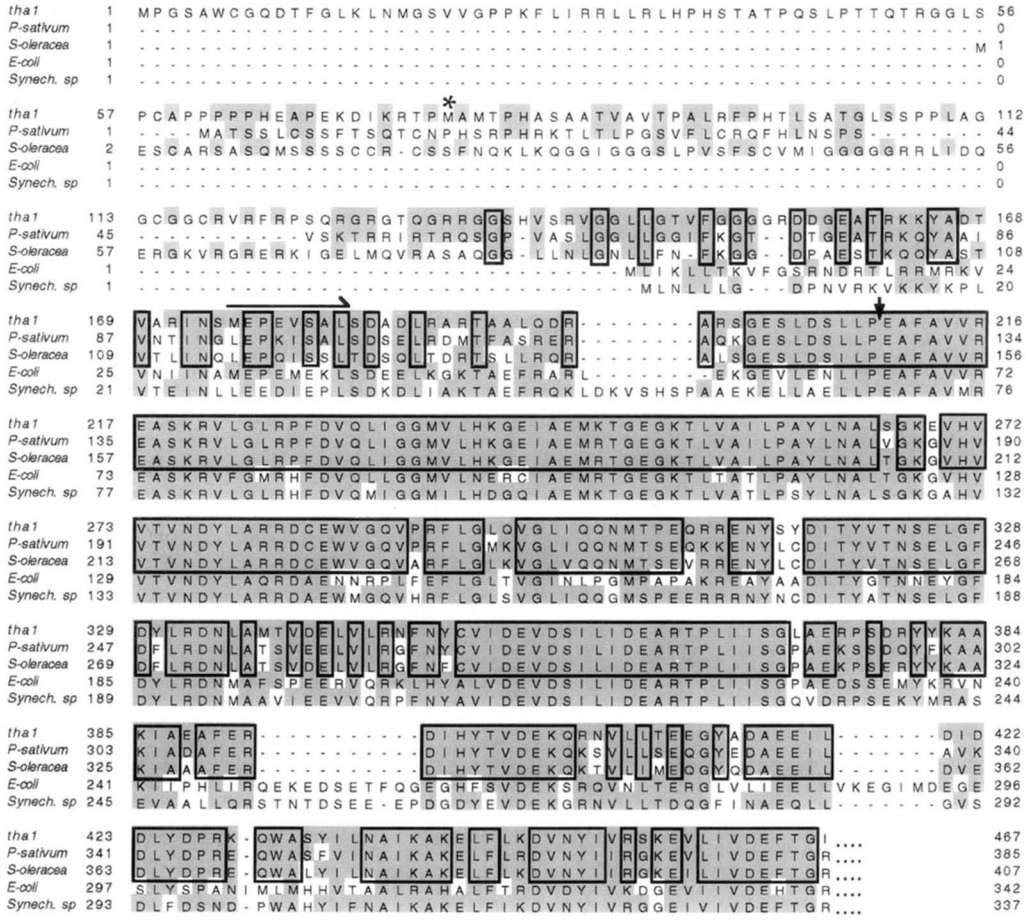

FIGURE 3.—Comparison of the deduced *tha1* gene product with other SecA homologues. The amino acid sequence shown for the *tha1* gene product was deduced from the combined sequence analysis of the genomic and cDNA clones. The genomic and cDNA sequence data have been deposited in GenBank/EMBL/DDBJ under accession numbers U71124 and U71123, respectively. The N-terminal methionine shown for *tha1* is encoded in genomic DNA but may not represent the authentic start codon; the putative *tha1* start codon (as described in RESULTS) is designated (*). The region corresponding to the primer used for the amplification of the *tha1* cDNA is indicated with a horizontal arrow. An intron in the *tha1* gene maps between the codons for amino acids 209 and 210 (↓). Amino acid identity displayed between the *tha1* gene product and the pea (*P. sativum*) and spinach (*S. oleracea*) chloroplast cp-SecA homologs is indicated by boxes. Amino acid similarity displayed between these proteins and the *E. coli* and Synechococcus SecA proteins is indicated by shading. Alignment and homology were determined as indicated in MATERIALS AND METHODS. The sequences shown correspond to approximately one third of the full-length proteins.

droxylated amino acids (20%), devoid of acidic residues, and contains an alanine at the second position. Together, these observations support the conclusion that the *tha1* gene encodes a chloroplast-localized SecA homologue.

The *tha1* gene is unique in the maize genome: The genomic clone encoding the first exon detects a single band on genomic Southern blots (Figure 1C and data not shown), indicating that the 5′-portion of the *tha1* gene has no close homologues in the maize genome. However, sequences that are most highly conserved between all *secA* genes occur in the second exon and further downstream. These highly conserved regions of the *tha1* cDNA (encoding amino acids 175–467 in Figure 3) were used to probe genomic Southern blots at reduced stringency (Figure 4). When DNA from the inbred line B73 was cut with the restriction enzyme *Sst*I, a single genomic fragment was detected, and when digested with *Hin*dIII, two genomic fragments were detected. We expected that *Hin*dIII would cut the genomic sequence corresponding to the probe into two fragments since the probe was known to contain an internal *Hin*dIII site (data not shown) and since the probe and the genomic DNA on the blot were derived from the same inbred line. Together, these results support the notion that there is only a single SecA homologue encoded in the maize genome.

Accumulation of cp-SecA in *tha1* mutants is very low: To use the *tha1* mutant productively in studies of cp-SecA function, it is important to know to what extent the *Mu* insertion decreases cp-SecA protein accumulation. This was addressed by quantifying both the *tha1* mRNA and cp-SecA protein in mutant leaves. Northern hybridizations were used to visualize *tha1* mRNA in *tha1-ref* or wild-type seedling leaves. A probe consisting of the 906-bp *tha1* cDNA revealed a mRNA of ~3.5 kb in

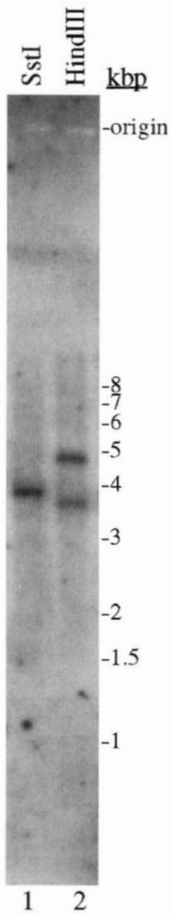

FIGURE 4.—Southern blot probed at reduced stringency with a highly conserved region of the *tha1* gene. DNA obtained from the inbred line B73 was digested with the indicated restriction enzyme. Hybridization was performed in 5× SSC at 54° and washes were with 0.5× SSC at 54°. This blot was probed with a DNA probe made from the entire 906-bp cDNA fragment derived from the *tha1* locus in B73.

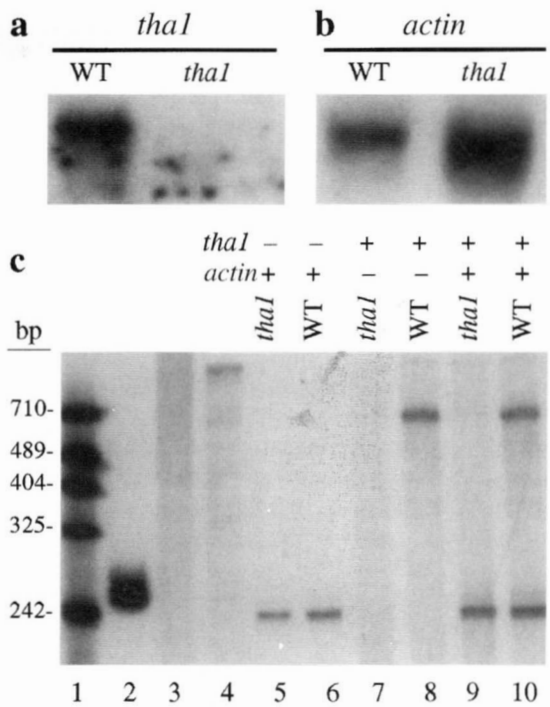

FIGURE 5.—Abundance of *tha1* mRNA in *tha1* leaf tissue. (A) Northern blot containing 3 μg of polyA+ -enriched RNA isolated from *tha1* and wild-type seedling leaves. The blot was probed with the 906-bp *tha1* cDNA. (B) The same blot was reprobed with a maize actin cDNA fragment. (C) RNAse-protection analysis. The probe for *tha1* mRNA was transcribed from a clone of the 719-bp *tha1* genomic fragment flanking the *Mu1* insertion (see Figure 1). The probe for actin mRNA was transcribed from a 232-bp partial actin genomic clone. The actin probe was synthesized such that its specific-activity was one-tenth that of the *tha1* probe, in order that the final signal intensities be comparable. Total leaf RNA (30 μg) from wild-type or *tha1* seedlings was hybridized with the *tha1* probe, the actin probe, or both, as indicated. Hybrids were digested with RNAse T1 and fractionated in a denaturing gel. Controls included a lane in which tRNA was substituted for maize RNA (lane 3), undigested actin probe (lane 2) and undigested *tha1* probe (lane 4). Lane 1 contains DNA size standards.

wild-type polyA+ -enriched leaf RNA (Figure 5A). This mRNA is sufficient in size to encode a protein of ~110 kD, the size of the previously described cp-SecA proteins (NAKAI *et al.* 1994; YUAN *et al.* 1994; BERGHOEFER *et al.* 1995). Even after long exposure, this mRNA was not detected in the *tha1-ref* sample. When an actin cDNA was used to reprobe the same blot, it became apparent that the *tha1-ref* sample contained considerably more mRNA than the wild-type sample (Figure 5B), making the absence of *tha1* mRNA still more significant.

The abundance of *tha1* mRNA was also assayed in RNAase-protection experiments (Figure 5C). Total leaf RNA from wild-type seedlings protected a probe fragment of ~700 nucleotides (based upon the mobility of DNA markers). This corresponds to the first protein-coding exon of the mRNA. An equivalent amount of total RNA from *tha1-ref* leaves did not protect the probe to detectable levels. A probe complementary to actin mRNA was protected to a similar extent in the two samples, demonstrating that equal amounts of mRNA were assayed. These results indicate that the *Mu1* insertion in *tha1-ref* causes a dramatic decrease in the accumulation of the *tha1* mRNA. The precise level of residual mRNA was impossible to determine since it was below the limit of detection in both experiments.

The amount of cp-SecA protein in *tha1-ref* chloroplasts relative to wild-type chloroplasts was quantified on a Western blot probed with an antiserum raised against a highly conserved portion of pea cp-SecA (NAKAI *et al.* 1994). This antiserum should recognize not just the *tha1* gene product but also the products of any nonallelic genes encoding cp-SecA. A prominent band of the expected size (110 kD) was detected in extracts of wild-type chloroplasts but was nearly undetectable in extracts of *tha1-ref* chloroplasts (Figure 6). Comparison with a dilution series of the wild-type sample demonstrated that the abundance of cp-SecA in *tha1-ref* chloroplasts is no more than a few percent of that in wild-type

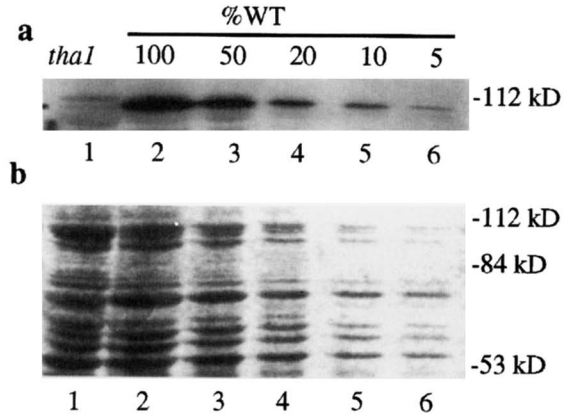

FIGURE 6.—Western blot showing loss of cp-SecA in *tha1* chloroplasts. Total proteins (7 μg) obtained from *tha1* or wild-type chloroplasts were loaded in lanes 1 and 2, respectively. Dilutions of the wild-type sample were analyzed in lanes 3–6. (A) The blot was probed with an antibody raised against a highly conserved fragment of pea cp-SecA (NAKAI *et al.* 1994). (B) The filter shown in A was stained with Ponceau S before antibody probing. This demonstrates that equal amounts of mutant and wild-type chloroplast proteins were analyzed.

chloroplasts. These results demonstrate that the *tha1* gene is the only significant source of cp-SecA in young maize leaves and that the *Mu1* insertion causes nearly a complete loss of cp-SecA in *tha1-ref* chloroplasts.

Cp-SecA functions in etioplasts: The role of cp-SecA has not been studied in plastid types other than chloroplasts. To assess its role in the biogenesis of etioplasts, we examined the phenotype of etiolated *tha1-ref* leaves (Figure 7). Protein was extracted from the leaf tips of two etiolated *tha1-ref* plants and from two normal siblings. Protein was then extracted from leaves of the same plants after they had been exposed to light for 24 hr.

To illustrate that the etiolated leaves had developed in the absence of light, we took advantage of the fact that the major light harvesting chlorophyll *a/b* binding protein (LHCP) fails to accumulate in etioplasts and accumulates rapidly upon exposure to light (NELSON *et al.* 1984). A strong signal was obtained with just 2.5% of the LHCP in the greened seedlings, but no signal was detected in the etiolated tissue (Figure 7D). Therefore, the etiolated leaves had not been exposed to significant light.

The protein deficiencies in etiolated and greened *tha1-ref* leaves were similar: a fivefold reduction in mature plastocyanin and cytochrome *f* was accompanied by an increase in the accumulation of the plastocyanin stromal intermediate (i-PC) (Figure 7, B and C). These results indicate that cp-SecA functions in the biogenesis of etioplasts and that the *tha1* gene (rather than a non-allelic gene encoding cp-SecA) provides cp-SecA to both chloroplasts and etioplasts.

The ratio of i-PC to mature PC was higher in the greened than in the etiolated mutant samples (Figure

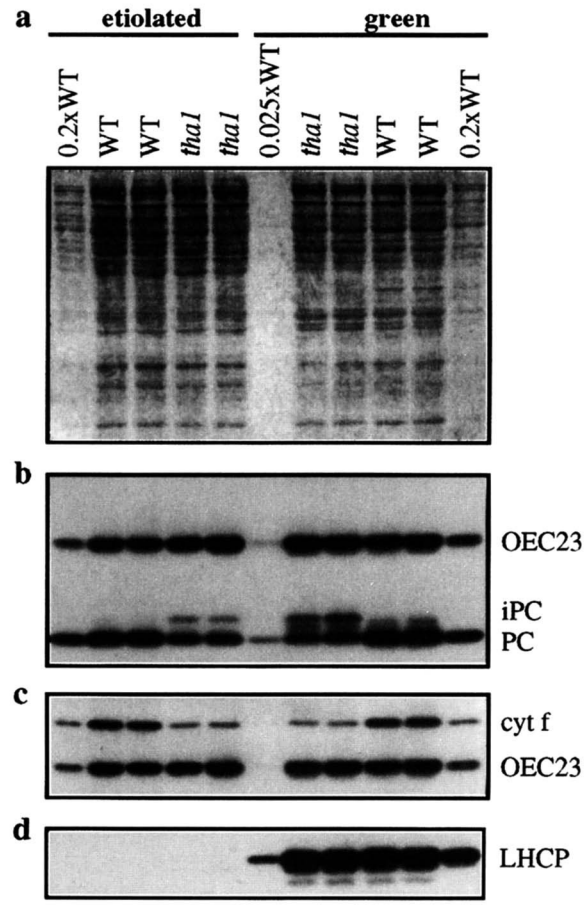

FIGURE 7.—Etiolated and green *tha1* leaves exhibit similar protein deficiencies. Protein was extracted from leaf tips excised from two etiolated *tha1* seedlings and two etiolated wild-type siblings. The plants were then exposed to light for 24 hr and protein was extracted from leaves of the same plants. Total leaf proteins (5 μg) or the indicated dilutions of a wild-type sample, were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. (A) Proteins bound to the filter were visualized by staining with Ponceau S. (B) The filter shown in panel (A) was probed with antibodies specific for plastocyanin and OEC23. (C) The filter shown in B was reprobed with antibody specific for cytochrome *f*. (D) The filter shown in C was stripped and reprobed with an antibody specific for LHCP, the major light harvesting chlorophyll *a/b*/ binding protein.

7B). We have observed that the accumulation of i-PC is variable, even among light-grown mutant seedlings (unpublished observations). The unusually high ratio of i-PC to mature PC observed in the greened samples shown here may have resulted from their unusual growth regime: the shift of etiolated leaves from dark to light may have overwhelmed the already compromised targeting machinery with newly synthesized substrates for transport.

The results of this experiment also suggest that OEC23 and OEC16 are efficiently translocated across internal etioplast membranes: these proteins accumulated to similar levels in etioplasts and chloroplasts and their stromal intermediates did not accumulate de-

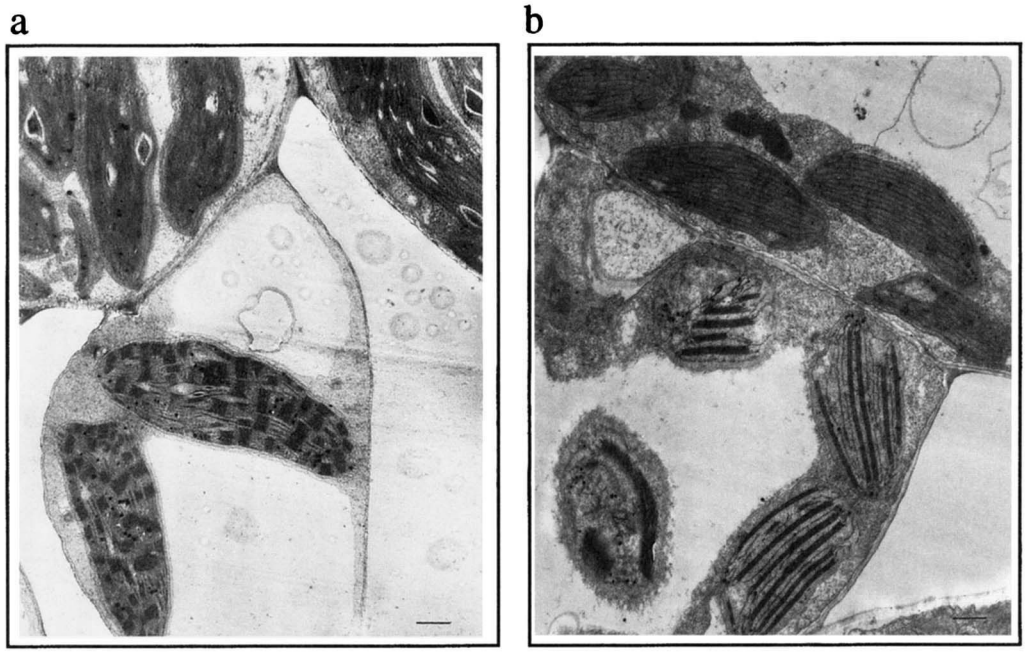

FIGURE 8.—Ultrastructure of *tha1* chloroplasts. (A) Section of a wild-type leaf. A mesophyll cell is shown on the bottom and two bundle sheath cells on the top. (B) Section of a *tha1* leaf. A mesophyll cell is shown on the bottom and a bundle sheath cell on the top. Bars, 1 μm.

tectably in etioplasts (Figure 7, B and C, and data not shown). This is intriguing in that these two proteins are strictly dependent upon a *trans*-thylakoidal delta pH for their translocation *in vitro* (reviewed by CLINE and HENRY 1996). These results suggest that either a sufficient pH gradient is generated in the absence of light and chlorophyll or that these proteins are targeted via an alternative mechanism in etioplasts.

Chloroplast ultrastructure in *tha1* mutants: Thylakoid membrane organization varies with growth environment and cell type. Mesophyll chloroplasts in maize contain clusters of stacked thylakoid membranes (grana) that are interconnected by unstacked lamellae (LAETSCH 1974). Chloroplasts in maize bundle sheath cells contain primarily unstacked thylakoid membranes that are distributed throughout the stroma and arranged in parallel to the long axis of the chloroplast.

The cp-SecA deficiency in *tha1-ref* mutants disrupts the structure of thylakoid membranes in mesophyll chloroplasts (Figure 8). Mesophyll thylakoid membranes are arranged in giant grana that often extend the entire length of the chloroplast and that exhibit an abnormally high electron density. These structures are reminiscent of those observed in the mesophyll chloroplasts of *hcf106* mutants (MARTIENSSEN *et al.* 1987, 1989). However, they are also quite similar to structures observed in several nonallelic barley mutants that lack photosystem II core particles specifically (SIMPSON and VON WETTSTEIN 1980; SIMPSON *et al.* 1989). This structure may be a consequence of lesions that lead to the loss of photosystem II core particles but not of the light harvesting complexes. Bundle sheath thylakoid membranes are organized similarly in wild type and *tha1* (Figure 8), despite the fact that the protein targeting defect is equally severe in bundle sheath and mesophyll chloroplasts (data not shown). In contrast, the bundle sheath thylakoids in *hcf106* are clearly aberrant in their organization (MARTIENSSEN *et al.* 1987, 1989).

DISCUSSION

Mutations in the nuclear genes *tha1* and *hcf106* block the translocation of distinct sets of proteins across the thylakoid membrane (VOELKER and BARKAN 1995). The proteins affected by *hcf106* require a ΔpH to cross the thylakoid *in vitro,* while the proteins affected by *tha1-ref* require ATP. The possibility that the pathway blocked in *tha1* mutants and the *secA/Y/E* pathway for protein secretion in *Escherichia coli* are closely related was first suggested by the fact that they are both ATP-dependent and sensitive to azide. Recently, this possibility gained further support by the discovery that cp-SecA, a chloroplast-localized homologue of the bacterial *secA* gene product, facilitates the translocation of plastocyanin and OEC33 across thylakoid membranes *in vitro* (NAKAI *et al.* 1994; YUAN *et al.* 1994). Our finding that *tha1* encodes a chloroplast-localized SecA homologue demonstrates that cp-SecA functions in targeting OEC33, plastocyanin and PSI-F *in vivo.* These results also demonstrate that our mutant screen and phenotypic analyses led us to an authentic component of the targeting machinery and give confidence that we can use a genetic approach to probe mechanisms by which proteins are targeted to the thylakoid membrane.

Our results indicate that the *Mu1* insertion in the *tha1-ref* allele causes a dramatic decrease in *tha1* expression. *Tha1* mRNA is not detectable in mutant tissue and immunoreactive cp-SecA protein accumulates to no more than a few percent of normal levels. The residual cp-SecA in *tha1-ref* chloroplasts may result from the

translation of residual mRNA that is synthesized despite the *Mu1* insertion. Alternatively, the *tha1-ref* allele may be null, the residual "cp-SecA" signal arising from nonspecificity of the antiserum, from a nonallelic gene in the maize genome (which seems unlikely considering the results of the genomic analysis) or from the presence of small revertant sectors. To be certain that we will be dealing with a null allele in future experiments, we are now seeking a derivative allele in which *Mu* excision was accompanied by a flanking deletion of essential *tha1* sequences.

Role of cp-SecA in the targeting of nuclear-encoded proteins to the thylakoid lumen: Previously, it was established that cp-SecA can facilitate the translocation of plastocyanin and OEC33 across isolated thylakoid membranes *in vitro* (NAKAI *et al.*, 1994; YUAN *et al.*, 1994). A role for cp-SecA in the targeting of PSI-F was also deduced, based upon the fact that PSI-F translocation is inhibited by azide (KARNAUCHOV *et al.* 1994; MANT *et al.* 1994). The *tha1* mutation causes a defect in the translocation of all three of these proteins (VOELKER and BARKAN 1995), demonstrating that cp-SecA is capable not only of promoting their translocation *in vitro*, but is actually required for normal rates of translocation *in vivo*.

Azide does not significantly inhibit the translocation of the nuclear-encoded proteins OEC16 and OEC23 across isolated thylakoids (reviewed by CLINE and HENRY 1996) nor is their translocation facilitated by cp-SecA *in vitro* (NAKAI *et al.* 1994; YUAN *et al.* 1994). The rates of translocation of OEC16 and OEC23 *in vivo* are not detectably reduced in *tha1-ref* (VOELKER and BARKAN 1995) despite its near absence of cp-SecA. Thus, these two nuclear-encoded proteins depend little, if at all, upon cp-SecA to cross the thylakoid *in vivo*.

Although cp-SecA clearly facilitates the translocation of several proteins *in vivo*, our results suggest that none of the proteins yet examined absolutely require cp-SecA. Mature, lumenal OEC33, plastocyanin, cytochrome *f*, and PSI-F accumulate in *tha1* mutants to 30–50% of their normal levels, despite their reduced rates of translocation (VOELKER and BARKAN 1995). There are several possible explanations for this observation. Our preferred hypothesis is that a redundant transport mechanism for these proteins is exploited in the absence of cp-SecA. A likely candidate for an alternative pathway is the OEC16/OEC23 pathway, since this is engaged by signal sequences that closely resemble those that target proteins to the SecA pathway. The analysis of double mutants with defects in both pathways should allow us to evaluate this possibility. It is also possible, however, that residual cp-SecA in mutant chloroplasts permits very slow translocation of these proteins, the translocated forms then accumulating to disproportionately high levels due to a decrease in their degradation rate relative to that in wild-type chloroplasts.

Role of cp-SecA in the targeting of chloroplast-encoded proteins: Most studies of thylakoid protein targeting have focused on nuclear-encoded proteins. Little information is available concerning the integration of the numerous thylakoid membrane proteins encoded by chloroplast genes. Of these, cytochrome *f* is the only one known to be synthesized with a typical cleavable signal sequence. Cytochrome *f* is an integral membrane protein with a single membrane-spanning domain, its N-terminus lying within the thylakoid lumen (GRAY 1992). Mutations in sequences encoding the cytochrome *f* signal sequence in Chlamydomonas (*Chlamydomonas reinhardtii*) interfered with the insertion of the protein into the membrane, establishing a functional role for the signal sequence *in vivo* (SMITH and KOHORN 1994). Our results indicate that cytochrome *f* requires cp-SecA for efficient insertion into the membrane. This is consistent with the observation that pea cytochrome *f*, when expressed in *E. coli*, inserts into the *E. coli* cytoplasmic membrane in a *secA*-dependent manner (ROTHSTEIN *et al.* 1985).

SMITH and KOHORN (1994) identified mutations in nuclear genes that suppressed the targeting defects caused by alteration of the cytochrome *f* signal sequence. In the context of our results, it would not be surprising if some of the nuclear suppressors alter the cp-SecA gene. The reduced accumulation of LHCP, a nuclear-encoded integral thylakoid protein, caused by one mutation in the cytochrome *f* signal sequence (SMITH and KOHORN 1994) suggested that cytochrome *f* and LHCP might engage a common component during their integration. This common component is unlikely to be cp-SecA since the *tha1* mutant has no apparent defect in the accumulation or integration of LHCP (VOELKER and BARKAN 1995) and purified cp-SecA does not facilitate the integration of LHCP into isolated thylakoid membranes (YUAN *et al.* 1994). Perhaps cytochrome *f* integration is facilitated by both cp-SecA and by 54CP, a homologue of SRP54 that functions in LHCP integration *in vitro* (FRANKLIN and HOFFMAN 1993; LI *et al.* 1995).

cp-SecA functions in a variety of plastid types: The plastids comprise a group of related organelles that contain chloroplast DNA and develop from a common progenitor. Plastid types other than chloroplasts contain internal membranes, although these are less abundant than the chloroplast thylakoid membrane. The localization of OEC33 within the lumen of an internal membrane system in etioplasts (RYRIE *et al.* 1984; HASHIMOTO *et al.* 1993) suggested the presence of a targeting machinery internal to etioplasts. Our results indicate that cp-SecA is a component of this machinery, since the *tha1* mutation affects etioplast and chloroplast protein accumulation in similar ways. Ectopic expression of plastocyanin in transgenic tomato plants resulted in the accumulation of mature plastocyanin in root and petal plastids (BOER *et al.* 1988), suggesting that the

targeting pathway mediated by cp-SecA is also functioning in these plastid types.

Mutants with defects in thylakoid protein targeting are useful not only for identifying components of the targeting machinery but also for establishing the roles of these components *in vivo*. Further study of *tha1* should elucidate roles of cp-SecA that are difficult to address *in vitro*. Loss-of-function mutants like *tha1* as well as mutants identified by their ability to suppress signal sequence mutations (SMITH and KOHORN 1994) will be essential tools for understanding mechanisms involved in thylakoid protein targeting.

We are grateful to LAURA ROY and MACIE WALKER for expert technical assistance and to SARAH HAKE, MICHAEL FREELING and coworkers for access to their *Mutator* stocks. We also appreciate gifts of plasmids encoding *Mutator* elements and actin from VICKI CHANDLER. We are especially grateful to TOSHIYA ENDO for the antibody to pea cp-SecA. The *tha1* locus was mapped in the University of Missouri-Columbia Maize RFLP Laboratory. Helpful comments on the manuscript were provided by LAURA ROY, DIANNA FISK, BRAD TILL, and BETHANY JENKINS. This work was supported by grant GM-48179 from the National Institutes of Health.

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Communicating editor: K. J. NEWTON