Abstract. Proteins are translocated across the chloroplast thylakoid membrane by a variety of mechanisms. Some proteins engage a translocation machinery that is derived from the bacterial Sec export system and require an interaction with a chloroplast-localized SecA homologue. Other proteins engage a machinery that is SecA-independent, but requires a transmembrane pH gradient. Recently, a counterpart to this ΔpH mechanism was discovered in bacteria. Genetic studies revealed that one maize protein involved in this mechanism, HCF106, is related in both structure and function to the bacterial tatA and tatB gene products. We describe here the mutant phenotype and molecular cloning of a second maize gene that functions in the ΔpH mechanism. This gene, thylakoid assembly 4 (tha4), is required specifically for the translocation of proteins that engage the ΔpH pathway. The sequence of the tha4 gene product resembles those of the maize hcf106 gene and the bacterial tatA and tatB genes. Sequence comparisons suggest that tha4 more closely resembles tatA, and hcf106 more closely resembles tatB. These findings support the notion that this sec-independent translocation mechanism has been highly conserved during the evolution of eucaryotic organelles from bacterial endosymbionts.

Key words: protein export • thylakoid • Mutator • maize • Sec-independent

The targeting of proteins to the chloroplast thylakoid membrane and the bacterial cytoplasmic membrane involves several conserved mechanisms (for reviews see Settles and Martienssen, 1998; Dalbey and Robinson, 1999; Keegstra and Cline, 1999). Chloroplasts contain homologues of the bacterial proteins SecA and SRP54 (cpSecA and cpSRP54), both of which function in the targeting of specific substrates to the thylakoid membrane. Other thylakoid proteins engage a targeting machinery that was, until recently, thought to be unique to the chloroplast. This mechanism, referred to as the ΔpH pathway because of its dependence upon a transmembrane pH gradient (Cline et al., 1992), functions in the absence of nucleoside triphosphates and soluble factors in vitro (for review see Schnell, 1998). NH2-terminal signal sequences target proteins to either the chloroplast ΔpH or cpSecA systems. These resemble bacterial signal sequences and are cleaved by a signal peptidase in the thylakoid lumen (for review see Schnell, 1998). Much attention has been focused on deciphering the features within the signal sequence that distinguish ΔpH from cpSecA substrates. One clear discriminant is the twin arginine motif: two arginine residues immediately precede the hydrophobic domain of the signal sequence in ΔpH pathway substrates, and both residues are essential to engage the ΔpH machinery (Chaddock et al., 1995; Henry et al., 1997). Previously, just one protein that functions in the thylakoid ΔpH system had been identified, HCF106 in maize (Voelker and Barkan, 1995; Settles et al., 1997).

Recently, a Sec-independent export mechanism was discovered in bacteria that is related to the thylakoid ΔpH system. This mechanism functions in the export of proteins that bind complex redox cofactors (for reviews see Settles and Martienssen, 1998; Dalbey and Robinson, 1999). That export of such proteins might involve a mechanism related to the thylakoid ΔpH system was first suggested by the fact that many secreted redox proteins have signal sequences with a twin arginine motif, as do ΔpH pathway substrates (Berks, 1996). This relationship was recently confirmed in several ways. First, the export of several E. coli proteins whose signal sequences have the twin arginine motif requires the function of the tatA and tatB genes, which have sequence similarity to maize hcf106 (Sargent et al., 1998; Weiner et al., 1998). Second, bacterial
twin arginine signal sequences direct the transport of proteins via the thylakoid ΔpH system in chloroplasts (M ori and Cline, 1998; Wexler et al., 1998). The bacterial version of this system has been named the tat' system because it is involved in twin arginine translocation (Sargent et al., 1998).

The ΔpH mechanism has attracted considerable attention because it differs in fundamental ways from the intensively studied Sec mechanism. For example, translocation via the ΔpH system in vitro requires neither nucleoside triphosphates nor soluble factors (for review see Schnell, 1998), and there is evidence that both the bacterial and thylakoid systems can accommodate proteins with tertiary structure (Creighton et al., 1995; Clark and Theg, 1997; Hynsd et al., 1998; Santini et al., 1998; Dalley and Robinson, 1999). Nonetheless, the thylakoid membrane remains highly impermeable to ions during the translocation of proteins via the ΔpH pathway (Teter and Theg, 1998).

The structure and mechanism of the ΔpH/tat translocation machinery are unknown. To identify additional proteins that are involved in this process, we sought new mutations in maize that specifically disrupt the thylakoid ΔpH system. We describe here the phenotype of one such mutation, which defines a new gene, thylakoid assembly 4 (tha4). The tha4 gene was cloned by transposon tagging, revealing a gene product that resembles the plant protein HCF106 and bacterial proteins implicated in the tat export mechanism.

Materials and Methods

Plant Material

tha4-m1 was recovered in a screen of F2 families derived from a maize line with active Mutator (Mu) transposons. Numerous tha4-m1/+ plants were propagated in parallel for several generations by crossing to inbred lines. Heterozygous plants were self-pollinated to recover homozygous mutant seedlings. tha4 mutant seedlings used for DNA and RNA extraction were identified initially by their subtle chlorophyll deficiency, and then confirmed by immunoblot analysis of leaf proteins. Plants used in these experiments (tha4) were formed with a nested probe, leading to the identification of clone A (see Fig. 4 A).

A genomic library derived from the maize inbred line B73 (a gift of Doug Rice, Pioneer Hi-Bred, Johnston, Iowa) was screened to obtain sequence information both upstream and downstream of clone A. A radiolabeled 190-bp fragment derived from gene-specific sequences in the 3′ untranslated region (UTR) of the tha4 cDNA (see next section for cDNA isolation) identified two overlapping genomic clones, which contained clone A sequences within 25- and 11-kb XbaI fragments, respectively. The 11-kb XbaI fragment was digested with SacI to yield a 2-kb fragment containing clone A sequences. The 2.5-kb XbaI and 2-kb SacI fragments were subcloned into a modified pBluescript SK+ vector and used as templates for sequencing. DNA sequences were analyzed by Y anling Wang in the Institute of Molecual Biology DNA Sequencing Facility (University of Oregon, Eugene, OR).

Primers used for PCR analysis of the tha4 locus in the revertant sector (see Fig. 4) were as follows: primer M, 5′ CGA AAT GGC CAC CTT GTA -CAC 3′; primer N, 5′ GGG ACAA CCA CCG GTA TAC 3′; and Mu primer, 5′ A GAGA AGCC A CGCCA WGC GCT Y ATT T 3′.

Isolation and Analysis of cDNA

To isolate a tha4 cDNA, a maize leaf cDNA library (Fisk et al., 1999) was screened by PCR using primers designed to amplify the 3′ ends of tha4 cDNA. For this purpose, tha4 gene primers were chosen that mapped to sequences in clone A encoding amino acids just downstream of the predicted transmembrane domain. The initial PCR used a tha4 gene primer (5′ C ACA GAC GCT GCC CCG GTA TCC 3′) and a vector primer (5′ A GGG TTT TCC CAG TCA CAG 3′) according to the following profile: 94°C/4 min, followed by 30 cycles of 94°C/1 min, 60°C/1 min, 72°C/2 min, and a final extension at 72°C/5 min. A second round of PCR was performed with a nested tha4 gene primer (5′ A TCG GCA A AGC CGT CAA-GAC 3′) and an EcoRI-oligo dT primer (5′ CGG AATT C(T) 3′) according to the profile: 94°C/4 min, followed by an initial two cycles of 94°C/1 min, 37°C/45 s, 72°C/2 min, followed by 30 cycles of 94°C/1 min, 60°C/45 s, 72°C/2 min, and a final extension at 72°C/5 min. Amplifications were performed in 50-μl reactions containing 50 mM KCl, 10 mM Tris-HCl, pH 9, 1% Triton X-100, 1.5 mM MgCl2, 0.2 mM dNTPs, 5 μl cDNA library stock or 1 μl of initial PCR, and 250 ng of each primer plus Taq DNA polymerase. The PCR product was cloned into pBluescript SK+ and its DNA sequence was determined. The cDNA library was screened using this PCR clone as a probe, yielding two types of cDNA, encoding highly similar proteins but with distinct 3′ UTR sequences. These revealed the presence of a closely related gene in the maize genome, which we named tha9. A thylakoid cDNA was distinguished from tha9 cDNA by four criteria: (1) their 3′ UTR detected an mRNA that accumulated to reduced levels in F2 mutant seedlings (see Fig. 5); (2) their 3′ UTR detected only authentic tha4 genomic clones (i.e., that matched clone A) when used to screen a genomic library of B73 DNA; (3) a nearly full-length, spliced tha4 cDNA obtained by reverse transcriptase-PCR (see below) was identical throughout a 382 bp region of overlap to genomic clone A; and (4) partial unspliced tha4 cDNA recovered from the cDNA library contained intron sequence that matched intron sequence in the tha4 clone obtained from the B73 genomic library.

Because library screens failed to yield full-length tha4 cDNA, a cDNA containing the entire coding region of the tha4 mRNA was obtained by reverse transcription–PCR amplification of poly (A)+ seedling leaf RNA from the inbred maize line B73 (Pioneer Hi-Bred). cDNA synthesis was catalyzed by M-MuLV reverse transcriptase (Promega) and primed with a 1843′ UTR gene-specific primer (5′ CTTCATA CGTAGAGCT 3′).
and resuspended in HS. Proteins from the salt-washed supernatants were as appropriate. In control experiments, to test the protease accessibility of Samples were centrifuged at 12,000 g, and 3 vol of proteinase K stop buffer (HS plus 10 mM EGTA). Inactivated by the addition of 1/20 vol of 40 mM PMSF, 1/10 vol of 0.1 M 0.2 mg/ml thermolysin or 0.03 mg/ml proteinase K on ice for 30 min. Ther-

...solved in GenBank/EMBL/DBJ under accession number A145755. The thaa cDNA sequence has been entered in Genbank/EMBL/DBJ under accession number A145756.

Sequence Alignments
Sequences of THA A (accession number A145755), THA 9 (accession number A145756), HCF106 (accession number A145756), TTA (accession number CA 06724), and TAT (accession number CA A06725) were aligned using ClustalW 1.7 (Thompson et al., 1994) and Boxshade (Bioinformatics Group, IJSR EC).

Results
A Mutation in the thaa Gene Disrupts the ΔpH–Dependent Thylakoid Targeting Pathway

The reference allele of thaa, thaa-m1: M11 (hereafter referred to as thaa-m1) was detected in a seedling screen of the F2 progeny of maize plants with active M1 transposons. Homozygous mutant seedlings were subtly chlorophyll-deficient and died after the development of three to four leaves. In these ways, thaa mutants resembled many previously described maize mutants that lack subsets of thylakoid membrane proteins (for review see Barkan, 1998). Immunoblot data presented previously (Roy and Barkan, 1998) showed that thaa-m1 mutants accumulate only 20% of the normal levels of the core subunits of photosystems I and II and the cytochrome b6f complex. However, the thylakoid ATP synthase and the major light harvesting chlorophyll a/b binding protein accumulate normally. The pig-

...tigation. To distinguish between these possibilities, the intrachloroplast location of the pre-

...southern hybridizations. The restriction mapping and sequencing of the HCF106 cDNA confirmed that the intrachloroplast location of the precursor was determined by fractionating thaa-m1 mutant chloroplasts to separate the stroma from the thylakoid membrane vesicles. Precursors of both OE23 and OE16 were enriched in the stromal fraction, whereas the mature

Figure 1. Immunoblot analysis of luminal proteins in thaa-m1 mutants. 5 µg of total leaf protein or the indicated dilutions of the wild-type (WT) sample were analyzed. Proteins were detected with monospecific antisera for OE33, OE23, OE16, or PC. A rrows indicate intermediate (i) or mature (m) forms of OE23 and OE16.
is often detected as a doublet, perhaps because it is encoded by a protease in the absence of intact membranes. Maize OE23 is high susceptible to proteases in thata4-m1 thylakoid membrane vesicles (Fig. 3 B). This fractionation behavior is the same as that described for HCF106 in wild-type chloroplasts (Settles et al., 1997; see Fig. 7). Therefore, tha4-m1 does not alter the accumulation of HCF106 or its insertion into the thylakoid membrane.

**Molecular Cloning of the tha4 Gene**

The 1.9-kb XhoI fragment was cloned from a size-enriched genomic library of tha4-m1 mutant DNA (Fig. 4 A, clone A). Southern blots of wild-type and thata4-m1 mutant DNAs were probed with the genomic sequence flanking the cloned Mu1 insertion, revealing that all mutant and no wild-type plants were homozygous for the cloned insertion (data not shown). Longer clones corresponding to this region were isolated from a genomic library of wild-type DNA. To test whether clone A contained the insertion that is the cause of the thata4-m1 mutant phenotype, the structure of the corresponding genomic region was monitored in a revertant sector that appeared on a thata4-m1 mutant leaf. DNA extracted from a dark green revertant sector and from the flanking, slightly paler mutant tissue was analyzed with PCR using primer pairs designed to select

forms were found predominantly in the thylakoid membrane fraction (Fig. 2). A small proportion of each precursor remained bound to the thylakoid membrane. To determine whether these bound precursors had been translocated across the membrane, the stromal face of the thylakoid vesicles was treated with carbonate, NaBr, or proteases. The carbonate and NaBr treatments caused some disruption of the vesicles, as revealed by the recovery of a small fraction of mature OE23 and OE16 in the supernatant; wild-type thylakoid membranes treated in the same fashion behaved similarly (see Fig. 7 B). Nonetheless, it is clear that the membrane-bound precursors were extrinsic proteins on the stromal face of the membrane: they were removed by treatment with carbonate or NaBr, and were selectively degraded by the proteases thermolysin or proteinase K (Fig. 2). These results provide strong evidence that the accumulation of incompletely processed OE23 and OE16 in thata4-m1 mutants results from a defect in their translocation to the thylakoid lumen.

Taken together, these results strongly suggest that tha4, like hcf106, functions in the ΔpH-dependent system for translocating proteins across the thylakoid membrane. The tha4 gene was mapped to chromosome 1L by crossing with a series of B-A translocation stocks (Beckett, 1978). In contrast, hcf106 maps to chromosome 2, indicating that tha4-m1 defines a new gene.

To address the possibility that tha4-m1 disrupts the ΔpH pathway by interfering with the accumulation of membrane-bound HCF106, the abundance and location of HCF106 were monitored in thata4-m1 mutants. HCF106 accumulates to normal levels in thata4-m1 mutants (Fig. 3 A). The same tha4-m1 chloroplast fractions used in Fig. 2 were probed with anti–HCF106 antibody (Fig. 3 B), revealing that HCF106 is tightly associated with the thylakoid membrane in thata4-m1 mutants. Furthermore, HCF106 is highly susceptible to proteases in tha4-m1 thylakoid membrane vesicles (Fig. 3 B). This fractionation behavior is the same as that described for HCF106 in wild-type chloroplasts (Settles et al., 1997; see Fig. 7). Therefore, thata4-m1 does not alter the accumulation of HCF106 or its insertion into the thylakoid membrane.

**Figure 2.** Localization of precursors to OE16 and OE23 in fractionated tha4-m1 chloroplasts. Chloroplasts (cp) from mutant seedlings were hypotonically lysed and centrifuged to separate thylakoid membrane vesicles (thylakoid) from stroma. To remove peripheral proteins on the stromal face of the membrane, the thylakoid fraction was washed with either Na2CO3 or NaBr. The resulting pellet (p) and supernatant (s) are indicated. Alternatively, the thylakoid fraction was treated with proteinase K or thermolysin with and without Triton X-100. Proteins were detected on immunoblots with monospecific antisera for OE23 or OE16. With the exception of lane 1 (0.25× cp), each lane contains protein isolated from the same number of chloroplasts. The stromal intermediate (i) and mature (m) forms of OE23 and OE16 are indicated. Triton was included in control protease reactions to demonstrate that mature OE23 and OE16 are susceptible to protease in the absence of intact membranes. Maize OE23 is often detected as a doublet, perhaps because it is encoded by a gene family (our unpublished results).

**Figure 3.** Immunoblot analysis of HCF106 in thata4-m1 mutants. (A) 5 μg of tha4-m1 and wild-type (WT) leaf protein were fractionated and probed with antiserum specific for HCF106. (B) The immunoblot shown in Fig. 2 was stripped and reprobed with antiserum specific for HCF106.
Primers M and N together, however, gave rise to an amplification product of 210 bp with a WT template DNA, the size predicted for the wild-type allele. A amplification of DNA from the mutant tissue on the sectored leaf (Fig. 4 B, tha4) with the Mu primer in conjunction with primers M or N resulted in the predicted DNA fragments of 223 and 136 bp, respectively. A s expected, the tha4 mutant DNA did not yield an abundant product when the gene-specific primers M and N were paired because PCR fails to amplify across intact Mu elements.

Revertant sectors are expected to be heterozygous and should, therefore, give rise to the products representing both alleles. With revertant DNA as a template, primer M or N paired with the Mu primer gave rise to the 223- or 136-bp fragments expected for the mutant allele (Fig. 4 B). The key finding was that the revertant DNA also contained an allele lacking the cloned Mu insertion; primer M paired with primer N yielded a robust amplification product. This product was slightly smaller than that resulting from a wild-type DNA template, indicating that it did not result from contamination with wild-type DNA. These results suggest that imprecise excision of Mu1 caused a small deletion of flanking genomic sequences, and that this excision, nonetheless, restored tha4 gene function. A s described below, the Mu insertion in tha4-m1 disrupts the untranslated sequence in the 5' portion of the tha4 gene, such that excision accompanied by a small deletion could well restore gene function. These results indicate that excision of the Mu1 insertion represented by clone A correlates with reversion to a wild-type phenotype, providing strong evidence that the clone contains a portion of the tha4 gene.

The genomic sequence flanking the cloned Mu1 insertion was used to obtain tha4 cDNA s. The tha4 cDNA encoded a continuous open reading frame of 170 amino acids. The Mu1 insertion disrupted sequences mapping 35 bp upstream of those encoding the predicted start codon (Fig. 4 C). A probe prepared from the unique 3' UTR sequences of the tha4 cDNA detected a leaf mRNA of ~900 nucleotides that accumulated normally in hcf106 mutants, but was barely detectable in tha4-m1 mutants (Fig. 5). The longest tha4 cDNA obtained began at the predicted start codon and included 740 nucleotides between the start codon and the beginning of the poly(A) tail. Given that
diagrammatically amplify either the mutant or wild-type allele (Fig. 4 B).

Control reactions first established that the predicted amplification products were obtained with homozygous mutant and wild-type DNA samples. A s expected, DNA from homozygous wild-type tissue (WT) gave no amplification products when the Mu primer was used in conjunction with either of the gene-specific primers, M or N.

Figure 4. Molecular cloning of the tha4 gene. (A) Map of the original genomic clone of tha4-m1. Clone A was a 1.9-kb XhoI fragment that included the 1.4-kb Mu1 transposon and 542 bp of flanking sequence. Comparison with cDNA sequence identified 129 bp of intron sequence within this clone. The bold line represents the translated sequence. The XhoI site on the left side of the clone was created upon insertion of the Mu1 transposon. (B) PCR analysis of the cloned genomic region in a somatic revertant sector. DNA was extracted from a revertant sector (sector), from mutant tissue on the same leaf (tha4), and from a +/+ sibling (WT). Control reactions lacked template DNA (no DNA). Arrows represent primer binding sites. The sizes of the amplification products predicted for each primer pair are illustrated. (C) Site of Mu1 insertion in tha4-m1. UTR untranslated and translated regions are represented by fine and bold lines, respectively. The Mu1 insertion is 35 bp upstream of sequences encoding the putative start codon. The known intron is diagrammed; other introns may be encoded in sequences downstream of the genomic clone. The sequence surrounding the Mu insertion site is shown beneath the map, with the box indicating the 9-bp that were duplicated upon Mu insertion. The putative start codon is shown in bold.

Figure 5. RNA gel blot showing loss of tha4 mRNA in tha4-m1 mutant. 20 μg of total leaf RNA from tha4-m1, wild-type (WT), or hcf106 seedlings was gel-fractionated, transferred to nylon membrane, and probed with a radiolabeled 190-bp DNA fragment that hybridizes specifically to the 3' UTR of the tha4 mRNA (top). The same filter was stained with ethidium bromide to visualize the 23S rRNA (bottom). 23S* is a fragment of the chloroplast 23S rRNA.
the RNA gel blot analysis indicated a length of ~900 nucleotides for the polyadenylated mRNA and that poly(A) tails commonly contain ~100 residues in plants, we predict that the 5' UTR of the tha4 mRNA contains 50–100 nucleotides. No in-frame ATGs are found in 300 bp of genomic sequence upstream of those encoding the predicted start codon. The Mu1 insertion, mapping 35 bp upstream of the predicted start codon, therefore, likely disrupts the 5' UTR of the tha4 gene.

**THA4 Resembles HCF106 and Bacterial Proteins Implicated in Sec-Independent Protein Export**

The deduced tha4 gene product (THA4) is 170 amino acids in length and has a single predicted membrane spanning domain. The ChloroP algorithm (Emanuelsson et al., 1999) predicts that THA4 is a chloroplast-localized protein. THA4 is related to maize HCF106 and to the products of the bacterial tatA and tatB genes (Fig. 6 A). TatA and TatB are bacterial proteins implicated in Sec-independent protein export (Sargent et al., 1998; Weiner et al., 1998) and hcf106 functions in the thylakoid ΔpH mechanism (Voelker and Barkan, 1995; Settles et al., 1997). The four proteins are closely related in their membrane-spanning domains (underlined in Fig. 6 A) and in the adjacent amphipathic helical domain (see Settles et al., 1997). The predicted mature form of THA4 is similar in size to TatA, whereas the predicted mature form of HCF106 is similar in size to TatB. THA4 and TatA lack the extended COOH-terminal acidic region found in both HCF106 and TatB. Previously, it was proposed that hcf106 is more closely related to tatA than to tatB (Sargent et al., 1998). However, these results suggest that hcf106 is more closely related to tatB and that tha4 is more closely related to tatA. In addition, an Arabidopsis cDNA sequence (accession number H37534) proposed previously to represent an hcf106 homologue (Settles et al., 1997) is, in fact, much more similar to tha4 (data not shown) and likely represents a tha4 ortholog.

cDNA library screens yielded two classes of cDNA, representing tha4 and a closely related gene, which we named tha9 (see Materials and Methods). Both genes were represented as cDNAs in a seedling leaf cDNA library, indicating that both are transcribed in seedling leaf tissue. The tha9 cDNA encodes a protein that is very closely related to THA4 (Fig. 6 B), and that is predicted by the ChloroP algorithm to be chloroplast-localized. The predicted mature form of THA9 is nearly identical to that of THA4 (sequence downstream of vertical arrows in Fig. 6 B); even the predicted transit peptides diverge to only a small degree. This degree of identity strongly suggests that these two proteins are localized similarly in the cell and that they have similar or identical functions.

**THA4 Is an Integral Thylakoid Membrane Protein with Its COOH Terminus Exposed to the Stroma**

The near identity of the mature regions of THA4 and THA9 precluded the generation of a THA4-specific antiserum. A polyclonal antiserum that would detect both THA4 and THA9 was generated to the COOH-terminal region of THA4 (Fig. 6 B, horizontal arrow). The antisera detected a protein in wild-type leaf tissue that migrated during SDS-PAGE with an apparent mass of 16 kD and accumulated to much reduced levels in tha4-m1 mutant leaf tissue (Fig. 7 A). The residual protein in the mu-
tA 4 is exposed to the stroma. The fractionation behavior of HCF106 was similar to that of tHA 4 (Fig. 7 B), consistent with the previous report that HCF106 is tightly associated with the membrane and is susceptible to proteases applied to the stromal face (Settles et al., 1997). Thus, tHA 4 and HCF106 are likely to be oriented similarly in the membrane, with their COOH-terminal acidic tails in the stroma.

The tha4 and ttha9 mRNAs Accumulate in Different Ratios in Different Tissue Types

Duplicate gene pairs analogous to taha/tha9 are common in maize as a consequence of the tetraploidy of the ancestral maize genome (Ahn and Tanksley, 1993). The near identity of the tHA 4 and tHA 9 amino acid sequences suggests that the two proteins have retained similar or identical biochemical functions. Nonetheless, tha9 function cannot fully compensate for the absence of tha4 since mutations in tha4 disrupt the ΔpH-dependent translocation mechanism in seedling leaves. To address the possibility that the two genes have acquired different patterns of regulation, their mRNAs were quantified in green seedling leaf tissue, etiolated seedling leaf tissue, basal leaf tissue, roots, endosperm, and immature ears (Fig. 8). These tissues differ with regard to their plastid populations; green seedling leaf contains mature chloroplasts; basal leaf is enriched in proplastids, the chloroplast progenitors found in undifferentiated cells; etiolated leaf contains etioplasts; which differentiate into chloroplasts upon exposure to light; and root, endosperm, and immature ear contain a variety of nonphotosynthetic plastid forms.

RNase protection assays were used to distinguish the tha4 and tha9 mRNAs. The probe generated from the tha4 cDNA was nearly completely protected by tha4 mRNA, as expected (Fig. 8 A, tha4 band). The tha9 mRNA protected smaller but still substantial fragments of the tha4 probe, because of their high degree of sequence complementarity (Fig. 8 A, tha9 bands); this permitted the simultaneous detection of both mRNA species in each lane. In addition, the tha9 mRNA was assayed with a probe generated from the tha9 cDNA (Fig. 8 B). The results of these two ttha9 mRNA assays were consistent and served to reinforce one another.

The level of the tha4 mRNA was substantially reduced in tha4-m1 mutants (Fig. 8 A, compare tha4 leaf and WT leaf samples), whereas the accumulation of tha9 mRNA was unaltered in tha4 mutants (Fig. 8, A and B). The ttha4 mRNA accumulated to similar levels in green seedling leaves, etiolated seedling leaves, basal leaf, and immature ear (Fig. 8 A). Its level was somewhat lower in endosperm and it was barely detectable in the root (Fig. 8 A).

The profile of tha9 mRNA accumulation differed significantly from that of taha. The ttha9 mRNA accumulated to the highest level in green leaf tissue, to slightly lower levels in etiolated leaf tissue, and to much lower levels in leaf base, endosperm, immature ear, and root. Most notably, the ratio of tha4 to ttha9 mRNA was much higher in leaf base and in immature ear than in green leaf (Fig. 8 A and B). The fact that tha4 mRNA is predominant in basal leaf tissue suggests that taha function is required early in the proplastid to chloroplast transition, when the elaboration of thylakoid membranes is initiated. The taha gene may
then provide supplemental protein in young and mature chloroplasts to maintain optimal function of the ΔpH-dependent translocation machinery.

**Discussion**

We have presented evidence that the maize tha4 gene functions in the ΔpH-dependent mechanism for the translocation of proteins across the chloroplast thylakoid membrane. THA 4 is the second plant protein to be identified that participates in this mechanism, the first being the related protein HCF106 (Voolker and Barkan, 1995; Settles et al., 1997). Both THA 4 and HCF106 are tightly associated with the thylakoid membrane; sequence analysis and protease sensitivity studies suggest they both have a single transmembrane domain and an acidic COOH-terminal tail on the stromal side of the membrane. However, the stromal domain of HCF106 is considerably longer and more acidic than that of THA 4.

THA 4 and HCF106 are related to the tatA and tatB genes found in all sequenced eubacterial genomes. tatA and tatB were recently implicated in a novel sec-independent mechanism for the export of periplasmic proteins that bind redox cofactors (for review see Dalbey and Robinson, 1999). A ligment of the most conserved regions of these proteins (the transmembrane and amphipathic helical domains) revealed a comparable degree of similarity between all protein pairs. However, these proteins fall into two classes based upon the length of their COOH-terminal hydrophilic tails: HCF106 and TatB have a long tail of nearly identical length, and THA 4 and TatA have a much shorter tail, also of nearly identical length (Fig. 6). On this basis, we propose that tha4 is more closely related to tatA and hcf106 is more closely related to tatB.

The similarity between the THA 4/TatA and HCF106/TatB sequences raises questions about the functional relationship between these genes. As is true of tha4 and hcf106, mutations in either tatA or tatB disrupt protein export (Sargent et al., 1998; Weiner et al., 1998), indicating that these genes have at least some distinct functions. The situation is still more complex because many bacterial genomes include a second tatA-like gene, termed tatE in E. coli, and mutations in tatE also disrupt export to some extent (Sargent et al., 1998). In maize, both tha4 and hcf106 are themselves members of duplicate gene pairs: from a maize leaf cDNA library, we recovered cDNAs encoding a protein with >90% identity to HCF106 (Barkan, A., unpublished results) as well as the tha9 cDNA described here, which is very closely related to tha4. That mutant phenotypes result from disruption of either hcf106 or tha4 is consistent with the notion that the members of each gene pair may not be completely redundant in their biochemical function. However, the near identity of the proteins in each pair leads us to favor the notion that the members of each pair play identical biochemical roles, but are subject to different patterns of regulation. This is supported by our finding that the tha4 and tha9 mRNA accumulate differentially in different plant tissues. In any case, a picture is emerging of a group of related proteins involved in this novel protein translocation mechanism. The biochemical role of these proteins is not known, although it has been proposed based upon their orientation in the membrane that they function as receptors (Settles et al., 1997). It will be fascinating to learn how these proteins are organized in the membrane and how they relate in a functional sense to one another.

Mutant phenotypes originally implicated THA 4 and HCF106 in the ΔpH-dependent translocation mechanism. Recently, conclusive evidence that these proteins function directly in translocation was obtained from in vitro import assays performed in the presence of anti–THA 4 or anti–HCF106 antibodies (Mori et al., 1999). Thus far, THA 4 and HCF106 are the only proteins known to participate in the ΔpH-dependent mechanism in chloroplasts. However, by comparison to the bacterial system, it seems likely that a plant homologue of the bacterial TatC protein is also involved. TatC is predicted to be a polytopic membrane protein and is encoded in the same operon as TatA.

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**Figure 8.** RNase protection analysis of tha4 and tha9 mRNA in different maize tissues. (A) Assays using a tha4 probe. 15 μg of total RNA from the indicated maize tissues was analyzed with an antisense probe generated from the tha4 cDNA. Control reactions contained 15 μg of tRNA plus 1 pg of a sense tha9 transcript generated by in vitro transcription of the tha9 cDNA (first lane) or 15 μg of tRNA alone (second lane). The band designated tha4 represents the tha4 mRNA; it is slightly smaller than the full-length probe, which included some vector sequence. The band designated tha4* also results from protection by the tha4 mRNA (note its absence in the tha4 mutant leaf sample); this band may result from a sequence polymorphism between the tha4 probe and tha4 mRNA, resulting in inefficient cleavage of the duplexed probe. The cluster of bands designated tha9 result from protection by the tha9 mRNA. (B) Assays using a tha9 probe. 10 μg of total RNA from the indicated maize tissues or 10 μg of tRNA (first lane) was analyzed with an antisense probe generated from the tha9 cDNA. The band designated tha9 represents the tha9 mRNA.
and TatC in E. coli (Sargent et al., 1998). A tatC deletion mutant has a severe defect in the export of tat substrates (Bogsch et al., 1998). Genes with similarity to tatC are found in the chloroplast genomes of certain algae and plant Cd NAs encoding tatC homologues have recently appeared in the databases. However, direct evidence for the role of these tatC homologues has not been reported. We recently recovered a maize mutant with a more severe defect in the Δ pH-dependent pathway than either hcf106 or thd4 mutants, and we have established that the mutation defines a third gene involved in this process (Pedersen, R., M. Walker, and A. Barkan, unpublished results). Whether this new mutation disrupts a TatC homologue or defines a novel component of this interesting protein translocation mechanism remains to be determined.

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