Tic21 Is an Essential Translocon Component for Protein Translocation across the Chloroplast Inner Envelope Membrane

Yi-Shan Teng,[a,b] Yi-shin Su,[b,1] Lih-Jen Chen,[b] Yong Jik Lee,[c,2] Inhwan Hwang,[c] and Hsou-min Li[b,3]

[a] Graduate Institute of Life Sciences, National Defense Medical Center, Taipei 112, Taiwan
[b] Institute of Molecular Biology, Academia Sinica, Nankang, Taipei 115, Taiwan
[c] Division of Molecular and Life Sciences, Center for Plant Intracellular Trafficking, Pohang University of Science and Technology, Pohang 790-784, Korea

An *Arabidopsis thaliana* mutant defective in chloroplast protein import was isolated and the mutant locus, *cia5*, identified by map-based cloning. CIA5 is a 21-kD integral membrane protein in the chloroplast inner envelope membrane with four predicted transmembrane domains, similar to another potential chloroplast inner membrane protein-conducting channel, At Tic20, and the mitochondrial inner membrane counterparts Tim17, Tim22, and Tim23. *cia5* null mutants were albino and accumulated unprocessed precursor proteins. *cia5* mutant chloroplasts were normal in targeting and binding of precursors to the chloroplast surface but were defective in protein translocation across the inner envelope membrane. Expression levels of *CIA5* were comparable to those of major translocon components, such as At Tic110 and At Toc75, except during germination, at which stage At *Tic20* was expressed at its highest level. A double mutant of *cia5* At *tic20-I* had the same phenotype as the At *tic20-I* single mutant, suggesting that CIA5 and At Tic20 function similarly in chloroplast biogenesis, with At Tic20 functioning earlier in development. We renamed CIA5 as *Arabidopsis* Tic21 (At Tic21) and propose that it functions as part of the inner membrane protein-conducting channel and may be more important for later stages of leaf development.

INTRODUCTION

Most proteins in chloroplasts are encoded by the nuclear genome and are synthesized in the cytosol as precursors with N-terminal targeting signals called transit peptides. Import of precursor proteins into chloroplasts is mediated by a protein translocon complex, which is composed of the Toc (for translocon at the outer envelope membrane of chloroplasts) and the Tic (for translocon at the inner envelope membrane of chloroplasts) proteins and stromal chaperones (for reviews, see Soll and Schleiff, 2004; Kessler and Schnell, 2006). Three Toc proteins, Toc159, Toc75, and Toc34, form the core Toc complex. Toc159 and Toc34 are homologous GTPases and function as the initial receptors for incoming precursors. Toc75 is the protein-conducting channel across the outer membrane. Toc75 is predicted to form a β-barrel–type channel similar to transporters found in the outer membrane of gram-negative bacteria and the Tom40 protein-conducting channel of the mitochondrial outer membrane.

The identity and composition of the protein-conducting channel across the chloroplast inner envelope membrane are much less clear. Two proteins, Tic20 and Tic110, have been suggested to function as the inner membrane channel. Tic20 was identified by direct cross-linking to importing precursors in pea (*Pisum sativum*) chloroplasts (Kouranov et al., 1998). It is deeply embedded in the inner membrane. It has some low-level similarity to prokaryotic amino acid permeases and is distantly related to the mitochondrial inner membrane Tim17-Tim22-Tim23 protein-conducting channel family, all of which have similar molecular weights and contain four predicted transmembrane domains (Sirrenberg et al., 1996). *Arabidopsis thaliana* has two genes encoding Tic20: At *Tic20-I* and At *Tic20-IV*. The At *Tic20-I* gene, compared with At *Tic20-IV*, encodes a protein that shares a higher degree of identity to pea Tic20. In vivo reduction of At Tic20 by expressing an antisense construct of At *Tic20-I* in *Arabidopsis* generated a range of phenotypes from seedling lethal, pale, and stunted growth to pale cotyledons but fully recovered mature leaves (Chen et al., 2002). At *Tic20-I* is expressed at its highest level during germination and at an equal level in etiolated and light-grown seedlings. These data indicate that At Tic20 is important for early plastid development (Chen et al., 2002). However, it has been reported that pea Tic20 is present at a 10-fold lower abundance compared with Toc75, arguing against a function for Tic20 as the general protein-conducting channel across the inner membrane (Vojta et al., 2004; Becker et al., 2005).

Tic110 is the major Tic component identified. It is predicted to have a small N-terminal hydrophobic domain and a large C-terminal hydrophilic domain. Incorporation of denatured
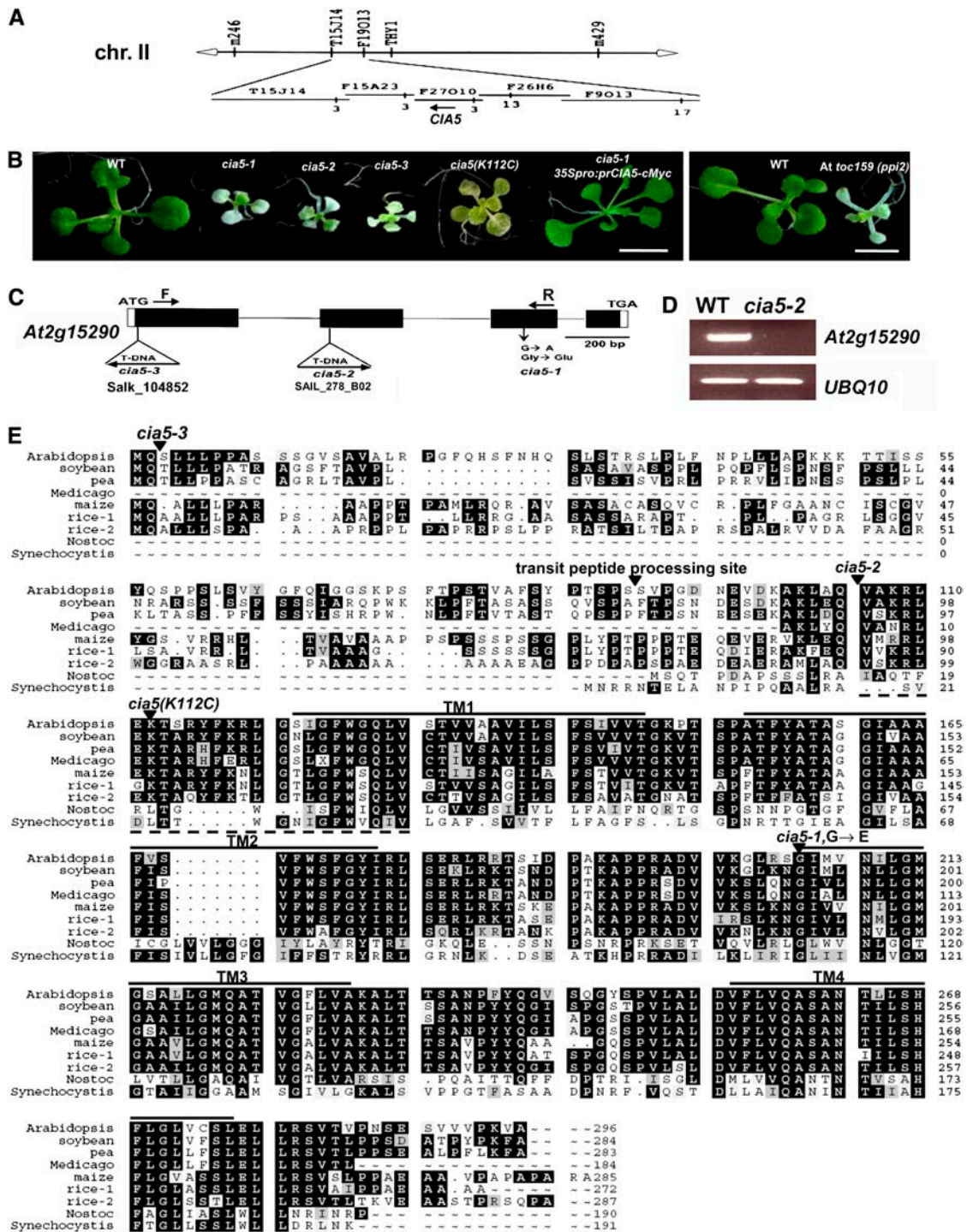
Figure 1. Molecular Cloning of *CIA5*.

(A) Chromosome location of *CIA5*. Vertical lines indicate the positions of PCR-based markers. Numbers under the lines indicate the numbers of recombinant plants. The direction of the *CIA5* transcript is marked by an arrow. chr., chromosome.

(B) Phenotypes of various mutants. All plants were grown on plates for 14 d. Bar = 0.5 cm.

(C) Gene structure of *CIA5* and mutation positions of *cia5* alleles. Open boxes, closed boxes, and lines between boxes indicate untranslated regions (UTRs), exons, and introns, respectively. F and R, *CIA5* forward and reverse primers, respectively, used in **(D)**.

(D) *cia5-2* is a null allele. mRNA isolated from leaves of 21-d-old wild-type and *cia5-2* mutant plants were used for RT-PCR analysis of *CIA5* expression. Locations of the primers used for RT-PCR are marked in **(C)**. *UBQ10* was amplified at the same time and used as a loading control.

(E) Sequence alignment of CIA5 from various species. The transit peptide processing site and the mutation positions of *cia5* alleles are marked by arrowheads. The putative transmembrane domains (TM) are marked by lines above the sequence. The dotted line indicates the peptide sequence from *Medicago* used for antibody generation. An available partial *Medicago* sequence was used for the alignment. Accession numbers for all sequences shown are listed at the end of Methods.

recombinant Tic110 into proteoliposomes gives rise to cation-selective channels (Heins et al., 2002). However, topology analyses in intact chloroplasts and expression in transgenic plants have indicated that Tic110 is anchored to the inner membrane with the N-terminal hydrophobic domain, and the large C-terminal domain is soluble and is located in the stroma (Jackson et al., 1998; Inaba et al., 2003, 2005). Part of the Tic110 stromal hydrophilic domain binds transit peptides directly and therefore may function as the stromal docking site for precursors when they are first translocated across the inner membrane into the stroma (Inaba et al., 2003). It is still likely that the membrane anchor of Tic110 participates in channel formation. Tic20 is detected in Tic/Toc supercomplexes in association with Tic110. It is therefore possible that the Tic channel is formed by interaction of Tic20 and Tic110 (Kessler and Schnell, 2006). However, the low-abundance Tic20 in leaves needs to be accounted for, possibly by the presence other channel components.

To further understand the mechanism of protein import into chloroplasts, we have designed a transgene-based screening strategy to isolate *Arabidopsis* mutants defective in chloroplast protein import (Sun et al., 2001). We have named these mutants *cia* (for chloroplast import apparatus). Characterization of the *cia5* mutants and the CIA5 protein indicates that CIA5 is an essential translocon component for protein translocation across the inner membrane. Expression data and single and double mutant phenotypes of *cia5* and At *tic20-I* suggest that CIA5 and Tic20 may perform similar functions and that CIA5 is more important for later and Tic20 is more important for earlier stages of leaf development.

RESULTS

Molecular Cloning of the *CIA5* Locus

Using a transgene-based screening strategy, possible chloroplast protein import mutants were selected based on hygromycin resistance in M2 seedlings due to the cytosolic accumulation of the chloroplast-targeted marker protein hygromycin phosphotransferase (Sun et al., 2001). One of the hygromycin-resistant M2 plants segregated one-quarter albino plants in the M3 generation (10 albino; 29 green). All the albino plants were hygromycin resistant, while the green plants showed a mixture of hygromycin-resistant and -sensitive phenotypes. Ten of the hygromycin-resistant green plants were propagated, and nine of them again segregated one-quarter albino plants in the M4 generation, and all the albino plants were hygromycin resistant. These results suggested that mutation in the same locus caused the albino and hygromycin-resistant phenotypes. The mutation conferred hygromycin resistance in the heterozygous state but caused the albino phenotype only in the homozygous state.

We isolated the *CIA5* locus by positional cloning. The mutation was initially mapped to the top arm of chromosome II between the cleaved-amplified polymorphic sequence markers (Konieczny and Ausubel, 1993) m246 and THY1 (Figure 1A). Additional cleaved-amplified polymorphic sequence markers were identified using sequences available at the time of cloning. Data from six recombinant plants delimited *CIA5* to the region contained by the right ends of BACs F15A23 and F27O10. All predicted open reading frames within this region were sequenced. The gene *F27O10.6* (*At2g15290*) in *cia5* contained a guanine-to-adenine mutation that changed a conserved Gly to Glu. To confirm that *At2g15290* was the mutant locus, a construct of *At2g15290* cDNA N-terminally fused to the coding region of a cMyc tag and placed under the control of the cauliflower mosaic virus 35S promoter (*35Spro: prCIA5-cMyc*) (due to the presence of a transit peptide, the full-length protein encoded by *At2g15290* was named prCIA5; see below) was transformed into heterozygous *cia5*, and all transformants were recovered to the wild-type phenotype (Figure 1B). Two T-DNA insertion mutants of *At2g15290*, SAIL_278_B02 and Salk_104852 (Alonso et al., 2003), were obtained. The mutants isolated from our screen, SAIL_278_B02 and Salk_104852, were renamed *cia5-1*, *cia5-2*, and *cia5-3*, respectively (Figures 1B and 1C).

All three alleles had identical phenotypes (Figure 1B). They were seedling lethal on soil but could produce variable numbers of albino leaves or even inflorescent tissues on synthetic media supplemented with sucrose. The youngest part of the plant at the center of the seedling was slightly green (Figure 1B). These phenotypes made *cia5* almost indistinguishable from the *Arabidopsis ppi2* mutant, which lacks the major chloroplast protein import receptor At Toc159 (Figure 1B; Bauer et al., 2000). RT-PCR analysis indicated that *cia5-2* was a null mutant (Figure 1D). Since all three alleles had identical phenotypes, it is most likely that all three alleles were null.

At2g15290 encodes a 297–amino acid polypeptide of 31 kD with a predicted chloroplast-targeting transit peptide (Figure 1E). The processing site was identified, by isolating and N-terminal sequencing the cMyc-tagged CIA5 from transgenic plants, as being located between residues 90 and 91 (Figure 1E). Homologues of CIA5 were found in higher plants, *Nostoc punctiforme PCC 73102* (hypothetical protein Npun02008398) and *Synechocystis* sp *PCC 6803* (hypothetical protein sll1656), and seem to be restricted to photosynthetic organisms. CIA5 had low sequence similarity to *Nostoc* proteins that are annotated to be members of the amino acid transporter and the permease component of the ABC-type sugar transporter families. Although no significant sequence similarity was found between CIA5 and Tic20, the mature region of CIA5 was predicted to have four transmembrane domains (Figure 1E) with a molecular mass of ~21 kD, similar to Tic20 (Chen et al., 2002) and the mitochondrial inner membrane protein-conducting channels Tim17, Tim22, and Tim23 (Sirrenberg et al., 1996).

CIA5 Is an Integral Inner Envelope Membrane Protein of Chloroplasts

The subcellular location of CIA5 was first investigated by transiently expressing a gene encoding the transit peptide of prCIA5 plus the first 14 amino acids of the mature protein fused to red fluorescent protein (RFP) (prCIA5TP-RFP) in isolated wild-type *Arabidopsis* protoplasts. The RFP fluorescence overlapped with the chlorophyll fluorescence (Figure 2A, top panel), indicating that the transit peptide of prCIA5 functioned as a chloroplast-targeting signal, and CIA5 was most likely a chloroplast protein. When similar experiments were performed with a construct encoding the transit peptide plus the first two transmembrane
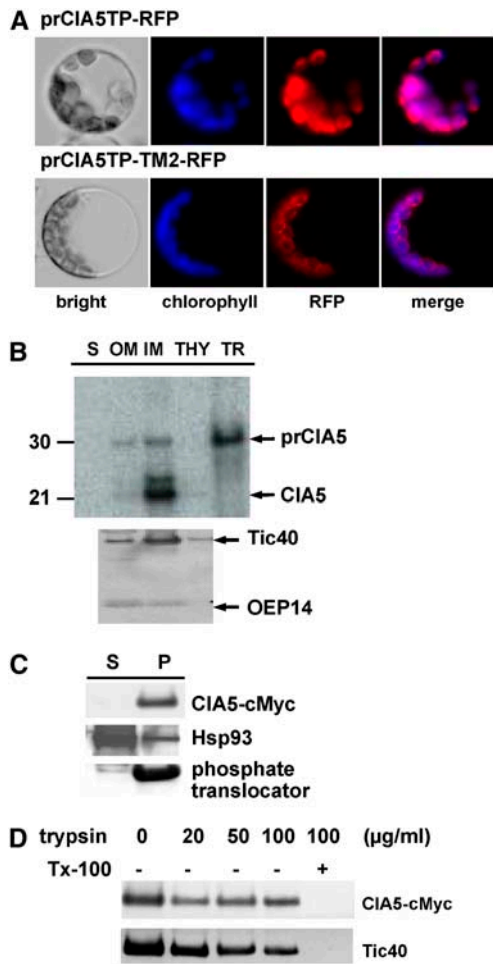
Figure 2. CIA5 Is an Integral Inner Envelope Membrane Protein of Chloroplasts.

(A) Subcellular localization of CIA5. prCIA5TP-RFP or prCIA5TP-TM2-RFP construct was transformed into wild-type protoplasts. bright, bright-field images; chlorophyll, chlorophyll autofluorescence (blue); RFP, RFP fluorescence (red); merge, overlap of the chlorophyll and RFP images.

(B) CIA5 was localized to the inner envelope membrane. Chloroplasts after import of [35S]prCIA5 were fractionated into the stroma (S), outer membrane (OM), inner membrane (IM), and thylakoid membrane (THY). Samples were analyzed by SDS-PAGE. An equal amount of proteins was loaded in each lane. A fluorograph of the gel is presented at the top. The same samples were analyzed by immunoblotting (bottom) for distribution Tic40 (Chou et al., 2003) and OEP14 (Li and Chen, 1996) in the membrane fractions as markers for inner and outer membranes, respectively. TR, in vitro–translated [35S]prCIA5.

(C) CIA5 was an integral membrane protein. Membranes from hypotonically lysed chloroplasts isolated from *35Spro:prCIA5-cMyc*–transformed plants were subjected to alkaline extraction and separated into soluble (S) and pellet (P) fractions. Samples were analyzed by immunoblotting with antibodies against proteins labeled at the right. Hsp93 is a stromal protein peripherally associated with the inner membrane (Nielsen et al., 1997).

(D) CIA5-cMyc was located in the inner membrane in vivo. Chloroplasts from *35Spro:prCIA5-cMyc*–transformed plants were treated with an increasing concentration of trypsin. Intact chloroplasts were reisolated and analyzed by immunoblotting with an antibodies against cMyc and Tic40. Tx-100, Triton X-100.

domains of prCIA5 fused to RFP (prCIA5TP-TM2-RFP), the RFP fluorescence was localized to the surface of chloroplasts (Figure 2A, bottom panel), suggesting that the addition of CIA5 trans-membrane domains had directed RFP to the envelope of chloroplasts. In agreement with these results, CIA5 has been identified in a proteomic study of *Arabidopsis* envelope membranes (Froehlich et al., 2003).

To further analyze to which membrane of the envelope CIA5 was localized, in vitro–translated [35S]prCIA5 was imported into isolated pea chloroplasts. Chloroplasts after import were fractionated into the stroma, inner and outer envelope membranes, and thylakoid membrane. As shown in Figure 2B, prCIA5 was ~31 kD, agreeing with its predicted molecular mass. After import, prCIA5 was processed to a mature protein slightly larger than 21 kD, and the mature protein was localized to the inner envelope membrane. A band migrating slightly slower than mature CIA5 was sometimes observed (Figure 2B, lane IM). The origin of this band was not clear. To investigate whether CIA5 was an integral membrane protein, chloroplasts were isolated from *35Spro:prCIA5-cMyc*–transformed plants. Membranes from hypotonically lysed chloroplasts were subjected to alkaline extraction. As shown in Figure 2C, CIA5-cMyc was resistant to alkaline extraction (Figure 2C), indicating CIA5 was an integral membrane protein. To further confirm the location of CIA5 in vivo, intact chloroplasts isolated from *35Spro:prCIA5-cMyc*–transformed plants were treated with increasing concentrations of trypsin. It has been shown that trypsin can penetrate the outer but not the inner envelope membrane of chloroplasts and can therefore only digest outer membrane proteins and protein exposed to the intermembrane space (Jackson et al., 1998). As shown in Figure 2D, CIA5-cMyc was resistant to trypsin digestion, confirming its inner envelope membrane location. The reduced signal intensity in samples treated with trypsin was most likely due to decreased protein loading as evidenced by a similar decrease in the amount of Tic40 detected.

Mutants of *cia5* Were Defective in Chloroplast Protein Import

To investigate if the *cia5* mutants were truly defective in chloroplast protein import, total leaf protein extracts were analyzed by immunoblots with antibodies against the 33-kD protein of oxygen evolving complex (OE33) and protochlorophyllide oxidoreductase (POR) of chloroplasts. Previous results indicated that, most likely due to the stability of their precursors (prOE33 and prPOR) and/or the sensitivity of the antibodies, accumulation of prOE33 and prPOR can often be detected in mutants defective in chloroplast protein import (Jarvis et al., 1998; Chou et al., 2003). As shown in Figure 3A, when an equal amount of total cellular proteins were analyzed, proteins in other organelles (e.g., the mitochondrial porin) appeared to be increased in the *cia5* mutant most likely due to a reduced amount of chloroplast proteins. Indeed the amount of mature OE33 and POR was greatly reduced in the mutant. By contrast, accumulation of prOE33 and prPOR was increased in the mutant, further suggesting a defect in chloroplast protein import.

To analyze the import defect in better detail, a gene encoding the transit peptide of the precursor to the small subunit of RuBP

carboxylase (prRBCS) fused with green fluorescent protein (GFP) (Lee et al., 2002) was transiently expressed in protoplasts isolated from the wild type, the At *toc159* knockout (*ppi2*) mutant, and the *cia5* mutant. In the wild type, the GFP fluorescence colocalized with the chlorophyll fluorescence (Figure 3B), indicating the import of GFP into chloroplasts. In the At *toc159* mutant protoplasts, while some GFP fluorescence was observed in chloroplasts, most was present in the cytosol. This result indicated a defect in targeting to chloroplasts and agreed with the function of At Toc159 as the initial receptor for chloroplast protein import. In contrast with the At *toc159* mutant, in *cia5* protoplasts, most GFP signal was observed around plastids, suggesting that *cia5* might be defective in a different step of the import process despite its phenotypic similarity to the At *toc159* mutant. Interestingly the GFP signals did not seem to exactly colocalize with the chlorophyll fluorescence but rather seem to concentrate on the periphery of chloroplasts, suggesting that a defect might exist for translocation into the stroma. Some
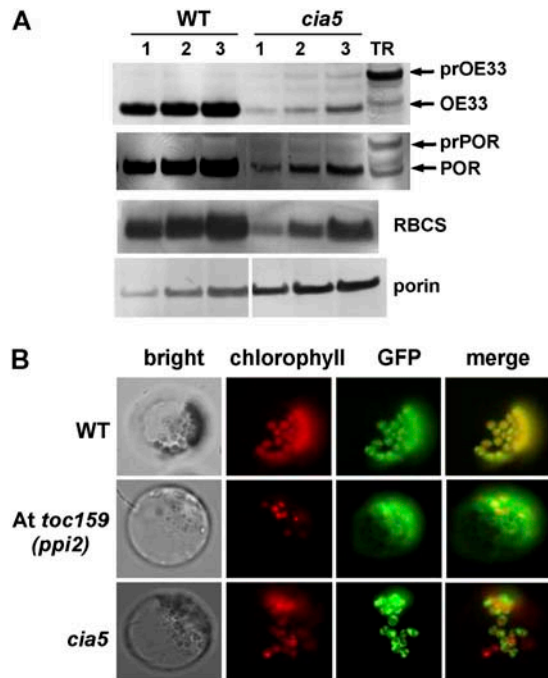
Figure 3. *cia5* Is Defective in Chloroplast Protein Import.

(A) *cia5* mutant had a reduced amount of chloroplast mature proteins and an increased amount of some precursor proteins. Total proteins extracted from 16-d-old wild-type and *cia5-2* mutant seedlings were analyzed by immunoblots with antibodies against OE33, POR, RBCS, and mitochondrial porin. For the analyses of OE33, 5, 10, and 20 μg of proteins were loaded in lanes 1, 2, and 3, respectively. Twice of those amounts was loaded for the analysis of POR, RBCS, and porin. TR, in vitro–translated prOE33 and prPORB.

(B) *cia5* had a different import defect from that of At *toc159*. The construct directing the expression of prRBCS transit peptide fused to GFP was transformed into wild-type, At *toc159*, and *cia5-1* protoplasts. bright, bright-field images; chlorophyll, chlorophyll autofluorescence (red); GFP, GFP fluorescence (green); merge, overlap of the chlorophyll and GFP images.
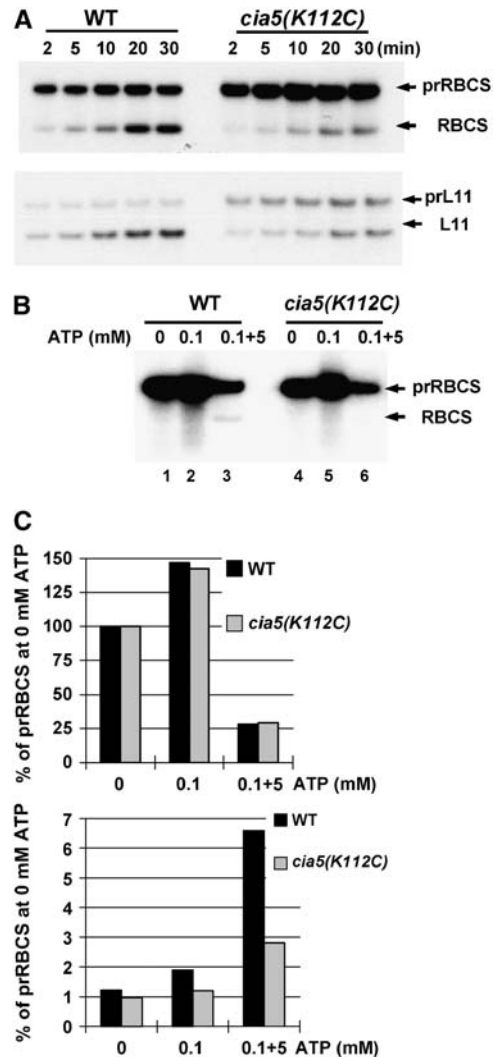

Figure 4. *cia5(K112C)* Mutant Chloroplasts Are Defective in Protein Translocation across the Inner Envelope Membrane.

(A) The *cia5(K112C)* mutant chloroplasts were defective in protein import. Chloroplasts isolated from the wild type and *cia5(K112C)* mutants were incubated with [³⁵S]prRBCS and [³⁵S]prL11. Intact chloroplasts were reisolated at each time point and analyzed by SDS-PAGE. The positions of precursor (prRBCS and prL11) and mature forms of each protein are indicated at right.

(B) The *cia5(K112C)* mutant chloroplasts were defective in translocation across the inner membrane. Chloroplasts isolated from the wild type and the *cia5(K112C)* mutant were kept on ice in the dark to deplete internal ATP. The energy-depleted chloroplasts were incubated with energy-depleted [³⁵S]prRBCS in the absence (0 mM, lanes 1 and 4) or presence (0.1 mM, lanes 2 and 5) of 0.1 mM ATP for 5 min in the dark at room temperature. The chloroplasts were pelleted by centrifugation. Half of the chloroplasts that had been incubated with 0.1 mM ATP were further incubated in import buffer containing 5 mM ATP for 15 min (0.1 + 5 mM ATP, lanes 3 and 6). Intact chloroplasts were reisolated and analyzed by SDS-PAGE. The positions of precursor (prRBCS) and mature RBCS are indicated at right.

(C) Quantification of the data shown in **(B)**. The amount of prRBCS present at 0 mM ATP was taken as 100%.
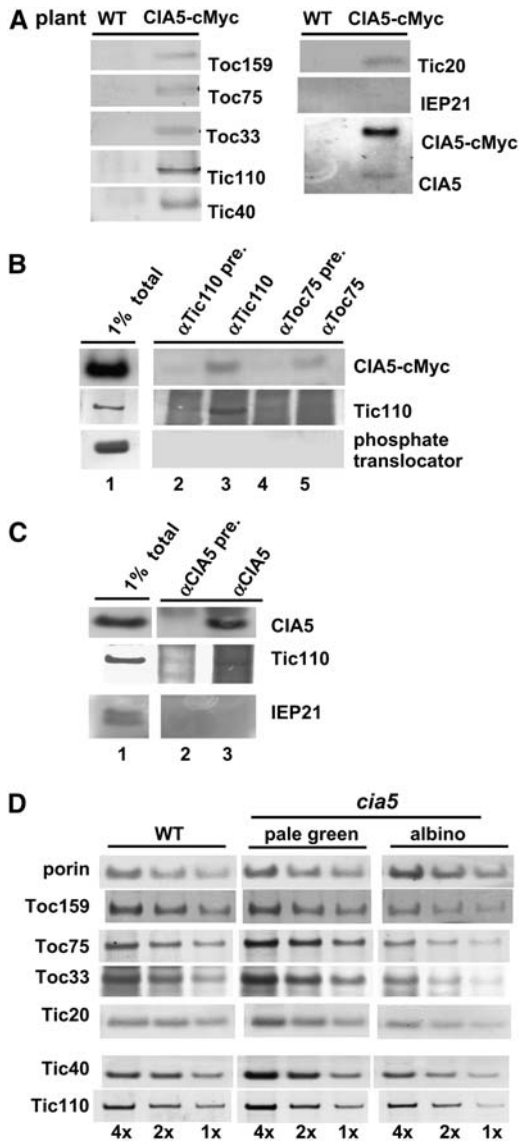
Figure 5. CIA5 Is a Component of the Translocon Complex.

(A) CIA5-cMyc was associated with known components of the translocon complex without the addition of cross-linkers. Total membranes of chloroplasts isolated from wild-type plants or from wild-type plants transformed with *35Spro:prCIA5-cMyc* were solubilized with 1% DM and immunoprecipitated with anti-cMyc-antibody–conjugated agarose beads. Precipitated proteins were eluted from the beads with glycine and analyzed by immunoblotting with antibodies against various proteins as labeled at right. CIA5-cMyc and endogenous CIA5 were detected using the anti-CIA5 antibody.

(B) CIA5-cMyc was associated with Tic110 and Toc75. Total membranes of chloroplasts isolated from wild-type plants transformed with *35Spro:prCIA5-cMyc* were solubilized with 1% DM and immunoprecipitated with antibodies against Tic110 (lane 3), Toc75 (lane 5), and corresponding preimmune sera (lanes 2 and 4). Lane 1, 1% of total solubilized membranes used for the immunoprecipitation. The amount of Toc75 precipitated was not analyzed due to close migration distance of Toc75 to the IgG heavy chain.

(C) Endogenous CIA5 was associated with Tic110. Inner membrane

plastids in *cia5* did not contain detectable GFP signals, suggesting a total loss of import ability. However, since the *cia5* mutant still contained some mature chloroplast proteins, including RBCS (Figure 3A), a residual level of protein import most likely still existed when *cia5* was grown on medium supplemented with sucrose.

The albino phenotype of *cia5* made it difficult to isolate plastids to study the import defect in details. We therefore tried to generate sublethal alleles of *cia5* by transforming *cia5-2* with prCIA5 that had mutations in various positions (see Methods). One of the transformants, *cia5(K112C)*, in which the Lys of residue 112 was mutated to Cys, showed a mosaic pale green phenotype (Figure 1B), and the transformant was viable and fertile on soil (data not shown). Chloroplasts were isolated from *cia5(K112C)* and wild-type seedlings and incubated with in vitro–translated [^{35}S]-labeled precursors of L11 (a component of the chloroplast 50S ribosomal subunit) and RBCS in an import time-course experiment. For both precursors, *cia5(K112C)* chloroplasts had a reduced amount of imported mature protein and an increased amount of precursors bound, although the import of prRBCS seemed to be more severely affected than prL1l in *cia5(K112C)* (Figure 4A).

We further pinpointed the stage of import in which *cia5(K112C)* chloroplasts were defective by performing a binding-chase experiment. Precursors were first incubated with isolated chloroplasts under 100 μM ATP to allow binding of precursors to the chloroplast envelope (Figure 4B, lanes 2 and 5). Chloroplasts were then reisolated and further chased with 5 mM ATP to allow bound precursors to be translocated across the inner membrane into the stroma (lanes 3 and 6). Compared with the wild type, *cia5(K112C)* chloroplasts were normal in allowing precursor binding under 100 μM ATP. After chasing with 5 mM ATP, in wild-type chloroplasts, some precursors were translocated into the stroma and processed to the mature form. In *cia5(K112C)* chloroplasts, almost no mature protein was produced (Figures 4B and 4C). This result indicated that when chloroplasts contained defective CIA5, these chloroplasts were specifically defective in protein translocation across the inner envelope membrane.

vesicles were isolated from pea chloroplasts and solubilized with 1% DM and immunoprecipitated with the antibody against CIA5 or its preimmune serum (pre.). Lane 1, 1% of total solubilized membranes used for the immunoprecipitation. All three lanes for each antibody hybridization in immunoblotting were run on the same gel and contrast adjusted the same way.

(D) *cia5* had a reduced amount of translocon components. Leaves from 21-d-old *cia5-2* mutant plants were separated into pale-green and albino parts. Total protein extracts were prepared from both parts and also from wild-types leaves of the same age. A twofold dilution series of samples was analyzed by immunoblotting with antibodies against proteins as labeled at left. Mitochondrial porin was used as a loading control. Due to the different efficiency of each antibody, the amount of proteins used in the 1x lane for the analyses of Toc75 and Tic20 was twofold that used for Toc33, eightfold that used for porin and Toc159, and 32-fold that used for Tic110 and Tic40. All blots for the same antibody were run on the same gel and images processed the same way.

CIA5 Is Part of the Translocon Complex

Because *cia5* mutant chloroplasts were defective in protein import, we next investigated if CIA5 was associated with other known translocon components. Chloroplasts were isolated from wild-type plants transformed with *35Spro:prCIA5-cMyc.* Total membranes of isolated chloroplasts were solubilized with 1% *n*-decyl-β-D-maltopyranoside (DM) and immunoprecipitated with an anti-cMyc antibody. As shown in Figure 5A, even without adding any cross-linker to stabilize complex–component interactions, CIA5-cMyc coimmunoprecipitated all the major Toc and Tic translocon components but not the inner membrane protein IEP21, which is not associated with the translocon (Kouranov et al., 1998). The absence of IEP21 indicated that although CIA5-cMyc was being overexpressed by the 35S promoter, it did not nonspecifically associate with other proteins. Interestingly, CIA5-cMyc also coimmunoprecipitated endogenous CIA5. This result suggested that at least part of CIA5-cMyc was assembled with endogenous CIA5, and CIA5 was most likely present in a multimeric form in vivo.

We further confirmed the presence of CIA5 in the translocon by reciprocal immunoprecipitation using antibodies against Toc75 and Tic110. Even in the absence of any cross-linker, both antibodies specifically immunoprecipitated CIA5-cMyc (Figure 5B). Although only a small percentage of CIA5-cMyc was precipitated, under the same conditions, the amount of Tic110 specifically precipitated by the anti-Toc75 antibody was almost not detectable (Tic110 in lane 5). Phosphate translocator, the most abundant protein in the envelope, was not precipitated by either antibody, confirming the specificity of the coimmunoprecipitation.

To further exclude the possibility that association of CIA5-cMyc with other translocon components was due to overexpression of CIA5-cMyc by the 35S promoter, we immunoprecipitated endogenous CIA5 from pea chloroplasts. Due to the low efficiency of our antibody, we first isolated inner membrane vesicles to enrich the amount of CIA5. As shown in Figure 5C, anti-CIA5 antibody specifically immunoprecipitated Tic110, while the nontranslocon protein IEP21 was not precipitated (Figure 5C, lane 3).

If CIA5 is part of the translocon complex, its absence may cause a reduction in other translocon components. Seedlings of the *cia5-2* mutant were dissected and separated into the young/pale-green part at the center of plants and the albino mature leaves. Total protein extracts were prepared from both parts and compared with the wild type by immunoblots. As shown in Figure 5D, the young/pale-green part of *cia5-2* had normal levels of translocon components. The albino mature leaves had a reduced amount of almost all translocon components tested.

CIA5 and Tic20 Have Complementary Expression Patterns and Mutant Phenotypes

The similarity of CIA5 to Tic20 in size and possible membrane topology raised the question of the functional specificity of each protein. To address this question, we first analyzed if any group of plastid proteins was preferentially depleted in *cia5* or *tic20* mutants. An At *Tic20-I* T-DNA insertion mutant (Salk_039676, Figure

6A) was obtained. The mutant produced no detectable amount of At Tic20-I, indicating that it was most likely a knockout mutant (Figure 6B). The At *tic20-I* knockout mutant was albino even in the youngest part of the seedling and often showed anthocyanin accumulation (Figure 6C). On synthetic medium supplemented
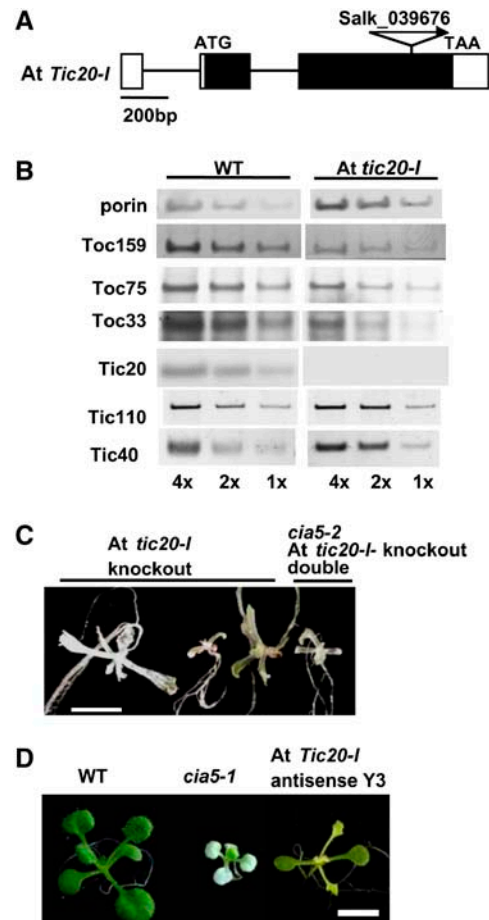
Figure 6. Relations between CIA5 and At Tic20.

(A) Gene structure of At *Tic20-I* and T-DNA insertion position of the At *tic20-I* knockout mutant. Open boxes, closed boxes, and lines between boxes indicate UTRs, exons, and introns, respectively.

(B) At *tic20-I* mutant had a reduced amount of some translocon components. Total proteins were extracted from leaves of the 21-d-old wild type and At *tic20-I* knockout mutant. A twofold dilution series of samples was analyzed by immunoblotting with antibodies against proteins as labeled at left. Mitochondrial porin was used as a loading control. Due to the different efficiency of each antibody, the amount of proteins used in the 1x lane for the analyses of Toc75 and Tic20 was twofold that used for Toc33, eightfold that used for porin and Toc159, and 32-fold that used for Tic110 and Tic40. All blots for the same antibody were run on the same gel and images processed the same way.

(C) Phenotypes of the At *tic20-I* knockout single mutant and *cia5-2* At *tic20-I* knockout double mutant. All plants were grown on plates for 18 d. The three plants on the left are At *tic20-I* knockout single mutants, and the plant on the right is a *cia5-2* At *tic20-I* knockout double mutant. Bar = 0.5 cm.

(D) Phenotype comparison of At *Tic20* antisense mutant Y3 and *cia5-2* mutant. All plants were grown on the plates for 14 d. Bar = 0.5 cm.

with sucrose, it could make a few irregular-shaped leaves and then the growth was arrested. Some seedlings stopped growing earlier than others, resulting in a range of different seedling size and morphology (Figure 6C). Protein composition analyses by immunoblots did not identify any clear difference between At *tic20-I* knockout and *cia5-2* (data not shown). Nonetheless, a clear interpretation of the results was hindered by the fact that the At *tic20-I* mutant had a reduced level of some major translocon components (Figure 6B); therefore, the possibility of secondary effects could not be excluded. The presence of At Tic20-IV may also provide a basal level of Tic20 function and prevent a complete loss of the proteins they translocate.

We then compared the expression patterns of *CIA5*, At *Tic20-I* (*At1g04940*), and At *Tic20-IV* (*At4g03320*) with those of At *Tic110* (*At1g06950*) and At *Toc75-III* (*At3g46740*), the gene encoding the functional Toc75 in *Arabidopsis* chloroplasts, using the Gene Chronologer tool in Genevestigator (Zimmermann et al., 2004). Gene Chronologer provides gene expression values throughout different stages of the *Arabidopsis* life cycle complied using available Affymetrix GeneChip data. However, due to mistakes in The Arabidopsis Information Resource (TAIR) annotation (see Methods for details), probe sets in the ATH1 Affymetrix GeneChip representing At *Tic20-I* actually correspond to a different gene. Therefore, no direct comparison of At *Tic20-I* to other genes can be made using Gene Chronologer, and the conclusion that the expression level of At *Tic20-I* is very low based on expression analyses using the ATH1 GeneChip (Vojta et al., 2004) should be reevaluated. However, from the Massively Parallel Signature Sequencing (MPSS) expression database (http://mpss.udel.edu/at/; Brenner et al., 2000), one oligonucleotide specifically hybridized to the 3′ UTR of At *Tic20-I*. The average abundance (transcript per million averaged from all libraries) of the At *Tic20-I* transcript, as represented by this oligonucleotide, is indeed much lower than that of Toc75 or Tic110 (Table 1), suggesting that there may indeed be a gap between the expression level of At *Tic20-I* and those of At *Tic110* and At *Toc75*.

From the Gene Chronologer analyses, the expression level of *CIA5* is comparable to those of At *Toc75-III* and At *Tic110* (Table 1) across almost all stages of development. However, the expression level of *CIA5* is lower at the earliest stage during germination. Interestingly, At *Tic20-IV* was expressed at one of its highest levels during germination. RT-PCR analyses using gene-specific primers have also shown that At *Tic20-1* is expressed at its highest level during germination (Chen et al., 2002). Therefore, the two At *Tic20* genes and *CIA5* seemed to have complementary expression patterns. The mutant phenotypes correlated with the complementary expression patterns. All the viable At Tic20 antisense plants had more severe phenotypes in cotyledons during germination or in the youngest part of the plants (Chen et al., 2002). For example, the best-characterized line Y3 had pale cotyledons and new leaves that gradually turned green as the cotyledons and leaves get older (Chen et al., 2002; Figure 6D). This is in contrast with *cia5*, whose leaves were still slightly green when first emerged but gradually became albino as the leaves mature. These results suggested that Tic20 might be more important early, and CIA5 more important later, in development.

We then generated a double mutant of *cia5-2* At *tic20-I* knockout. The double mutant had identical phenotypes to the At *tic20-I* knockout single mutant (Figure 6C). This result suggested that At Tic20-I and CIA5 function in the same aspect of chloroplast biogenesis, and At Tic20-I functions earlier in development than CIA5. It is possible that in the At *tic20-I* knockout mutant, the lack of At Tic20-I had caused irreversible damages to plastids and arrested plastid development. Adding or deleting the function of CIA5 later in development had no additional effect.

DISCUSSION

Several lines of evidence indicated that CIA5 is an essential translocon component for protein import into chloroplasts. Null *cia5* mutants were lethal and accumulated unprocessed chloroplast precursor proteins. Chloroplasts from *cia5* mutants were specifically defective in protein translocation across the inner membrane. CIA5 was specifically copurified with other major translocon components, and *cia5* mutant chloroplasts had reduced levels of other translocon components. Furthermore, the expression level of *CIA5* is comparable to other major translocon components. Because CIA5 is located in the inner membrane, it

Table 1. Transcript Abundance Data in Gene Chronologer and MPSS

Genevestigator Gene Chronologer

		102	512	396	114	97	246	403	80	56	
No. of Arrays											
Age (d)		1	6	14	18	21	25	29	36	45	MPSS TPM[a]
At *Toc75-III* (*At3g46740*)	Mean	5534	4475	3473	3972	5309	3205	3247	2916	3403	91
	SE	266	84	73	155	130	73	88	127	257	
At *Tic110* (*At1g06950*)	Mean	5102	3549	2713	2669	4309	2232	2625	2283	3086	130
	SE	212	73	58	129	103	66	77	82	239	
CIA5 (*At2g15290*)	Mean	2861	4340	3415	4857	4320	3903	4100	3140	2612	125
	SE	112	93	90	175	134	76	76	120	138	
At *Tic20-IV* (*At4g03320*)	Mean	1938	772	956	493	129	579	649	718	3560	0
	SE	220	32	59	69	12	58	63	90	319	
At *Tic20-I*											38

[a] TPM, average normalized abundance in all libraries (transcript per million).

should be called a Tic protein. On SDS-PAGE, pea CIA5 migrated in between pea Tic20 and Tic22, very close to Tic22 (data not shown). We have therefore renamed CIA5 as *Arabidopsis* Tic21 (At Tic21).

CIA5/At Tic21 is deeply embedded in the inner membrane, and its cyanobacterial homologues have some similarities to proteins that are annotated as amino acid transporters and sugar permeases. These data suggest that CIA5/At Tic21 may function as a component of the protein-conducting channel across the inner membrane. There are at least five proteins that are ~20 kD with four potential transmembrane domains and have been shown to be components of protein-conducting channels: chloroplast Tic20, mitochondrial inner membrane Tim17, Tim22, and Tim23, and endoplasmic reticulum Derlin-1, a channel component for retrotranslocation of misfolded proteins from the endoplasmic reticulum to the cytosol (Ye et al., 2004). Proteins in this group are smaller than most characterized channel/transporter proteins (Dahl et al., 2004), and it is not clear how they are related to other channel proteins. Our preliminary data indicated that *Synechocystis* CIA5 homologue sll1656 could not complement *cia5* even when overexpressed in the inner envelope membrane of *Arabidopsis* chloroplasts (L.-J. Chen and H.-m. Li, unpublished results). This result indicates that the function of CIA5/At Tic21 has diverged from its cyanobacterial homologues. It would be interesting to analyze the function of the cyanobacterial homologues and therefore have a better understanding of the evolutionary origin of chloroplast protein import machinery.

The function of At Tic20 as the inner membrane channel was doubted mainly due to its low expression level compared with other translocon components (Vojta et al., 2004). The expression was analyzed using leaves from 4-week-old plants (Vojta et al., 2004). However, very early in development during seed germination the expression level of both At *Tic20* genes are at their highest level. By contrast, the expression level of CIA5/At *Tic21* is comparable to those of At *Tic110* and At *Toc75-III* in most stages of vegetative tissue development but is slightly lower during germination. Phenotypes of the *cia5-2* At *tic20-I* knockout double mutant also suggested that At Tic20 and CIA5/At Tic21 function at the same aspect of chloroplast biogenesis, with At Tic20 functioning earlier in development. In the At Tic20 antisense plants, it is possible that the presence of remaining At Tic20 keeps plastid development from being aborted. Through the functioning of CIA5/At Tic21, possibly together with other translocon components, chloroplast development could gradually recover later in development. In the *cia5* mutants, At Tic20 may support early plastid development and result in the green color in very young tissues. As leaves mature and the expression of At Tic20 drops, if no functional CIA5/At Tic21 is provided to sustain chloroplast development, the leaves turn albino.

If both At Tic20 and CIA5/At Tic21 function as components of the Tic channels, it is not clear whether they function as independent channels. They may each associate with other known or even yet unknown Tic proteins to form independent channels. However, even though they seem to have different expression patterns, it is still possible that they interact structurally or functionally. It is also not clear whether they have any specificity in the proteins they translocate. Although we have not found preferential depletion of certain proteins when comparing the *cia5-2*

and At *tic20-I* knockout mutants, it is still possible that At Tic20-I has preference for certain proteins important for early plastid biogenesis and that CIA5/At Tic21 has preference for proteins important for chloroplast expansion and maintenance. Mutation in either gene may arrest plastid development and cause a reduction in other translocon components and lead to a similar reduction in all major chloroplast proteins.

METHODS

Molecular Cloning of *CIA5*, Growth of Plants, Chloroplast Isolation, and Import into Chloroplasts

The *cia5-1* mutant was crossed to the Landsberg *erecta* ecotype, and DNA was isolated from 871 F2 mutant seedlings for mapping of the *CIA5* locus. Growth of pea (*Pisum sativum* cv Little Marvel), isolation of chloroplasts from 9- to 11-d-old pea seedlings, and in vitro transcription and translation of precursor proteins were performed as described (Perry et al., 1991). All molecular characterizations of the *cia5* mutants were performed with lines that had been backcrossed at least twice. Growth of *Arabidopsis thaliana* on Murashige and Skoog synthetic medium, isolation of *Arabidopsis* chloroplasts, and protein import into *Arabidopsis* chloroplasts were performed as described (Sun et al., 2001), except plants were grown for 21 d. The binding and chase experiment and trypsin treatment of intact chloroplasts were performed as described (Chou et al., 2003).

Fractionation of chloroplasts (Li and Chen, 1996) and alkaline extraction of chloroplasts after import (Tranel et al., 1995) were performed using published procedures. After alkaline extraction, the extracted pellet and the soluble fractions were separated by centrifugation at 125,000*g* for 45 min using a Beckman TLA 45 rotor. The pellet was directly dissolved in SDS-PAGE sample buffer. The supernatant was first precipitated by trichloroacetic acid before dissolving the precipitate with sample buffer.

Plasmid Construction

At2g15290 full-length cDNA was amplified by PCR from first-strand cDNA of *Arabidopsis* leaf mRNA (Sun et al., 2001) with a forward primer adding a *Bam*HI site and a reverse primer adding an *Eco*RI site and mutating the stop codon to Glu to the PCR fragment. The amplified fragment was subcloned into the *Bam*HI-*Eco*RI site of the plasmid pBluescript-cMyc (Hung et al., 2004) with five tandem copies of cMyc. The resulting plasmid was named pBluescript-prCIA5-cMyc. The prCIA5-cMyc fragment was then amplified from pBluescript-prCIA5-cMyc with a forward primer adding a *Bam*HI site and a reverse primer adding a *Bgl*II site to the PCR fragment. The fragment was cloned into the *Bam*HI-*Bgl*II site of the binary vector pCAMBIA1390 containing a 35S promoter. The resulting plasmid was named pCAMBIA1390-*35Spro:prCIA5-cMyc*. For in vitro transcription, *At2g15290* cDNA was amplified with a forward primer adding an *Xho*I site and a reverse primer adding an *Eco*RI site to the PCR fragment. The fragment was cloned into the *Xho*I-*Eco*RI site of pSP72 (Promega). The resulting plasmid was named pSP72-prCIA5. For transient expression in protoplasts, a fragment containing the coding region of prCIA5 amino acids 1 to 104 (transit peptide plus the first 14 amino acids of the mature protein) or amino acids 1 to 191 (transit peptide plus the first two transmembrane domains of the mature protein) was amplified from pSP72-prCIA5 using the SP6 primer and a reverse primer adding a *Bam*HI site. The PCR fragment was cut with *Xho*I and *Bam*HI and subcloned into the *Xho*I-*Bam*HI site of p326RFP (Clontech). Plasmid containing cDNA for prL11 (SSP 09645) was obtained from the Kasuza DNA Research Institute (Asamizu et al., 2000). The K112C site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) on the plasmid pBluescript-prCIA5-cMyc. The

fragment encoding prCIA5(K112C) was then excised with *Bam*HI and *Xho*I and subcloned into the *Bam*HI-*Sal*I site of the binary vector pCHF1. The construct was transformed into heterozygous *cia5-2*. Transformants that were homozygous for both *cia5-2* T-DNA insertion and pCHF1-prCIA5(K112C)-cMyc transgene were selected by segregation of the Basta-resistant gene (*cia5-2* T-DNA insertion) and G418-resistant gene (pCHF1) and confirmed by PCR amplification of both markers.

Transient Expression of Fusion Proteins in *Arabidopsis* Protoplasts

Plasmids were introduced by polyethylene glycol–mediated transformation (Jin et al., 2001) into protoplasts prepared from leaf tissues of *Arabidopsis*. The expression of proteins was monitored at various time points after transformation. Images of GFP or RFP in intact protoplasts were obtained from protoplasts in incubation medium on a glass slide covered with a cover slip. Images were taken under a fluorescent microscope (Axioplan 2; Carl Zeiss) equipped with a ×40/0.75 objective (Plan-NEOFLUAR) and a cooled CCD camera (Senicam; PCO Imaging) at 20°C. The filter sets used were XF116 (exciter, 474AF20; dichroic. 500DRLP; emitter, 510AF23) and XF117 (exciter, 540AF30; dichroic, 570DRLP; emitter, 585ALP) (Omega Optical, Brattleboro, VT). Photoshop (version 7.0; Adobe Systems) was used to process the images.

Antibodies and Immunoprecipitation

The antibody against CIA5 was generated against a peptide corresponding to the N-terminal sequence of *Medicago truncatula* CIA5 (VANRLEK-TARHFERLGSLGFWGQLV; Figure 1E). The antibody recognized both pea and *Arabidopsis* CIA5. Attempts to generate anti-CIA5 antibody using peptide sequences corresponding to the N terminus of pea or *Arabidopsis* CIA5 were not successful. Monoclonal antibody against cMyc and agarose-conjugated anti-cMyc antibody were purchased from Santa Cruz Biotechnology. Antibodies against Tic and Toc components were prepared as described (Chou et al., 2003; Tu et al., 2004). Antibodies against POR were generated against *Escherichia coli* overexpressed and purified full-length *Arabidopsis* PORB precursor (At4g27440). Antibody against Tic20 was generated against a peptide (ASKDVPSSFRFPPMTKKPQ) corresponding to the N terminus of mature At Tic20-I (Chen et al., 2002). The antibody recognized both pea and *Arabidopsis* Tic20. The antibody against the phosphate translocator was generated against a peptide (YRSIFSKKAMTDMDSTNVC) corresponding to residues 264 to 281 of *Arabidopsis* phosphate/triose-phosphate translocator (At5g46110). The anti-Tic20 and antiphosphate translocator antibodies were generated by BioSource. Coimmunoprecipitation with translocon components was performed as described (Chou et al., 2003), except no cross-linker was added, membranes were solubilized with 1% DM instead of 1% Triton X-100, and immunoprecipitates were eluted with 0.2 M glycine, pH 2.0. Pea chloroplast inner envelope membrane vesicles were isolated as described (Tu et al., 2004).

Miscellaneous Procedures

During the analyses of At Tic20-I expression, we found that, for At *Tic20-I*, in the Munich Information Center for Protein Sequences (MIPS) database the original gene number *At1g04940* has been split into two genes, with *At1g04940* assigned to At Tic20-I and *At1g04945* assigned to another gene encoding an unknown protein. In the TAIR-NCBI V6 November, 2005 annotation, the transcript for At Tic20-I has been mistakenly deleted, and the gene number *At1g04940* is assigned to the *At1g04945* unknown protein gene in MIPS. Unfortunately, all 11 probes in the ATH1 Affymetrix GeneChip representing the original *At1g04940* are located in the gene *At1g04945* and cannot be used to represent the expression of At *Tic20-I*.

The *cia5-2* At *tic20-I* double mutant was generated by crossing heterozygous plants of the two single mutants. F1 seedlings heterozygous for T-DNA insertion in both genes were selected by PCR analyses for the T-DNA insertions. All F2 seedlings were genotyped by PCR for the T-DNA insertions. RT-PCR analyses were performed as described (Sun et al., 2001) except purified poly(A) mRNA instead of total RNA was used as templates for first-strand cDNA synthesis. Total plant protein extracts for immunoblotting analyses were prepared by homogenizing plate-grown seedlings in extraction buffer (300 mM Tris-HCl, pH 8.5, 8% SDS, 1 mM EDTA, 1 mM PMSF, 1 mM pepstatin, and 1 mM leupeptin). Samples were analyzed by SDS-PAGE, blotted onto polyvinylidene difluoride membranes, and detected by alkaline phosphatase–conjugated secondary antibodies. All electrophoresis was performed as described (Tu and Li, 2000). Insertion mutant information was obtained from the SIGnAL website at http://signal.salk.edu and further confirmed by DNA sequencing. The *ppi2* mutant (CS11072) was obtained from the ABRC at Ohio State University.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY057510 (*Arabidopsis* CIA5), BQ297283 (1 to 201 amino acid residues of soybean [*Glycine max*] CIA5), AW706279 (113 to 284 amino acid residues of soybean CIA5), BF004538 (*Medicago* CIA5), DQ535894 (pea CIA5), BT017168 (maize [*Zea mays*] CIA5), AK069783 (rice [*Oryza sativa*] CIA5-1), AK073270 (rice CIA5-2), ZP_00105923 (*Nostoc* CIA5), and BAA16830 (*Synechocystis* CIA5).

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