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

Yi-Shan Teng, Yi-shin Su, Lih-Jen Chen, Yong Jik Lee, Inhwan Hwang, and Hsou-min Li

**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 Tic proteins, Tic159, Tic75, and Tic34, form the core Tic complex. Tic159 and Tic34 are homologous GTPases and function as the initial receptors for incoming precursors. Tic75 is the protein-conducting channel across the outer membrane. Tic75 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 (Sirenberg et al., 1996). *Arabidopsis thaliana* has two genes encoding Tic20: AtTic20-I and AtTic20-IV. The AtTic20-I gene, compared with AtTic20-IV, encodes a protein that shares a higher degree of identity to pea Tic20. In vivo reduction of AtTic20 by expressing an antisense construct of AtTic20-I resulted in a 10-fold lower abundance compared with Tic75, arguing against a function for Tic20 as the general protein-conducting channel across the inner membrane (Vojta et al., 2004; Becker et al., 2005).

**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, AtTic20, 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 AtTic110 and AtToc75, except during germination, at which stage AtTic20 was expressed at its highest level. A double mutant of *cia5* AtTic20-IV had the same phenotype as the AtTic20-I single mutant, suggesting that CIA5 and AtTic20 function similarly in chloroplast biogenesis, with AtTic20 functioning earlier in development. We renamed CIA5 as *Arabidopsis* Tic21 (AtTic21) 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.

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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 tic20-1 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 it 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 proplastids. 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
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(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 and phosphate translocator. 

(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.

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 (pOE33 and pPOR) and/or the sensitivity of the antibodies, accumulation of pOE33 and pPOR 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 pOE33 and pPOR 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.

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%.
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-translation-[35S]-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 resolated 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.

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 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-type plants 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 Tic75 and Tic20 was twofold that used for Tic33, eightfold that used for porin and Tic159, 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 with 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.

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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 Tic33, eightfold that used for porin and Tic159, 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 tic20-1 knockout and cia5-2 (data not shown). Nonetheless, a clear interpretation of the results was hindered by the fact that the At tic20-1 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 compiled 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 quite 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 tic20-1 knockout. The double mutant had identical phenotypes to the At tic20-1 knockout single mutant (Figure 6C). This result suggested that At Tic20-I and CIA5 function in the same aspect of chloroplast biogenesis, and At Tic20-1 functions earlier in development than CIA5. It is possible that in the At tic20-1 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 |
| No. of Arrays | Age (d) | 1 | 6 | 14 | 18 | 21 | 25 | 29 | 36 | 45 | MPSS TPMa |
| 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 | |

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 translocon proteins. Our preliminary data indicated that *Synechocystis* CIA5 homologue sll1656 could not complement cia5 even when overexpressed in the inner envelope membrane of *Arabidopsis* plastids (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 Tic11 and At Tic75-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 function 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-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., 1996) were performed using published procedures. After alkaline extraction, the extracted pellet and the soluble fractions were separated by centrifugation at 125,000g 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 BamHI site and a reverse primer adding an EcoRI site and mutating the stop codon to Gibus to the PCR fragment. The amplified fragment was subcloned into the BamHI-EcoRI 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 BamHI site and a reverse primer adding a BglII site to the PCR fragment. The fragment was cloned into the BamHI-BglII site of the binary vector pCAMBIA1390 containing a 35S promoter. The resulting plasmid was named pCAMBIA1390-35S::prCIA5-cMyc. For in vitro transcription, At2g15290 cDNA was amplified with a forward primer adding an Xhol site and a reverse primer adding an EcoRI site to the PCR fragment. The fragment was cloned into the Xhol-EcoRI 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 BamHI site. The PCR fragment was cut with Xhol and BamHI and subcloned into the Xhol-BamHI 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 pCIA5(K112C) was then excised with BamHI and Xhol and subcloned into the BamHI-SalI 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-pCIA5(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 (VANLKE-TARHFERLGLSLGFWGQLV; 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 agaroase-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 (ASKDVPSRSFFPPMTKKQ) corresponding to the N terminus of mature At Tic20-1 (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/fructose-phosphate translocators (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 Tic20-1 expression, we found that, for At Tic20-1, 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 Tic20-1 and At1g04945 assigned to another gene encoding an unknown protein. In the TAIR-NCBI V6 November, 2005 annotation, the transcript for At Tic20-1 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 Tic20-1.

The cia5-2 At tic20-1 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, bloated 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 (GS11072) 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).

**ACKNOWLEDGMENTS**

We thank Chi-Chou Chiu for his assistance in gene structure analyses of At Tic20-1 and the Affymetrix Gene Expression Service Lab of Academia Sinica for providing the probe information of At1g04940 on the ATH1 chip. We thank Danny Schnell for the Y3 At Tic20 antisense line and the anti-IEP21 antibody, Ming-Yan Kuo for the preparation of the anti-POR antibody and the pea CIA5 cDNA clone, Hwa Dai for the monoclonal antibodies against maize mitochondrial porin, and Neil Hoffman for the antibody against OE33. We thank the Salk Institute Genomic Analysis Laboratory for providing the sequence-indexed Arabidopsis T-DNA insertion mutant cia5-3 and At tic20-1, Syngenta for cia5-2, the Kasuza DNA Research Institute for plasmid encoding pL1,1, and the ABRC for ppp2. Funding for the SIGnAL indexed insertion mutant collection was provided by the National Science Foundation. We thank Kentaro Inoue, Chi-Chou Chiu, Ching-Chih Chu, and Pai-Hsiang Su for critical reading of the manuscript. This work was supported by grants to H.-M.L. from the National Science Council (NSC 94-2321-B001-010) and the Academia Sinica of Taiwan and grants to I.H. from the Creative Research Initiatives Program of the Ministry of Science and Technology of Korea.

Received May 19, 2006; revised July 11, 2006; accepted July 13, 2006; published August 4, 2006.

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Tic21 Is an Essential Translocon Component for Protein Translocation across the Chloroplast Inner Envelope Membrane
Yi-Shan Teng, Yi-shin Su, Lih-Jen Chen, Yong Jik Lee, Inhwan Hwang and Hsou-min Li
Plant Cell 2006;18;2247-2257; originally published online August 4, 2006;
DOI 10.1105/tpc.106.044305

This information is current as of September 7, 2017

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