In Vivo Analysis of the Role of atTic20 in Protein Import into Chloroplasts

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The import of nucleus-encoded preproteins into plastids requires the coordinated activities of membrane protein complexes that facilitate the translocation of polypeptides across the envelope double membrane. Tic20 was identified previously as a component of the import machinery of the inner envelope membrane by covalent cross-linking studies with trapped preprotein import intermediates. To investigate the role of Tic20 in preprotein import, we altered the expression of the Arabidopsis Tic20 ortholog (atTic20) by antisense expression. Several antisense lines exhibited pronounced chloroplast defects exemplified by pale leaves, reduced accumulation of plastid proteins, and significant growth defects. The severity of the phenotypes correlated directly with the reduction in levels of atTic20 expression. In vitro import studies with plastids isolated from control and antisense plants indicated that the antisense plastids are defective specifically in protein translocation across the inner envelope membrane. These data suggest that Tic20 functions as a component of the protein-conducting channel at the inner envelope membrane.

INTRODUCTION

Nucleus-encoded preproteins are imported into chloroplasts via the cooperative action of multimeric complexes (translocons) in the outer and inner chloroplast envelope membranes, designated the Toc and Tic complexes, respectively (Schnell et al., 1997; Keegstra and Cline 1999; Bauer et al., 2001). Components of the Toc and Tic machinery associate physically to form a membrane supercomplex that facilitates the direct translocation of preproteins from the cytoplasm (Nielsen et al., 1997; Schnell et al., 1997; Kouranov et al., 1998) to the stroma. One function of the translocons is to maintain the critical permeability barrier of the organelle to ions and metabolites during the protein import reaction. Membrane translocation of preproteins is proposed to proceed through selectively permeable protein-conducting channels (van den Wijngaard and Vredenberg, 1997) formed by components of the Toc and Tic complexes.

Covalent cross-linking (Ma et al., 1996) and electrophysiological (Hinnah et al., 1997) studies suggest that Toc75, an integral outer membrane component, participates in protein conductance at the outer membrane. Toc75 is predicted to form a β-barrel structure in the outer membrane (Sveshnikova et al., 2000), providing a potential pore for the transport of unfolded preproteins. The nature of the protein-conducting channel of the Tic apparatus has not been defined. The activity of an inner membrane anion channel, PIRAC (protein import–related anion channel), is associated with protein import (van den Wijngaard and Vredenberg, 1997, 1999; van den Wijngaard et al., 1999, 2000), supporting the concept that protein translocation at the Tic machinery occurs through a regulated channel that opens in response to preproteins during import.

Tic20 is a 20-kD integral inner membrane protein containing four predicted α-helical transmembrane segments and short N- and C-terminal soluble domains (Kouranov et al., 1998). Tic20 was identified as a Tic component using covalent cross-linking studies with preproteins trapped at various stages during in vitro import in isolated pea chloroplasts (Ma et al., 1996; Kouranov and Schnell, 1997). Cross-linking to pea Tic20 (psTic20) was detected at an intermediate stage in import after the preproteins had inserted across the outer membrane and increased at later stages in import when the preproteins had inserted across the inner membrane translocon (Kouranov and Schnell, 1997). These results, coupled with the topology of psTic20, led us to propose that it constitutes part of the protein-conducting channel at the inner membrane. Interestingly, psTic20 and Tim17, a component of the protein-conducting channel of the mitochondrial inner membrane translocon, are distantly
related to a class of prokaryotic branched-chain amino acid transporters (Rassow et al., 1999; Reumann et al., 1999). The similarities in size and primary sequence among this group of membrane proteins support the hypothesis that Tic20 participates in preprotein translocation.

psTic20 associates with two additional Tic components, psTic22 and psTic110. The function of psTic22 is unknown, but its localization to the intermembrane space and interactions with translocating preproteins suggest that it participates in coordinating the interaction of the Toc and Tic complexes during import (Kouranov et al., 1998). psTic110 associates with plastid ClpC (Akita et al., 1997; Nielsen et al., 1997) and Cpn60 (Kessler and Blobel, 1996) and is proposed to concentrate these two stromal chaperones at the site of protein import to facilitate membrane transport and subsequent folding of the import substrate. The three Tic components (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998), along with a fourth component, psTic40 (Stahl et al., 1999), associate with the Toc complex to form a Toc-Tic supercomplex that corresponds to functional protein import sites across the double membrane envelope.

We have altered the expression levels of the Arabidopsis ortholog of psTic20, atTic20, in an effort to determine whether the activities of Tic20 correlate with a role in inner membrane translocation. The expression of an atTic20 antisense gene results in a reduction in atTic20 expression and pronounced pale phenotypes in transgenic Arabidopsis. Plastids from atTic20 antisense plants are defective in their ability to import preproteins, leading to pronounced defects in chloroplast biogenesis. These results support the identification of Tic20 as a Tic component and provide additional evidence for its role as a component of the protein-conducting channel of the inner membrane preprotein translocon.

RESULTS

Identification and Expression Pattern of the atTic20 Gene

An examination of the available Arabidopsis genomic sequence data revealed the existence of a single gene, designated atTic20, exhibiting a high degree of identity to psTic20. The deduced amino acid sequences of the proteins from the two species exhibit 58% overall identity and 73% identity in the sequences corresponding to the mature polypeptides (Figure 1A). In contrast to the known psTic20 transit sequence of 83 amino acids, the atTic20 protein is predicted to contain an N-terminal transit sequence of 102 amino acids.
amino acids. The precursor form of atTic20 was targeted to isolated chloroplasts and processed to its predicted size, confirming it as a chloroplast protein (Figure 1B). Newly imported atTic20 was insensitive to protease digestion in intact chloroplasts and fractionated with the chloroplast membrane fraction (Figure 1B), consistent with its localization to the inner envelope membrane. On the basis of this high degree of identity, we designate atTic20 as the ortholog of psTic20.

Previous studies have shown that the activities and levels of the protein import apparatus correlate with plastid development, peaking during periods of rapid growth and plastid proliferation (Dahlin and Cline, 1991; Jarvis et al., 1998). As a central component of the plastid protein import apparatus, the expression of atTic20 is expected to follow a similar profile. As a first step in the in vivo analysis of atTic20, we examined the developmental and tissue-specific expression profiles of the atTic20 gene. Wild-type Arabidopsis (Wassilewskija) plants were grown under short day conditions (8 hr of light and 16 hr of dark). Total RNA was extracted at different ages between 5 and 60 days after germination from the aboveground portion of the plants, and the relative abundance of atTic20 mRNA was determined by quantitative comparative reverse transcriptase-mediated polymerase chain reaction (RT-PCR) using 18S rRNA as an internal control. The ratio of the amount of the atTic20 RT-PCR product to the 18S rRNA RT-PCR product was used as an arbitrary measurement unit. The 5’ sense primer for amplifying atTic20 cDNA spans the splice site of two exons of the atTic20 gene that are separated by a 247-bp intron, thereby preventing the amplification of any genomic DNA that might contaminate the RNA preparations accidentally.

As shown in Figure 2A, the atTic20 mRNA levels were high at 5 days after germination, when cotyledons were expanding, and decreased to a lower level at 10 days, when the cotyledons stopped expanding and the primary leaves began to develop. The expression of atTic20 increased as the primary leaves expanded and additional rosette leaves developed, with levels peaking at 25 days. The atTic20 mRNA levels declined after 30 days as the plants matured and began to bolt. These results clearly indicate that the expression of atTic20 mRNA correlates with developmental stages involving cell growth and increases in chloroplast size and numbers.

To obtain the tissue-specific expression profile of atTic20, the mRNA levels in roots, rosette leaves, cauline leaves, stems, flowers, and siliques of mature plants as well as green and etiolated seedlings were examined. atTic20 was expressed in all tissues examined, although the relative levels varied among different tissues (Figure 2B). The levels of atTic20 were highest in rapidly growing tissues, such as stems, developing flowers, and siliques. Furthermore, atTic20 mRNA was found at equivalent levels in both etiolated and light-grown seedlings, suggesting a role of the protein in early chloroplast development.

To confirm that atTic20 levels correlate with atTic20 protein levels, we immunoblotted extracts from different ages and several tissues of Arabidopsis seedlings with an antibody to an N-terminal peptide derived from atTic20. Figure 2C demonstrates that atTic20 was present in all tissues and ages examined. The levels of atTic20 ranged from their

![Figure 2A](image1.png)
![Figure 2B](image2.png)
![Figure 2C](image3.png)

**Figure 2. Expression Pattern of atTic20.**

(A) Temporal expression pattern of atTic20 mRNA. Total RNA was extracted from wild-type Arabidopsis plants at the ages indicated. The relative atTic20 mRNA levels were determined by comparative RT-PCR using 18S rRNA as the control (see Methods). The levels of atTic20 mRNA are expressed as the ratio of the ethidium bromide stain intensity of the atTic20 and 18S rRNA PCR products. atTic20 expression was highest during periods of rapid growth and declined as plants matured.

(B) Tissue distribution of atTic20 mRNA expression. Total RNA was extracted from the indicated tissues of 40-day-old light-grown or 5-day-old etiolated plants. The relative atTic20 mRNA level in each tissue was determined by comparative RT-PCR as described in (A). atTic20 mRNA was detected at varying levels in all tissues examined.

(C) Distribution of the atTic20 protein. Total membrane fractions (75 μg of protein each) from etiolated, green, and root tissues or green tissue harvested at the ages indicated were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-atTic20 IgG. atTic20 protein was detected in all tissues examined, with the highest levels found in young green tissues. Error bars in (A) and (B) indicate ±SD.
highest levels in green tissues, to moderate levels in etiolated plants, to their lowest levels in roots. atTic20 was most abundant in the early stages of plant development (5 and 25 days after germination), with lower levels apparent in mature plants (60 days after germination). These data correlate with the mRNA levels detected by RT-PCR and reflect the relative levels of plastid development in these tissues. These results indicate that atTic20 is expressed throughout the plant and is not restricted to photosynthetic tissues, consistent with its proposed role as a general component of the import machinery.

**Generation and Phenotypic Analysis of atTic20 Antisense Plants**

The atTic20 cDNA coding region (825 bp) was cloned into a binary vector, pCAMBIA3300-1, in the antisense orientation with respect to the 35S promoter of cauliflower mosaic virus. Transformation by floral dipping of wild-type Arabidopsis with this construct renders the transformants resistant to the herbicide BASTA (phosphinothricin). Several hundred BASTA-resistant plants were obtained from the T1 generation. Approximately 40% of the BASTA-resistant plants appeared indistinguishable from the control plants transformed with the vector alone (data not shown). Thirty percent of the T1 transformants possessed pale cotyledons but recovered fully and were indistinguishable from control plants at later stages of development (data not shown). Another 10% of the BASTA-resistant T1 transformants exhibited severe pale phenotypes (Figure 3A, Y4). These plants lacked any apparent chlorophyll and failed to survive on soil or Suc-supplemented agar plates beyond the cotyledon stage of development (data not shown). The inviability of these plants likely is attributable to a severe reduction in atTic20 expression, suggesting that atTic20 is required for viability.

The remaining T1 transformants exhibited a variety of pale phenotypes throughout development. Seedlings of three viable lines that represent the spectrum of pale phenotypes are shown in Figure 3A. Approximately 15% of the T1

![Figure 3. Phenotypes of atTic20 Antisense Plants.](image)

(A) Comparison of 5-day-old control (C), Y2, Y3, Y4, and Y19 antisense lines grown on soil. The Y4 and Y19 lines exhibit marked pale phenotypes at early stages of plant development, whereas the Y3 and Y2 lines are only slightly pale.

(B) Comparison of 40-day-old control (C), Y2, Y3, and Y19 antisense lines grown on soil. A 40-day-old Y3 plant homozygous for the atTic20 antisense gene (Y3A) is shown in the center of the left panel and shown enlarged 2.5 times at right. The true leaves of Y2, Y3, and Y19 antisense plants exhibit a variety of pale phenotypes and moderate growth defects. Y3 homozygous plants (Y3A) exhibit a severe pale phenotype and growth defect.

(C) Confirmation of antisense gene incorporation into the genome of lines exhibiting antisense phenotypes. Genomic DNA from the indicated antisense (Y19, Y2, Y3, and Y4) and control (C) plants was extracted and subjected to PCR using primers p3300s and p3300a that are specific for regions of the pCAMBIA3300-1 vector flanking the atTic20 antisense gene. Lane 1 contains DNA molecular mass markers (M). The 1.0- and 0.75-kb markers are indicated at left.
plants, represented by lines Y2 and Y19, exhibited very pale cotyledons. These lines were viable on soil, but the green tissues remained notably pale throughout the life cycle of the plants (Figure 3B, Y2 and Y19). The phenotypes of these two lines remained stable in three subsequent generations that have been examined.

Another 5% of the T1 seedlings exhibited mildly pale phenotypes in their cotyledons (Figure 3A, Y3), but their developing leaves and stems were significantly pale (Figure 3B, Y3). These seedlings were viable but retained a pale phenotype throughout development. The Y3 line represents this population. Interestingly, plants from the T2 generation of the Y3 line exhibited a Mendelian 1:2:1 segregation of pale phenotypes. Twenty-five percent of the plants exhibited a phenotype indistinguishable from wild-type plants and were BASTA sensitive. Fifty percent of the plants exhibited a significantly pale phenotype similar to the T1 plants. The remaining 25% exhibited a much more severe pale phenotype (Figure 3B, Y3A). We designate these plants Y3A. In addition to a nearly albino pigmentation, the true leaves of the Y3A plants had exaggerated serrated edges. One hundred percent of the progeny of the Y3A plants exhibited the severe phenotype in the three subsequent generations that have been examined. The phenotypic segregation profile of the Y3 line is explained most easily if the T1 generation was hemizygous for a single antisense gene insertion and the Y3A plants corresponded to plants homozygous for the insertion. The increase in the severity of the pale phenotype in Y3A could be explained by increased antisense expression from the additional antisense gene. DNA gel blot analysis of the plants from the Y3 line supported the existence of a single antisense gene insertion in these plants (data not shown).

The incorporation of the antisense atTic20 construct into the genome of the four antisense lines presented in Figure 3A was confirmed by PCR amplification of total genomic DNA using a primer pair corresponding to the pCAMBIA3300-1 sequences adjacent to the antisense Tic20 cDNA cloning site. As shown in Figure 3C, genomic DNA from all four BASTA-resistant lines yielded the expected ~900-bp PCR product, whereas the control sample failed to give an amplification product.

For the purpose of analyzing the antisense phenotypes, we focused our studies on the three viable lines that exhibited the most pronounced pale phenotypes (Y2, Y3, and Y19). To test whether the phenotypes of the antisense lines result from a decrease in atTic20 expression, we determined the levels of atTic20 mRNA and protein in these plants. Plants at 30 days after germination were chosen for analysis because the highest levels of atTic20 mRNA are detected in true leaves of control plants at this stage of development (Figure 2A). Figure 4 shows that atTic20 mRNA levels were reduced markedly in the three antisense lines. The levels of atTic20 mRNA were lowest in the Y3 line, representing ~25% of control levels, whereas atTic20 mRNA levels in the Y2 and Y19 lines were reduced to 65 and 50% of control levels, respectively (Figure 4A). Immunoblots of extracts from control and antisense lines with anti-atTic20 IgG confirmed that the levels of atTic20 in chloroplast envelopes were reduced in all three antisense

**Figure 4.** Suppression of atTic20 mRNA and Protein Expression in Antisense Arabidopsis Plants.

(A) atTic20 mRNA levels in antisense plants. Total RNA was extracted from the leaves of 30-day-old control or antisense (Y2, Y3, and Y19) plants. The relative amount of atTic20 mRNA in each RNA sample was determined by comparative RT-PCR using 18S rRNA as the internal control. The graph at bottom represents the average of four experiments. The gel at top shows an ethidium bromide–stained agarose gel from one representative experiment. The levels of atTic20 mRNA were reduced significantly in all three antisense lines.

(B) atTic20 protein levels in antisense plants. Immunoblots of chloroplast envelope membranes (25 μg of protein each) from control and antisense (Y2, Y3, and Y19) plants with anti-atTic20 and anti-atToc33 IgGs. The levels of atTic20 and atToc33 in antisense samples are expressed as a percentage of the levels measured in samples from control plants. The gel at top shows one representative immunoblot of four used for the quantitative analysis. The immunoblots confirm that atTic20 expression is reduced in all three antisense lines, whereas atToc33 is not affected significantly. Error bars indicate ±SD.
lines, with the lowest levels observed in Y3 plants (Figure 4B). The level of atToc33, a component of the outer envelope translocon, was not altered significantly in the antisense lines (Figure 4B), suggesting that antisense expression selectively alters atTic20 levels. Thus, the reduced levels of atTic20 correlate directly with the severity of the pale phenotypes in the antisense lines, supporting the link between atTic20 expression and plastid biogenesis.

To quantitatively measure the pale phenotype of the mutant lines, the chlorophyll content of the aboveground portion of control and antisense atTic20 (Y2, Y3, and Y19) plants at 5-day intervals with 80% acetone. Chlorophyll content was quantified by absorption at 652 nm by the method of Arnon (1949). Each data point represents the mean of four or more measurements. The chlorophyll content of all three antisense lines was reduced throughout development, consistent with their pale phenotypes. Error bars indicate ± SD.

To quantitatively measure the pale phenotype of the mutant lines, the chlorophyll content of the aboveground portion of control and antisense plants grown under short-day conditions was determined at 5-day intervals. The chlorophyll content of control plants did not change dramatically during the first 20 days of development but increased dramatically between 20 and 25 days (Figure 5). The chlorophyll content of the antisense lines was consistently lower than that of control plants (Figure 5). The chlorophyll content of the Y2 and Y19 lines began at very low levels, consistent with their pale phenotypes at the early seedling stage. Although these levels increased with development, both lines reached a maximum chlorophyll content equivalent to one-half that of control plants at 30 days. In contrast, the Y3 line remained pale at all stages of development, with no appreciable increase in chlorophyll content upon maturity. The chlorophyll content of Y3 plants was less than 25% of the levels of control plants at 25 days (Figure 5).

All three lines exhibited measurable growth defects. The wet weights of 40-day-old Y2, Y19, and Y3 lines averaged 50, 30, and 55% less, respectively, than control plants (Figure 3B). The Y3A plants averaged 85% lower wet weights compared with control plants (Figure 3B). Growth of the Y2, Y3, Y3A, and Y19 lines on agar plates supplemented with Suc did not alter the visible phenotypes or growth characteristics of the plants (data not shown). Therefore, the pale phenotypes were not simply the result of a photosynthetic defect but likely were caused by a general defect in plastid function.

**Chloroplast Content and Morphology in Antisense Plants**

To assess the effects of atTic20 levels on chloroplast biogenesis directly, we examined the morphology of chloroplasts from the primary leaves of 10-day-old antisense plants by electron microscopy. The chloroplasts from the antisense plants exhibited marked alterations in thylakoid membrane abundance and organization (Figure 6). The amount of thylakoid membrane in chloroplasts from all three lines was reduced significantly. Furthermore, fewer grana were observed in the chloroplasts from the antisense plants (Figures 6B to 6D) compared with the extensive, orderly stacked network of control thylakoids (Figure 6A). Examination of multiple sections indicated that plastids from the antisense plants generally were smaller than control plastids. However, the number of chloroplasts per cell did not appear to be altered significantly. These results confirm that atTic20 is required for chloroplast development and maintenance and that reduction in atTic20 levels causes defects in plastid development.

**Figure 5.** Comparison of Chlorophyll Content in Antisense and Control Plants.

Chlorophyll was extracted from the aboveground portions of control and antisense atTic20 (Y2, Y3, and Y19) plants at 5-day intervals with 80% acetone. Chlorophyll content was quantified by absorption at 652 nm by the method of Arnon (1949). Each data point represents the mean of four or more measurements. The chlorophyll content of all three antisense lines was reduced throughout development, consistent with their pale phenotypes. Error bars indicate ± SD.

**Figure 6.** Ultrastructure of Chloroplasts from atTic20 Antisense Plants.

Control (A), Y3 (B), Y2 (C), and Y19 (D) antisense plants were grown on agar plates containing 1% Suc. Electron microscopic samples were prepared from the pale leaves of antisense plants and the corresponding green leaves of control plants of the same age. The antisense lines exhibited a decrease in thylakoid membrane development consistent with their pale phenotypes. Bars = 400 nm.
Both the pale phenotype and the abnormal chloroplast ultrastructure of antisense plants were in agreement with a defect in protein import across the chloroplast envelope. To explore this possibility, we examined the abundance of nucleus-encoded chloroplast proteins in antisense and control plants by comparative immunoblotting. We examined the levels of two major nucleus-encoded chloroplast proteins in the antisense lines, the major light-harvesting complex protein (LHCP) and the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (SSU). Both proteins were strongly light induced and expressed predominantly in chloroplasts. In addition, we examined the levels of the \( \alpha \)-subunit of the E1 subunit of pyruvate dehydrogenase (PDH\( \alpha \)), an enzyme expressed constitutively in all plastid types (Johnston et al., 1997). All three lines showed marked decreases in the accumulation of LHCP, SSU, and PDH\( \alpha \) (Figure 7A). The levels of all plastid proteins were lowest in the Y3 line, consistent with its pronounced phenotype. All three proteins were detected as their mature forms, indicating that they have been imported into chloroplasts (Figure 7A). In no case did we observe the accumulation of precursor forms of any of the three proteins. The levels of actin were not altered in the antisense lines, indicating a specific reduction in plastid-specific proteins (Figure 7A). These data suggest that reduced levels of atTic20 result in the inefficient import and accumulation of plastid preproteins, resulting in growth defects, underdeveloped thylakoid membranes, and reduced photosynthetic capacity.

We also examined the abundance of protochlorophyllide oxidoreductase (POR) in 5-day-old etiolated seedlings. POR is the major nucleus-encoded plastid protein in etioplasts of dark-grown plants (Griffiths, 1978). The abundance of POR was reduced in all three antisense lines compared with control plants (Figure 7B). Thus, a decrease in atTic20 expression appears to have a general effect on the accumulation of plastid proteins at all developmental stages.

In contrast to the levels of chloroplast proteins in mature plants (Figure 7A), the levels of POR were lowest in the Y2 and Y19 lines, whereas Y3 plants had a more modest reduction in POR accumulation. These data suggest a differential expression of the antisense transgenes during development. Consistent with this proposal, we measured the levels of atTic20 mRNA by comparative RT-PCR and found that they were higher in etiolated Y3 plants than in etiolated Y2 and Y19 plants (data not shown). These data are consistent with the more pronounced phenotype of the Y2 and Y19 lines at early stages of plant development compared with the Y3 line (Figure 3A).

We examined the levels of atTic110, atToc75, atToc33, and atToc159 in each antisense line to determine whether the effects on plastid protein accumulation were specific for decreases in atTic20 or were caused by a secondary effect.
on the accumulation of other components of the import apparatus. atTic110 (Lubeck et al., 1996) and atToc75 (Tranel and Keegstra, 1996) contain transit peptides and appear to involve components of the Toc/Tic system for their import, whereas atToc33 (Chen and Schnell, 1997) and atToc159 (Muckel and Soll, 1996) insert into the outer membrane independent of a transit sequence. All three Toc components were detected in antisense plants at nearly the same levels as in control plants (Figure 7C), indicating that the outer membrane translocon is intact. The levels of atTic110 were increased slightly compared with control plants in all three antisense lines. These data indicate that the defect in the accumulation of nucleus-encoded proteins in the antisense plants is attributable directly to reduced levels of atTic20 and not to indirect effects on the biogenesis of other components of the import apparatus.

**Chloroplast Protein Import into Antisense Plastids**

To obtain direct evidence that the reduction in atTic20 expression affects chloroplast protein import, we determined the ability of plastids isolated from antisense plants to import preproteins. We focused our studies on the Y3 line because it exhibits the most consistent and severe phenotype. As a first step, we investigated the import of the precursor to SSU (preSSU). Chloroplasts isolated from 2- to 4-week-old control or Y3 antisense plants were incubated with chloroplasts (50 µg of chlorophyll) from control or Y3 antisense plants in a standard protein import reaction for 30 min. The samples were divided in half and treated with 100 µg/ml thermolysin (+ T-lysin) or buffer (− T-lysin) on ice for 30 min. The chloroplasts were reisolated through 40% Percoll silica gel and analyzed directly by SDS-PAGE and phosphorimaging. One-tenth of the 35S-preSSU in vitro translation product (St.) added to each reaction is shown in lane 1. The level of preSSU import was reduced in Y3 antisense chloroplasts.

**Figure 8. Import of preSSU into Isolated Chloroplasts of Antisense Plants.**

Chloroplasts were prepared from 2- to 4-week-old control or Y3 seedlings grown on agar plates containing 1% Suc. (A) Import of preSSU in control and antisense plants. In vitro–translated 35S-preSSU was incubated with chloroplasts (50 µg of chlorophyll) from control or Y3 antisense plants and a standard protein import reaction for 30 min. The samples were divided in half and treated with 100 µg/ml thermolysin (+ T-lysin) or buffer (− T-lysin) on ice for 30 min. The chloroplasts were reisolated through 40% Percoll silica gel and analyzed directly by SDS-PAGE and phosphorimaging. One-tenth of the 35S-preSSU in vitro translation product (St.) added to each reaction is shown in lane 1. The position of precursor (preSSU) and mature (SSU) proteins is indicated at left. The gel at the top of each panel shows one representative fluorograph of the four used for the quantitative analysis. Error bars indicate ± SD.

(B) Time course of 35S-preSSU import into isolated chloroplasts from control or Y3 antisense plants. Import was performed as in (A). Samples corresponding to 25 µg of chlorophyll were collected at the times indicated, treated with 100 µg/ml thermolysin on ice for 30 min, reisolated through 40% Percoll silica gel, and analyzed directly by SDS-PAGE and phosphorimaging. One-tenth of the 35S-preSSU in vitro translation product (St.) added to each reaction is shown in lane 1. The positions of precursor (preSSU) and mature (SSU) proteins are indicated at left. The plastids from Y3 antisense plants exhibited a slower rate of preSSU import than control plants.
plasts to translocate preproteins at the outer membrane. The incubation of isolated chloroplasts with preproteins in the presence of 0.1 mM ATP results in the formation of an early import intermediate that has inserted across the outer but not the inner membrane (Schnell and Blobel, 1993; Young et al., 1999). We used these conditions as a quantitative measure of protein translocation through the Toc complex. Figure 9 shows that the levels of the early import intermediate were indistinguishable in Y3 and control chloroplasts. These results indicate that preprotein binding to and insertion into the outer membrane translocon is not affected in the atTic20-deficient chloroplasts.

Second, we tested the ability of the Y3 chloroplasts to import the early intermediate across the inner membrane. Chloroplasts from control and Y3 plants were incubated with $^{35}$S-preSSU under conditions that lead to the formation of the early import intermediate (Figure 10, lanes 1 and 3). Once again, the levels of the early import intermediate were indistinguishable in control and Y3 chloroplasts. The chloroplasts were reisolated and suspended in import buffer containing 2 mM ATP to promote translocation across the inner membrane (Figure 10, lanes 2 and 4). Under these conditions, control chloroplasts imported $\sim$81% of the early intermediate into the stroma, where it was processed to its mature form (Figure 10, cf. lanes 1 and 2). In contrast, Y3 plastids imported $\sim$20% of the early import intermediate over the same period (Figure 10, cf. lanes 3 and 4). Thus, the Y3 chloroplasts are selectively defective in preprotein translocation across the inner membrane. These data are consistent with the hypothesis that atTic20 functions as a component of the protein-conducting channel in the inner membrane translocon.

**DISCUSSION**

We obtained multiple lines of transgenic plants that have significantly lower expression levels of atTic20 by expressing an antisense atTic20 gene in Arabidopsis. The atTic20 antisense plants exhibited a variety of defects, including pale to nearly albino leaves, retarded growth (Figure 3), abnormal plastid ultrastructure (Figure 6), and reduced accumulation of nucleus-encoded plastid proteins (Figure 7). Plastids from the most severely affected antisense line, Y3, exhibited a specific defect in preprotein translocation across the inner envelope membrane (Figure 10). Together, these phenotypes strongly suggest that Tic20 plays a central role in preprotein import into plastids by participating in translocation across the inner membrane.

A variety of different pale phenotypes were observed among the atTic20 antisense plants (Figure 3). The variation in the developmental onset of the pale phenotypes in the antisense lines likely is the result of variation in the cell- or tissue-specific profile of antisense expression as a result of the position of the T-DNA insertions in the genome. Such variation is well documented for other antisense approaches (Gutensohn et al., 2000; Kumar and Soll, 2000). Nevertheless, the pale phenotypes in all of the antisense lines are most pronounced in rapidly growing tissues (Figure 3). The expression of two additional import components, atToc33 and atToc75, has been shown previously to correlate with rapid growth and development in green tissues (Jarvis et al., 1998; Gutensohn et al., 2000). The demand for protein import is highest in these tissues because of the rapid development and increased division of chloroplasts. The levels of these import components decrease as leaves mature, and demand for protein import primarily is for organelle maintenance. We observed a similar expression profile for atTic20 (Figure 2). Therefore, the correlation between rapid growth and the pale phenotype in the atTic20 antisense plants is consistent with the role of atTic20 in protein import.

The phenotypes of the atTic20 antisense plants are similar to the phenotypes of previously characterized Arabidopsis mutants of plastid import components. Arabidopsis lines
lacking the major Toc34-like protein, atToc33, or expressing an antisense atToc33 gene exhibit a marked pale phenotype early in development but recover fully in a manner similar to the Y2 and Y19 antisense atTic20 lines (Jarvis et al., 1998; Gutensohn et al., 2000). They also show abnormal chloroplast morphology, including a reduction in thylakoid membrane development, accumulation of nucleus-encoded chloroplast proteins, and overall plastid size. A mutant lacking atTic20, pp2, exhibits a severe albino phenotype and is not viable on soil (Bauer et al., 2000a). The plastids from the pp2 mutant contain significantly less LHCP, SSU, and other nucleus-encoded plastid proteins. This phenotype is similar to the extremely pale phenotype exhibited by the atTic20 antisense plants that fail to develop beyond the cotyledon stage (Figure 3A, Y4). Antisense tobacco plants with reduced levels of the stromal processing protease also exhibit dramatic growth defects, a chlorotic phenotype, and reduced thylakoids (Wan et al., 1998).

The import defect exhibited by the atTic20 antisense plants appears to affect the accumulation of an array of nucleus-encoded plastid proteins (Figure 7). In addition to reduced levels of chloroplast-specific proteins such as SSU and LHCP, the antisense lines contained reduced levels of POR, a protein expressed predominantly in etioplasts, and PDHα, an enzyme expressed constitutively in all plastid types. These data, in conjunction with the observation that growth on Suc did not alter the antisense phenotype, indicate that atTic20 is a general component of the Tic apparatus that is required for the import of proteins in all plastid types. This is in contrast to atToc33 and atToc159, which appear to be involved specifically in chloroplast development (Jarvis et al., 1998; Bauer et al., 2000a). The atTic20 null mutant, pp2, is not viable on soil but survives if the photosynthetic defect is bypassed by supplementing the plants with Suc (Bauer et al., 2000a; Yu and Li, 2001). The two Toc proteins are encoded by small gene families that are expressed differentially during development (Jackson-Constan and Keegstra, 2001). atTic20 is expressed in all tissues examined and is likely to be required at all developmental stages.

There are conflicting reports in the literature using in vitro import assays regarding whether or not prePORA is imported into chloroplasts by the general Toc-Tic machinery (Aronsson et al., 2000; Reinbothe et al., 2000). Although our data do not exclude the possibility that some stage of POR import may involve components other than the known Toc and Tic proteins, it is clear that atTic20 participates in POR accumulation in vivo. Therefore, translocation of POR across the inner envelope membrane is likely to involve at least this component of the Tic translocon.

In vitro protein import assays using isolated chloroplasts from the Y3 line provided direct evidence that atTic20 is a core component of the Tic translocon. The import kinetics of preSSU in Y3 plastids were significantly slower than control chloroplasts (Figure 8). Translocation of preSSU across the outer envelope membrane did not appear to be affected because the formation of a preSSU early import intermediate that is trapped in transit through the Toc complex is comparable in both Y3 and control plastids (Figure 9). This is consistent with the observation that Toc components were expressed at normal levels in antisense plants. In contrast, the efficiency of translocation of the early intermediate across the inner envelope was reduced approximately twofold to threefold over control levels (Figure 10). These data provide direct evidence for the participation of atTic20 in preprotein translocation across the inner membrane. Previous studies have demonstrated that psTic20 cross-links to trapped import intermediates that are inserted partially across the inner membrane (Kouranov and Schnell, 1997). These data, in conjunction with the data from the analysis of the antisense plants, support the hypothesis that atTic20 is a component of the protein-conducting channel of the Tic apparatus.
The levels of other Toc and Tic components examined were not reduced in the antisense lines, although at least one of these components, atTic110, is proposed to use the general import pathway (Stahl et al., 1999). In ppi2, an atToc159 null mutant, the levels of the import components examined, including atTic110, were normal (Bauer et al., 2000a). In that case, the authors proposed that the import of the Toc/Tic components uses a different preprotein receptor (i.e., atToc132 or atToc120) and thus a different Tic translocon. Here, we extend this hypothesis and speculate that the import of atTic110 relies not only on a distinct Tic translocon but also on a distinct Tic translocon. In fact, the mitochondrial inner membrane translocon components Tim17, Tim23, and Tim44 and some other inner membrane proteins are imported by a distinct Tim complex (Bauer et al., 2000b). The unique Tim complex contains Tim22, a protein structurally related to Tim23 and Tim17. A similar system might exist in chloroplasts. Indeed, in addition to the atTic20 gene, two Arabidopsis genes encoding proteins with some similarity to atTic20 exist (Jackson-Constan and Keegstra, 2001). These two genes might represent distinct Tic complexes that could account for the import of specific classes of plastid proteins. The atTic20 antisense lines provide an experimental system in which to examine the diversity of translocation pathways across the inner envelope membrane.

One unexpected result is that the abundance of atTic110 was increased slightly in the antisense plants (Figure 7). The increased levels of atTic110 may reflect an attempt by the plants to compensate for a defective inner membrane translocon by increasing the expression of the Tic components. It will be of interest to examine the levels of atTic22 and other Tic components when reagents become available to determine if their targeting or expression is affected in the atTic20-deficient lines.

METHODS

Plant Material and Growth Conditions

All experiments were performed with Arabidopsis thaliana ecotype Wassilewskija. Plants were grown at 21.5°C with 8 hr (short-day condition) or 16 hr (long-day condition) of daylight as indicated in the text. For growth on agar plates, seed were surface-sterilized and sown on 0.9% agar containing 0.5 times concentrated Murashige and Skoog (1962) medium, 1% (w/v) Suc, and/or 50 g/mL BASTA (phosphinothricin; Sigma Chemical, St. Louis, MO).

Construction of pCAMBIA3300-1/Antisense atTic20

The atTic20 expressed sequence tag clone F1H7T7 was obtained from the Arabidopsis Biological Research Center (Columbus, OH). The region corresponding to the atTic20 open reading frame was amplified from F1H7T7 by polymerase chain reaction (PCR) using the atTic20s primer (forward primer, 5′-GGATCCCTTA-GTCGTACGGAAATC-3′) and the atTic20a-BamHI primer (reverse primer, 5′-GGATCCTTA-GTCGTACGGAAATC-3′). The binary vector pCAMBIA3300-1 was digested with NcoI and treated with the Klenow fragment of DNA polymerase to generate blunt ends. The purified atTic20 open reading frame PCR product and blunt-end pCAMBIA3300 were ligated, and antisense atTic20 clones were confirmed by DNA sequencing. The pCAMBIA3300-1 vector was a kind gift from Dr. Felix Kessler (Swiss Federal Institute of Technology, Zurich, Switzerland).

Plant Transformation and Selection

The pCAMBIA3300-1/antisense atTic20 plasmid was transformed into Agrobacterium tumefaciens (GV3101) and introduced into Arabidopsis plants using the floral dip protocol (Clough and Bent, 1998). BASTA-resistant plants were confirmed for T-DNA transformation by PCR of genomic DNA of the transformants (McKinney et al., 1995). The confirmed antisense plants were grown to maturity under long-day conditions. Plants from the T1 seed of the transformed plants were assumed to be derived from independent T-DNA insertion events for purposes of phenotype characterization.

Chlorophyll Extraction and Quantification

Chlorophyll was extracted from total aboveground tissue or leaf tissue of antisense plants (T2 generation) and control plants of the same age by grinding in 80% acetone. The chlorophyll concentrations were determined by measuring absorbance at 652 nm (Arnon, 1949) and are expressed as μg chlorophyll/mg wet weight. The chlorophyll contents represent the means of four to seven samples.

Comparative Reverse Transcriptase–Mediated PCR

Total RNA was extracted from total aboveground tissue or leaf tissue of antisense plants (T2 generation) and control plants of the same age using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized using random hexamer primers and 1 μg of total RNA with the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The cDNA was diluted eight times, and 1 μL of the diluted cDNA was used as a template for each 50-μL PCR reaction. Comparative reverse transcriptase (RT)–mediated PCR was performed using thespecific primers for Tic20 and plant 18S rRNA as an internal control (QuantumRNA Plant 18S Internal Standards Kit; Ambion, Austin, TX). The gene-specific primer pairs to atTic20 were as follows: forward, atTic20-5′-CCCTTGGTTTG-TGAAGGCTTTTTT-3′; reverse, atTic20-3′-GGAGTCATAAGGATCCAGGATACTG-3′. The linear range of the comparative PCR was determined to be 22 to 32 cycles, and the optimal ratio of the 18S rRNA and atTic20 primers was determined to be 6:4. Ethidium bromide–stained PCR products were quantified using a Kodak Digital DC120 EDAX digital camera system (Eastman Kodak, Rochester, NY). Each mRNA level represents the mean of four RT-PCR experiments.

Protein Extraction and Immunoblotting

Total protein was extracted directly in boiling SDS-PAGE sample buffer from total aboveground tissue or leaf tissue of antisense plants.
and control plants of the same age (Bauer et al., 2000a) for all immuno- 
noblots except those probed with anti-atTic20 serum. For anti- 
atTic20 immunoblots, total membrane fractions or total chloroplast 
envelope membrane fractions were prepared from antisense or con- 
trol plants as described previously (Ma et al., 1996). Samples corre- 
sponding to equivalent amounts of protein were resolved by SDS- 
PAGE and transferred to nitrocellulose membranes. Immunoblotting 
was performed as described previously (Ma et al., 1996). Immuno- 
blot signals were determined using a STORM chemiluminescence 
detector (Molecular Dynamics, Sunnyvale, CA) and by densitometric 
scanning of x-ray films. The amount of protein used for all immuno- 
noblots was chosen to ensure that the chemiluminescence signal for 
each antigen was a linear function of protein content. The relative 
level of each protein in the antisense plant extracts was expressed as 
a percentage of the chemiluminescence signal measured for the cor- 
responding protein in control plant extracts.

Antisera to atTic20 were raised against an N-terminal peptide 
(SKDVPSSFRFPMTKK). Antiser to atToc33 and Arabidopsis 
protochlorophyllide oxidoreductase (POR) were raised against full- 
length atToc33 and amino acids 70 to 234 of prePORA that had been 
expressed in Escherichia coli. Antiserum to the minor subunit of ribu- 
lose-1,5-bisphosphate carboxylase/oxygenase (SSU) was prepared 
from purified ribulose-1,5-bisphosphate carboxylase/oxygenase as 
described previously (Schnell et al., 1991). Antiserum to the major 
light-harvesting complex protein (LHCP) was a kind gift of Dr. 
Douglas Randall (University of Missouri, Columbia, MO).

Electron Microscopy

Leaflets were fixed with 2.5% glutaraldehyde in 0.05 M sodium ca- 
codylate, pH 7.4, under vacuum for 3 hr and washed three times with 
0.05 M sodium cacodylate, pH 7.4. Fixed samples were treated with 
1% osmium tetroxide in 0.05 M sodium cacodylate, pH 7.4, for 2 hr 
and washed three times with 0.05 M sodium cacodylate, pH 7.4. The 
samples were dehydrated by the following treatments: incubation in 
70% ethanol for 10 min, incubation in 100% ethanol for 10 min, and 
incubation twice in 100% propylene oxide for 15 min. EMbedding 
embedding mixture (Electron Microscopic Sciences, Fort Washing- 
ton, PA) was prepared according to the manufacturer’s instructions. 
Dehydrated samples were infiltrated with one-third concentrated 
EMbed812 (in propylene oxide) for 3 hr, two-thirds concentrated 
EMbed812 overnight, and 100% Embed812 for 1.5 hr before embed- 
ding in EMbed812 by incubation at 60°C for 2 hr. Seventy-nanome- 
ter sections of the samples were prepared and dried on 150-mesh 
copper grids and poststained with uranyl acetate and lead citrate as 
described previously (Smith and Croft, 1991). The grids were dried 
and observed using a Philips–Tecnai (Eindhoven, The Netherlands) 12 
transmission electron microscope.

Arabidopsis Chloroplast Preparation

Two- to 4-week-old Arabidopsis seedlings were grown on 0.9% agar 
plates containing 1% Suc and 0.5 times concentrated Murashige and 
Skog medium including 50 μg/mL BASTA for antisense lines. The 
preparation of chloroplasts was performed as described by Fitzpatrick 
and Keegstra (2001). Briefly, aboveground tissue was collected and 
chopped into small pieces in digestion buffer (400 mM sorbitol, 0.5 
mM CaCl₂, and 20 mM Mes-KOH, pH 5.2). The plant tissue was 
treated with 4% Cellulase and 0.8% Macerozyme (Yakult Honsha, 
Tokyo, Japan) in digestion buffer at room temperature under moder- 
at temperature for 3 to 4 hr to generate protoplasts. The protoplasts 
were separated from the plant tissue by filtering through 200-μm nylon 
mesh and washed with protoplast resuspension buffer (400 mM sor- itol, 0.5 mM CaCl₂, and 20 mM Mes-KOH, pH 6.0). The protoplasts 
were lysed in breakage buffer (300 mM sorbitol, 5 mM EDTA, 5 mM 
EGTA, 10 mM NaHCO₃, 0.1% BSA, and 20 mM Tricine-KOH, pH 8.4) 
by filtering sequentially through 20- and 10-μm nylon mesh. Chloro- 
plasts were isolated and purified on Percoll as described previously 
(Ma et al., 1996).

In Vitro Import Assay

Chloroplasts were prepared from 2- to 4-week-old atTic20 antisense 
or control plants. The amounts of chloroplasts prepared from dif- 
ferent lines were normalized to total protein content. Formation of 
early import intermediates and import assays were performed as 
described previously (Young et al., 1999; Chen et al., 2000) using 
For import, chloroplasts were incubated with 2 mM ATP under light 
at room temperature for the times indicated in Figures 8 and 10, re- 
purified on 40% Percoll silica gel, and subjected to SDS-PAGE. 
Thermolysin treatments were performed on ice for 30 min using 
100 μg thermolysin/mL as described previously (Schnell et al., 1994). 
For binding and chase experiments, chloroplasts containing 130 
μg of protein were preincubated in the dark at 26°C for 10 min to de- 
plicate endogenous ATP. The depleted chloroplasts were incubated 
for 10 min with 0.1 mM ATP and 35S-preSSU to form the early import 
intermediate. The chloroplasts were purified on 40% Percoll silica 
gel, and one-half was dissolved directly in SDS-PAGE sample buffer. 
The remaining chloroplasts were resuspended in import buffer con- 
taining 2 mM ATP and incubated for 15 min at 26°C. The chloroplasts 
then were collected with a brief spin and analyzed by SDS-PAGE. All 
samples were quantified using a Phosphorimager (Molecular Dy- 
namics). The concentration of 35S-preSSU in Figure 9 was calculated 
from the known specific radioactivity of the 35S-Met used in the 
translation reaction. The phosphorimaging intensity of serial dilutions 
of 35S-Met was converted to a molar amount based on its specific 
radioactivity (1000 Ci/mmol). This factor was used to determine the 
specific radioactivity of the 35S-preSSU by comparing the phospho- 
rimaging intensities of 35S-Met and 35S-preSSU.

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