Supplemental Material

Figure S1. The levels of substrate proteins of SRP/ALB3 and TATC were reduced in SCY2 RNAi plant.

Figure S2. Chloroplast lysis methods produce inside-out inner envelope vesicles and right-side out thylakoid vesicles.

Figure S3. Properly integrated ALB3, SCY1, and FTSH12 are resistant to 0.1 M NaOH extraction and produce diagnostic protease protected degradation products.

Figure S4. Properly integrated FTSH12 produces a protease-resistant degradation product of ~39 kD that likely represents its two TMs and intervening loop exposed to the inter-envelope space.

Figure S5. 30% NaOH/70% Na_2CO_3 can be used for diagnosis of TIC40 integration.

Figure S6. Membrane integration of FTSH12 is time dependent.

Figure S7. Quantitative analysis of integrated SCY1 and ALB3 in antibody inhibition assays.

Figure S8. Quantitation of membrane integrated mTIC40 in antibody inhibition assays.

Table S1. Primers used in this study

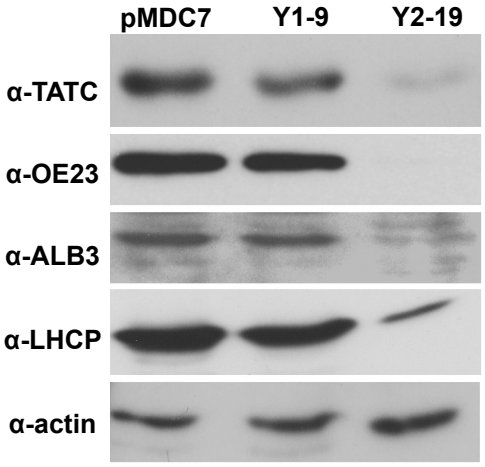

Figure S1. The levels of substrate proteins of SRP/ALB3 and TATC were reduced in SCY2 RNAi plants.

Total protein extracts from 6 d estrogen-induced seedlings were subjected to SDS-PAGE and immunoblotting with various antibodies. Actin was used as the internal control for equal protein loading.

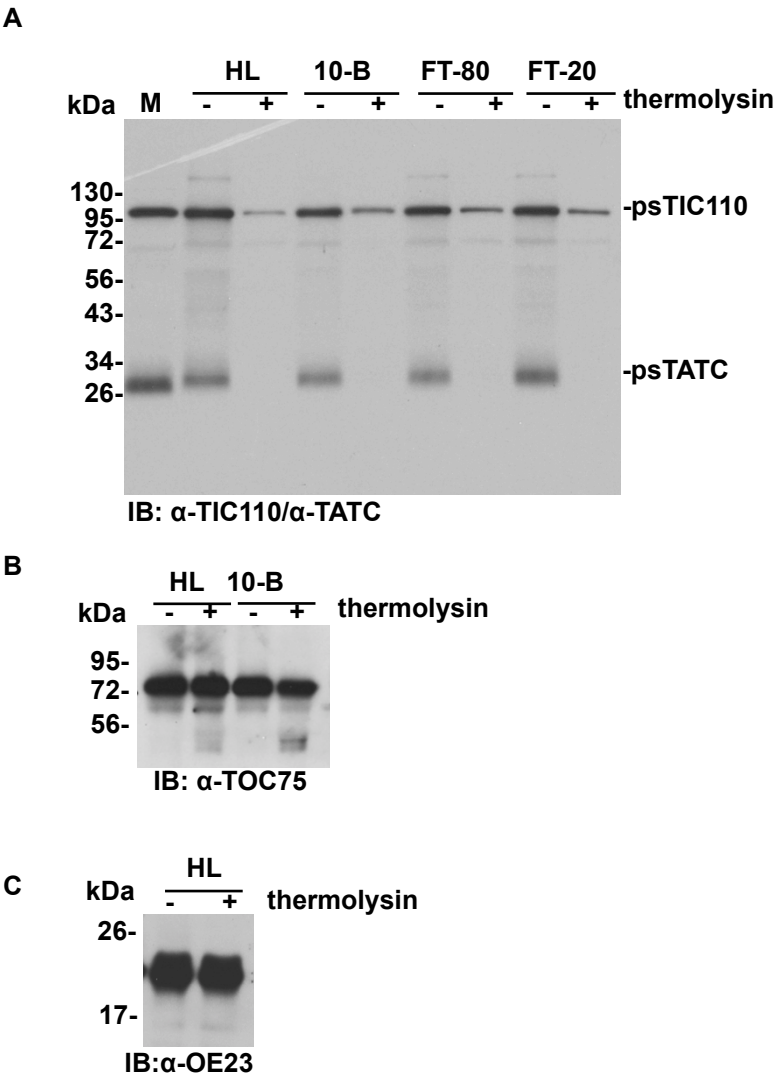

Figure S2. Chloroplast lysis methods produce inside-out inner envelope vesicles and right-side out thylakoid vesicles.

Intact pea chloroplasts were lysed as follows: HL: hypotonic lysis chloroplasts were resuspended in 10 mM Hepes-KOH pH 8.0 for 5 min followed by addition of 1 volume of 2x IB. 10-B (mechanical lysis): chloroplasts were incubated in 0.6 M sucrose on ice for 10 min to plasmolyze the inner envelope from the outer envelope and were then ruptured by 50 to 75 strokes with ten-Brock homogenizer and the percent ruptured monitored by phase contrast microscopy. FT-80 (freeze-thaw at -80°C): chloroplasts in 0.6 M sucrose were frozen at -80°C for 30 min and then thawed by brief incubation at 37°C. This cycle was repeated once (Chu and Li, 2011). FT-20 (Freeze-thaw at -20°C): chloroplasts in 0.6 M sucrose were frozen at -20°C for 1h and then thawed on the bench (20-25°C)(Cline et al., 1981). Broken chloroplasts in 0.6 M sucrose were diluted with 1 volume of 10 mM Hepes-KOH. Membranes were recovered by centrifugation at 40,000 rpm in a TLA55 rotor for 30 min at 2°C and were then suspended in an equivalent volume of import buffer. Aliquots were treated without or with thermolysin at 0.1 µg per µg of Chl for 40 min at 5°C. Reactions were terminated with EDTA and treated membranes recovered by centrifugation as above and then subjected to analysis by SDS-PAGE and immunoblotting. Note, subsequent experiments showed that centrifugation in a micro centrifuge at top speed for 30 min was sufficient to pellet envelope and thylakoids, whereas 40,000 rpm TLA55, 30 min was necessary to pellet alkaline extracted membranes.

Li et al. Figure S3

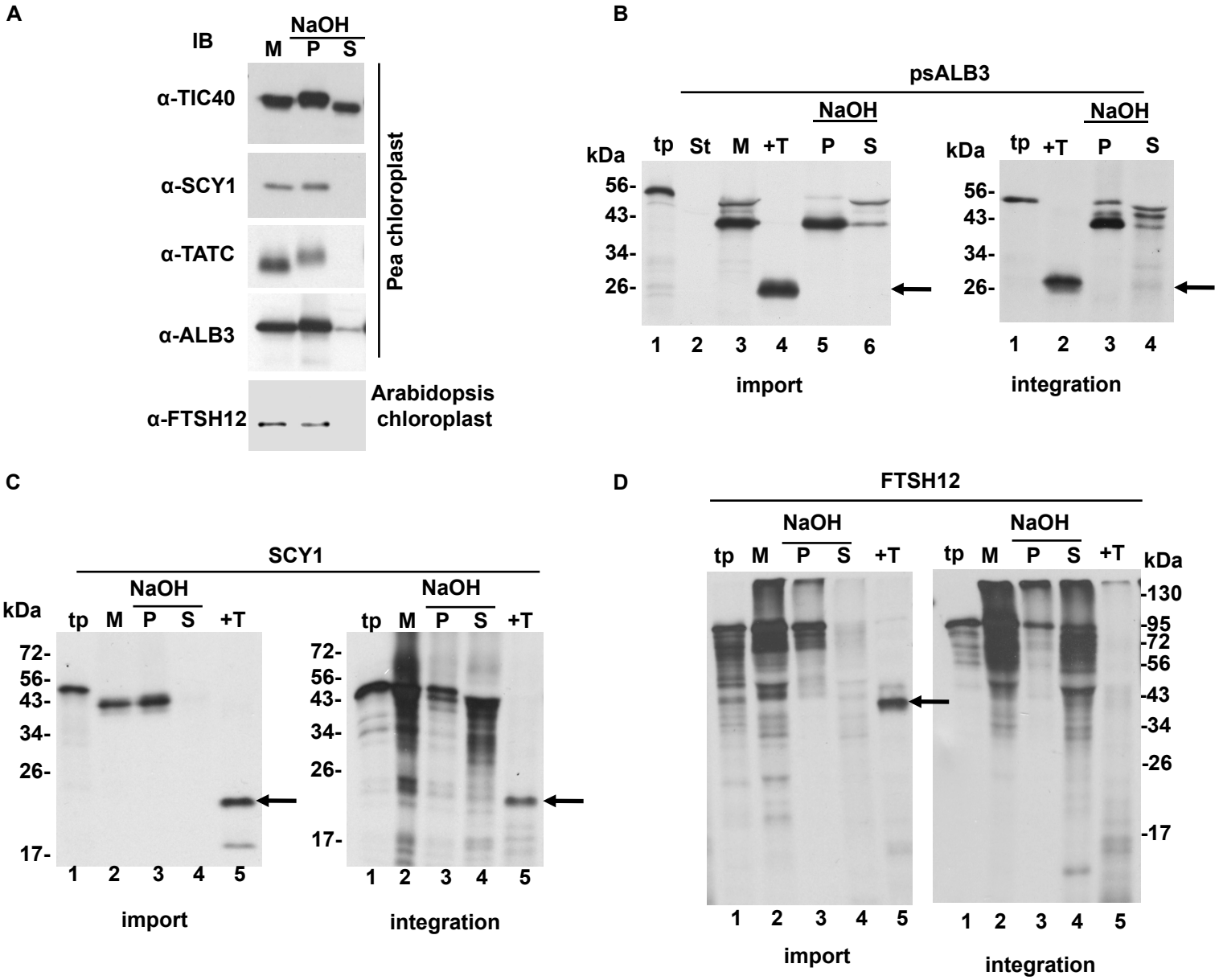

Figure S3. Properly integrated ALB3, SCY1, and FTSH12 are resistant to 0.1 M NaOH extraction and produce diagnostic protease protected degradation products.

A. Chloroplasts were isolated from pea or Arabidopsis seedlings, the chloroplasts were hypotonically lysed with 10 mM HEPES-KOH pH 8.0, readjusted to 0.33 M sorbitol and the membranes pelleted at 40,000 rpm in a TLA55 rotor for 40 min. One aliquot was analyzed directly (M) and a second aliquot was subjected to extraction with ice cold 0.1 M NaOH for 30 min on ice followed by centrifugation at 40,000 rpm, TLA55. The proteins in the supernatant were precipitated by 20% TCA (Chu and Li, 2011). All samples were dissolved in the starting volume in SDS-SB containing 8 M urea and subjected to immunoblotting. **B-D.** In *vitro* translated precursors for psALB3, SCY1, and FTSH12 were incubated with intact pea chloroplasts and 2.5 mM ATP in the light at 25°C for 50 min. Intact chloroplasts were recovered on Percoll cushions, lysed hypotonically, and the membranes pelleted as above. The supernatant is stroma (St). Suspended membranes were divided into 3 equal aliquots. One was analyzed directly (M), one was extracted with 0.1 M NaOH as above into pellet (P) and supernatant (S) fractions, and one was treated with thermolysin (T+) at 0.5 μg μg^{-1} of Chl for 30 min at 4°C. All samples were loaded in stoichiometric amounts. Integration assays were conducted similarly but with hypotonically lysed chloroplasts and 10x excess of SE. Recovered membranes were analyzed directly, extracted with NaOH, or treated with thermolysin. Arrows indicate protected protease degradation products.

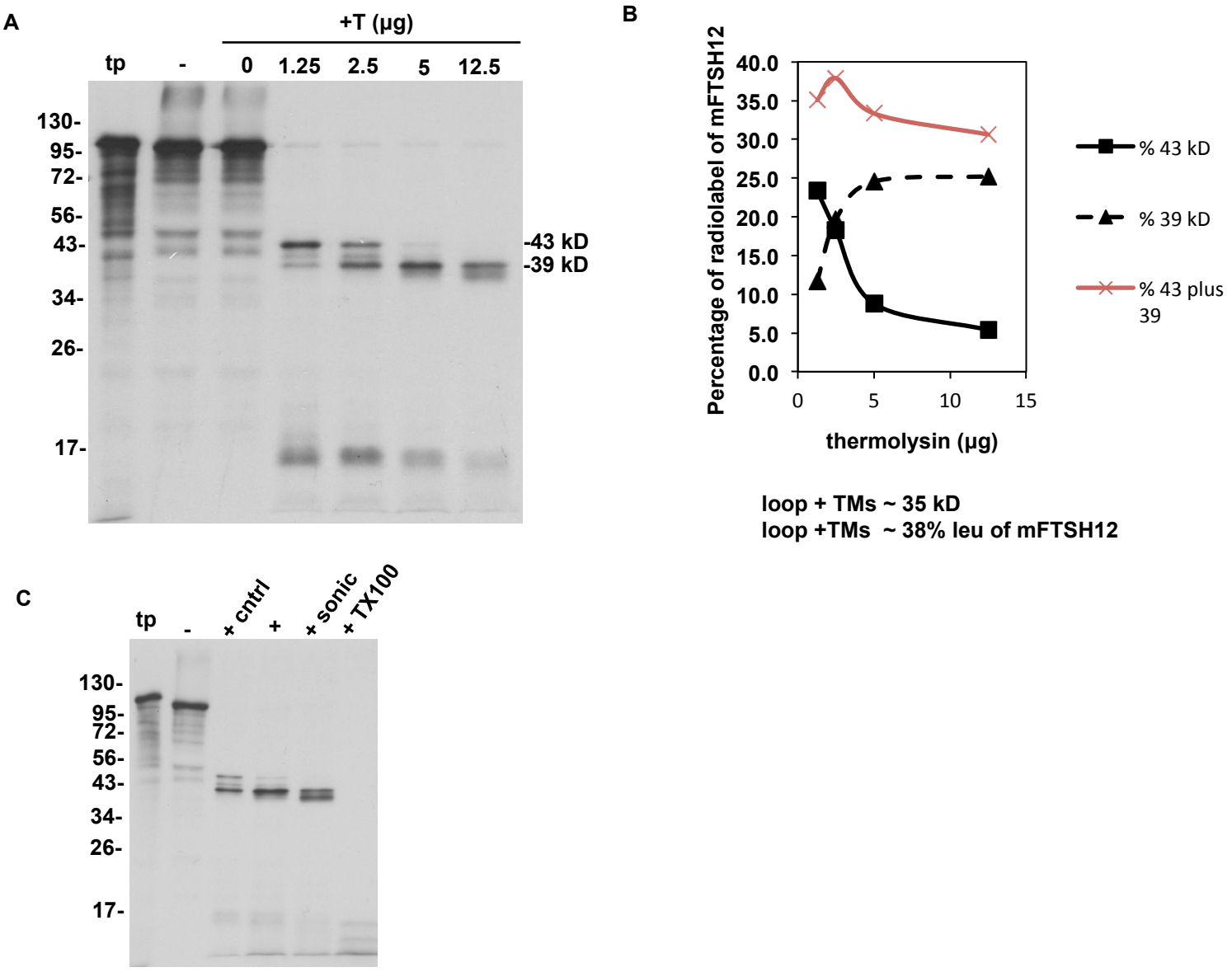

Figure S4. Properly integrated FTSH12 produces a protease-resistant degradation product of ~39 kD that likely represents its two TMs and intervening loop exposed to the inter-envelope space.

A. and B. pre-FTSH12 translated in vitro with ^{3}H leu was imported into pea chloroplasts, recovered chloroplasts were lysed, and the membranes recovered by centrifugation and resuspended in IB. Aliquots (125 µL, containing 25 µg of chlorophyll) were treated with varying amounts of thermolysin protein for 30 min at 5 °C. Protease reaction was stopped by addition of EDTA to 10 mM and washing with an excess of 14 mM EDTA in IB. Samples were separated by SDS-PAGE and subjected to fluorography. The radioabel of bands was determined by scintillation counting of proteins extracted from gel slices (Cline, 1986) and plotted as a percentage of the total radiolabel of undigested mFTSH12. The size and leucine content of the TMs plus loop were predicted by analysis on Membrane Protein Explorer.
C. The 43/39 kD degradation products are protected from thermolysin by the membrane bilayer. Pre-FTSH12 was imported into chloroplasts and membranes were obtained by lysis and centrifugation of recovered chloroplasts followed by re-suspension in IB. Two aliquots were treated without (-) or with (+cntrl) thermolysin for 30 min at 5 °C and terminated as is normal for these assays (Methods). Three other aliquots 6 times concentrated were treated with equivalent thermolysin either undisturbed (+), with bath sonication during treatment (+sonic) or in which the membrane was dissolved with Triton X-100 (TX100). These latter 3 treatments were terminated with EDTA to ~85 mM followed by addition of 100 °C 2x SSB and 4 min heating at 100 °C.

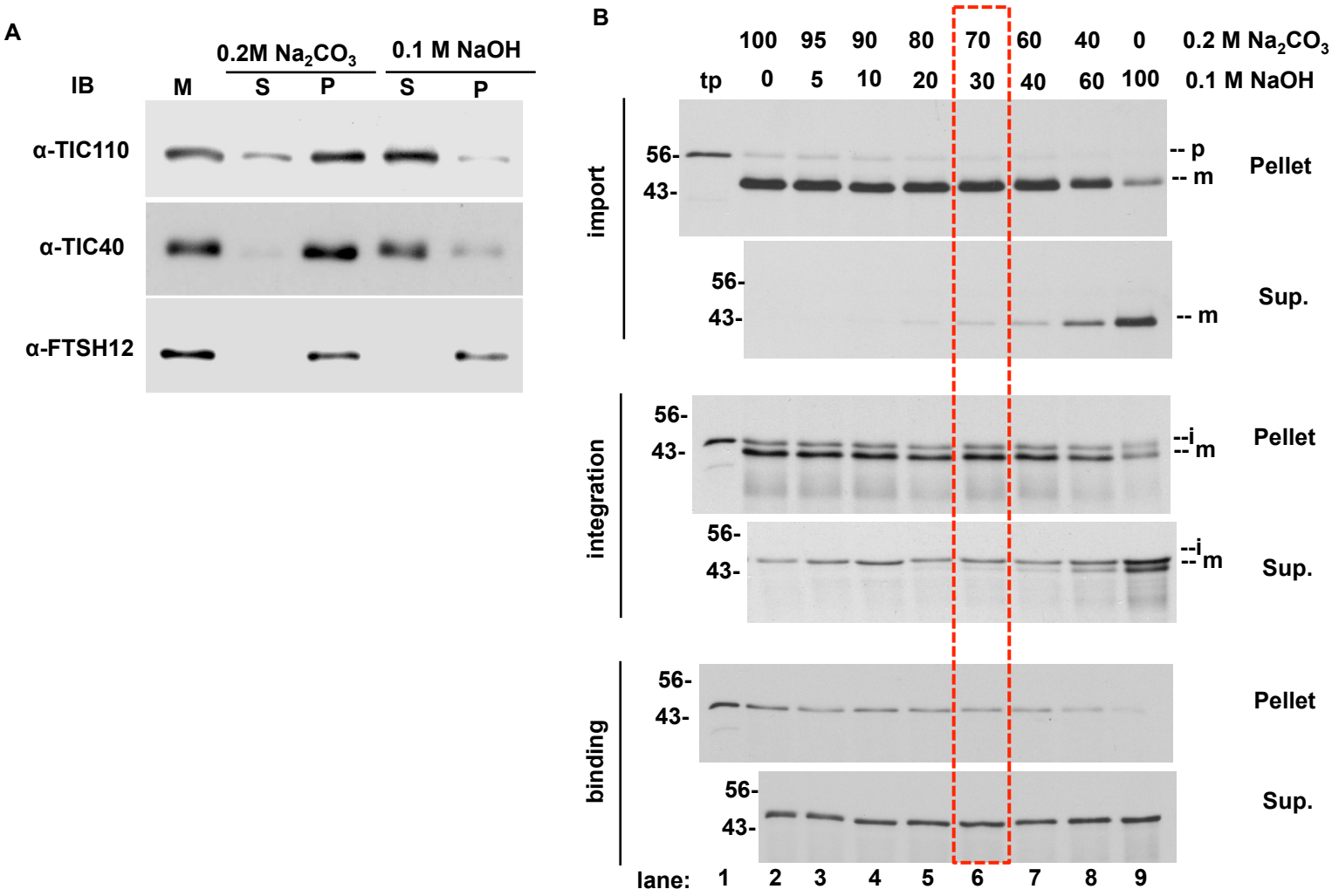

Figure S5. 30% NaOH/70% Na_2CO_3 can be used for diagnosis of TIC40 integration.

A. Membrane fractions (M) of Arabidopsis chloroplasts were isolated and extracted with 0.2 M Na_2CO_3 or 0.1 M NaOH. The resulting supernatant (S) and pellet (P) were subjected to SDS-PAGE and immunoblotting with anti-TIC110, anti-TIC40 or anti-FTSH12 sera. **B.** Extraction of membrane bound or integrated TIC40. **Top panels**: *In vitro* translated preTIC40 was incubated with pea chloroplasts and 2.5 mM ATP in import buffer for 30 min in light at 25°C. Intact chloroplasts were repurified and lysed with 10 mM Hepes-KOH, readjusted to import buffer, and membranes recovered by micro centrifugation at top speed for 20 min. Equal aliquots were mixed 150 µL of different combinations of 0.2 M Na_2CO_3 and 0.1 M NaOH for 30 min on ice. Membranes were recovered by ultra centrifugation and proteins of the supernatant were recovered by 20% TCA precipitation. **Middle panels**: *In vitro* translated iTIC40 was mixed with chloroplast lysate, a 10x excess of SE, and 2.5 mM ATP for 50 min in light at 25°C. Membranes were recovered by centrifugation and extracted with alkali as described above. Samples were analyzed as in the upper panel. **Lower panels**: In *vitro* translated iTIC40 was incubated as above (middle panel) except that ATP was omitted and that the translation mixture, lysate, and 10x SE were pretreated with apyrase for at least 20 min before transfer of reactions to 25°C and light. Extraction was as above. The positions of preTIC40, iTIC40, and mature TIC40 are designated to the right of the panels as p-, i-, and m-, respectively.

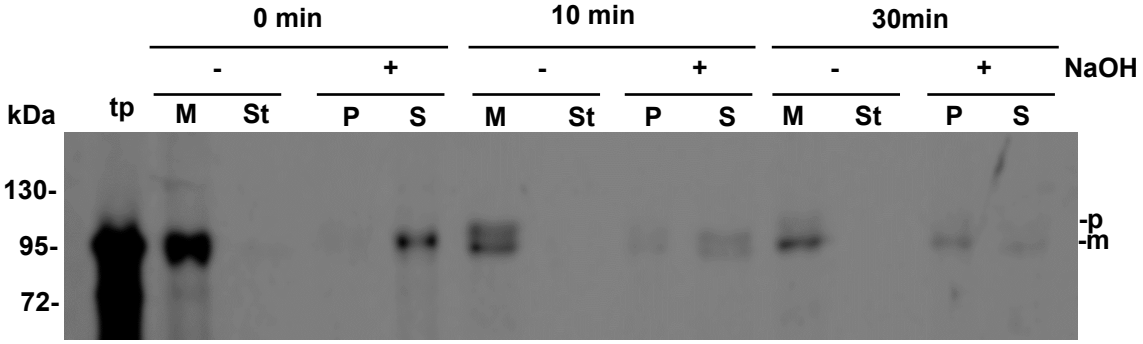

Figure S6. Membrane integration of FTSH12 is time dependent.

In vitro translated full length FTSH12 was diluted and incubated with intact pea chloroplasts in darkness on ice for 15 min as described in Methods. After incubation, chloroplasts were pelleted at 1000 x *g* for 5 min at 2°C and hypotonically lysed with 10 mM Hepes-KOH pH 8.0 at 2 mg mL^{-1} of Chl on ice in darkness, then adjusted to 1 x IB. The lysate was divided into 3 aliquots. One (0 min) was immediately centrifuged at 100,000 x *g* for 20 min at 2°C to separate membrane (M) and stroma (St) fractions. The stroma fractions were precipitated with 20% TCA and the membrane fraction was extracted with 0.1 M NaOH. Then the NaOH resistant pellet (P) and NaOH extracted soluble (S) were separated and all samples were analyzed with SDS-PAGE and fluorography as described in Methods. The other two aliquots of the lysate were supplied with 5 mM ATP, 10 x SE and incubated at 25°C for 10 and 30 min in light, respectively. After incubation, both samples were processed in the same way as for the 0 min sample. p: precursor; m: mature form.

Li et al. Figure S7

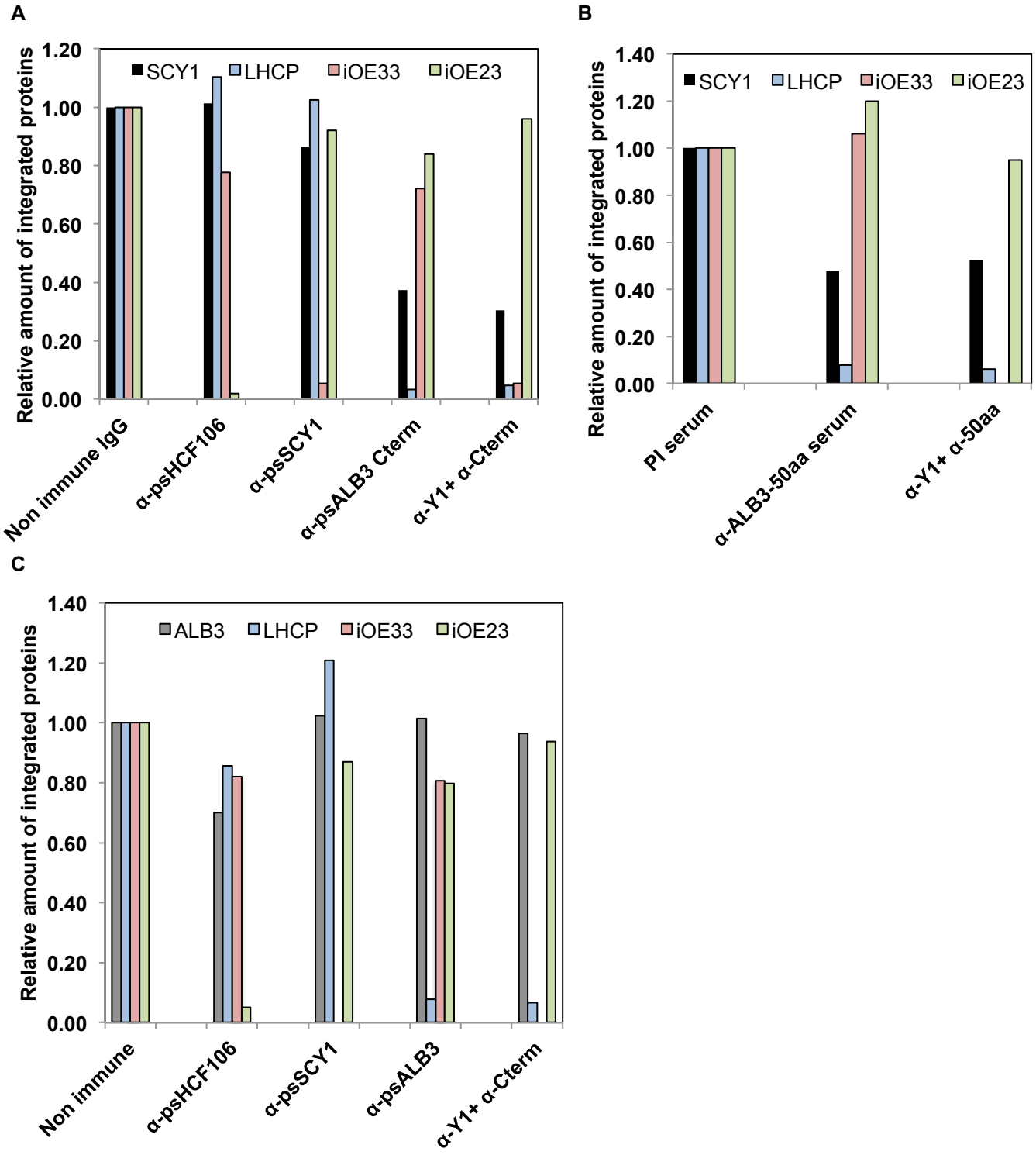

Figure S7. Quantitative analysis of integrated SCY1 and ALB3 in antibody inhibition assays

The signals of SCY1-DP, ALB3-DP, LHCP-DP, mOE23 and mOE33 in thermolysin-treated membrane samples recovered from antibody inhibition assays (Fig.10) were quantified from scanned X-ray films by densitometric analysis with Quantity One software (Bio-Rad) and normalized to related control samples (non immune IgG for **(A) and (C)** and pre-immune serum for **(B)**).

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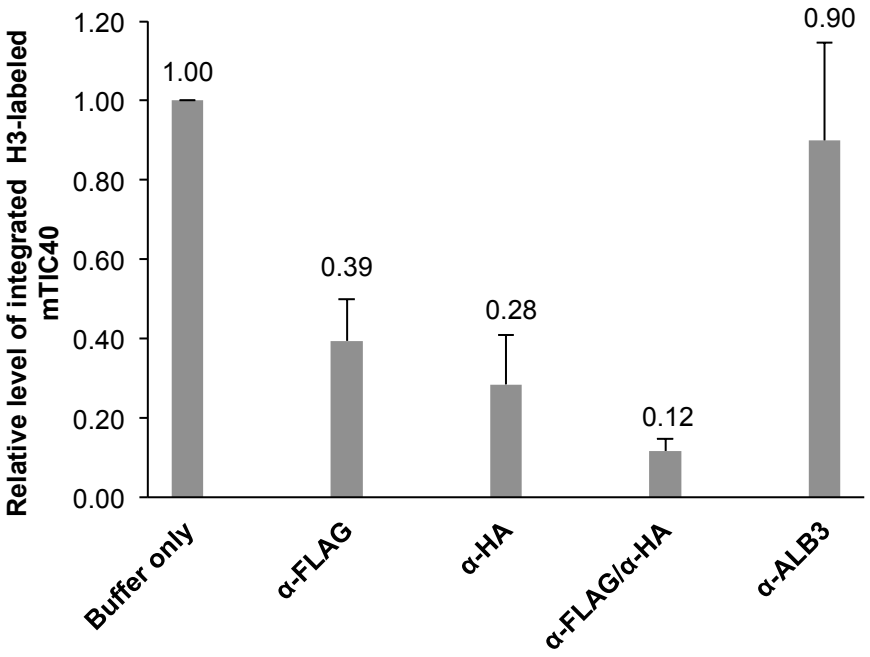

Figure S8. Quantitation of membrane integrated mTIC40 in antibody inhibition assays.

Fluorogram signals of mTIC40 in pellet fractions recovered from OH^-/CO_3^{2-} extraction in antibody inhibition assays were quantified from scanned x-ray films by densitometric analysis with Quantity One software (Bio-Rad). Each bar presents the mean value (numbers on the top of each bar) of three independent experimental results that were normalized to control samples (Buffer only). Error bar: s.d..

Table S1. Primers used in this study

Primers for Hairpin RNA construction

Name	**Sequence (5' to 3')** The Gateway recombination site and corresponding sequence are underlined.
Outside cpSecY forward	GCATGATTGATGATGGTTGC
cpSecY SOE reverse	GCAACTTTGGATAGACTTGAGC
cpSecY SOE forward	GCTCAAGTCTATCCAAAGTTGC
Outside cpSecY reverse	GCGAGGCATAATTGAGCGG
B1-cpSecY forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTCT TCAGCTGC TATTGAGGACAGTTCC
B5r-cpSecY reverse	GGGGACAACTTTTGTATACAAAGTTGCCTGA AAAACTTT GCTTGTTAGACTATATAAGCATACC
B5-cpSecY forward	GGGGACAACTTTGTATACAAAAGTTGCTGAA CATATACT ATCCCGAGTACCAAGAGG
B2-cpSecY reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCT TCAGCTGC TATTGAGGACAGTTCC
B1-cpSecY2 forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTGT GTTTTGAC CTTGATAATGTTTTTGCAGGAGACCC
B5r-cpSecY2 reverse	GGGGACAACTTTTGTATACAAAGTTGCTCAC CCAGTTCC TCTATCAAAAACATAAAATGTTAACTACAAAT AC
B5-cpSecY2 forward	GGGGACAACTTTGTATACAAAAGTTGATGAG AACACTC ACCAACAGAACCCGAGACAAAGC

Primers for RNAi plant screening

Name	**Sequence (5’ to 3’)**
MDC7 forward	CGCTGAAGCTAGTCGACTCTAGC
MDC7 reverse	CGAAAGCTGGGAGGCCTGGATCG

cpSecY hairpin screen forward	GGTATGCTTATATAGTCTAACAAGC
cpSecY hairpin screen reverse	GCTTGTTAGACTATATAAGCATACC
cpSecY2 hairpin screen forward	GATAGAGGAACTGGGTGAG
cpSecY2 hairpin screen reverse	CTCACCCAGTTCCTCTATC

Primers for qRT-PCR

Name	**Sequence (5' to 3')**
Ubiquitin10 qRT-PCR forward	GGCCTTGTATAATCCCTGATGAATAAG
Ubiquitin10 qRT-PCR reverse	AAAGAGATAACAGGAACGGAAACATAGT
cpSecY2 qRT-PCR forward	GCTGGAATGCAACCTGTTCTC
cpSecY2 qRT-PCR reverse	AGGTGAACCCAGAATACTTGCAA
cpTatC qRT-PCR forward	CAACGCCGGAGCAAAGG
cpTatC qRT-PCR reverse	TGGTGAATCGTCGTCATTGAG
TIC40 qRT-PCR forward	AAGAGGTAATGGATGTGTTCAACAAG
TIC40 qRT-PCR reverse	GCTTTTTCAACCCGTCATTCC
TIC110 qRT-PCR forward	CATTTCTTCTGGAGTGGATGGTT
TIC110 qRT-PCR reverse	AGACATGGCAGTCTCTCTGGATAA
TOC75-III qRT-PCR forward	ACCTCTAGCCGTAGCCTCAGTCT
TOC75-III qRT-PCR reverse	CAGAACCGACGGAAGATTCG
cpSecY1 qRT-PCR forward	CGGACGACGTGAGTGAACAA
cpSecY1 qRT-PCR reverse	CAGGTCGGACTAGAGGGATTGA
FtsH12 qRT-PCR forward	TGAGGCTGGTCATATAGTGTTGGC
FtsH12 qRT-PCR reverse	ACCGATACAGCAGTTTCCTTGC

Primers for gene cloning

Name	**Sequence (5' to 3')**
AtTic40-1F	GCGAATTCATGGAGAACCTTACCCTAGTTTC

AtTic40-1R	GCGGGTACCTCAACCCGTCATTCCTGGGAAG
FtsH12-1F	GCGAATTCATG GAG ATT GCA ATT TCG TAT AAA C
FtsH12-1R	GCGTCGACCTAGCTTCTGTGGAGTGGC
AtAlb3-1F	CGC GAA TTC ATG AGT GGA GTA GGA GGA GGA GGA A
AtAlb3-1R	CGC TCT AGA CTA TAC AGT GCG TTT CCG CTT CG
AtAlb3-2F	CGG AAT TC ATG T TCA GCT TAA ACG AGA TTC CTC CTT TC
AtTic110-1F	GAGGTACCATGAATCCCTCACTCGTCACCGCC
AtTic110-His-R	GCGTCTAGATTACACCACCACCACCACCACAAAGACGAAATTGCCCTCTTCTGC
AtFtsh12-TPdel-EcoRI	GCTTATCTCTTCAGAATTCGCCATGGTGAAAAGATCAAAAAGTTTTGG
AtcpScY1-delTP-EcoRI5’	CTTCTTCTTCAGAATTCGCGATGGCCTCTCGTCTCAATCACAAATC
iTic40Xba5’	CCTAATATTTCTAGAATGGCCTCCAAAATATCTGCCTCTGC