Supplemental Methods

Schematic of cassette components and clones used

Restriction sites used: SpeI, BglII, and XbaI
SpeI: ACTAGT encodes amino acid sequence **TS**
BglII: AGATCT encodes amino acid sequence **RS**
XbaI: TCTAGA encodes amino acid sequence **SR**

XbaI and SpeI recognition sites have compatible cohesive ends and create a hybrid site when ligated. The hybrid site TCTAGT encodes amino acid sequence **S**S.

Unstructured linker series:
GS3: TS$(GGGGS)_1(GGGGS)_2(GGGGS)_3$RS

GS9: TS$(GGGGS)_1(GGGGS)_2(GGGGS)_3(GGGGS)_4(GGGGS)_5(GGGGS)_6(GGGGS)_7(GGGGS)_8(GGGGS)_9$RS

GS15: TS$(GGGGS)_1(GGGGS)_2(GGGGS)_3(GGGGS)_4(GGGGS)_5(GGGGS)_6(GGGGS)_7(GGGGS)_8(GGGGS)_9(GGGGS)_{10}(GGGGS)_{11}(GGGGS)_{12}(GGGGS)_{13}(GGGGS)_{14}(GGGGS)_{15}$RS

Locations of changes made to pOE17:
Native transit peptide/mature domain junction AA: M…*SQAARA*/ETVKTIKIGA…AKLG*
Transit peptide (Tp moiety): ***M…SQAARA***/TS
N-terminal of mature domain (mOE17 moiety): RSETVKTIKIGA…AKLG*
C-terminal of mature domain (pOE17 moiety): **M…SQAARA/.....LGDVLAKLG**TS

Location of changes made to protA:
Original pS/ProtA sequence: M……QCIDSGGVTPAANA/AQHDEAQQN…*
N-terminal of protA mature domain (protA moiety): RSAQHDEAQQN…*

/: denotes site of signal peptidase processing cleavage
*: denotes stop codon of mature domain
...: denotes continuation of native sequence between amino acid residues shown

Tp-protA:
M…SQAARA/GTQCIDSGGVTPAANA/ AQHDEAQQN…*

Tp-GS_n-mOE17 series:
M…SQAARA/TS$(GGGGS)_n$RSETVKTIKIGA…*

Tp-GS15-47AA Stop:
M…SQAARA/TS$(GGGGS)_{15}$RSETVKTIKIGAPPPPSGGLPGTLNSDQARDFDLPLKERFYLQPLPPA*

Tp-GS_nProtA series:
M…SQAARA/TS$(GGGGS)_n$RSAQHDEAQQN…*

pOE17-GS_n-protA series:
M…SQAARA/.....LGDVLAKLGTS$(GGGGS)_n$RSAQHDEAQQN…*

pOE17-GS_n-mOE17 series:
M…SQAARA/.....LGDVLAKLGTS$(GGGGS)_n$RSETVKTIKIGA…*

pOE17-GS15-mOE17-G3-mOE17:
M…SQAARA/.....LGDVLAKLGTS$(GGGGS)_{15}$RSETVKTIKIGA…AKLGSS$(GGGGS)_3$RSETVKTIKIGA…AKLG*

Tp-GS15-mOE17-G3-mOE17:
M…SQAARA/TS$(GGGGS)_{15}$RSETVKTIKIGA…AKLGSS$(GGGGS)_3$RSETVKTIKIGA…AKLG*

Supplemental Table I

precursor	est length (nm)	est area $(nm)^2$	completed transport (%) mean	SEM
Tp-protA	18.5	55.6	40.9	9.5
Tp-GS3-protA	19	55.94	52.5	6.9
Tp-GS9-protA	29	62.2	10.9	4. 6
Tp-GS15-protA	39	68.5	4.2	2.9
pOE17	3.9	21.2	100	3.9
Tp-GS3-mOE17	8.9	24.3	100	11
Tp-GS9-mOE17	18.5	30.6	95.3	11.7
Tp-GS15-mOE17	28.9	36.9	90.6	4.3
Tp-GS15-$(mOE17)_2$	38.8	61.2	56.3	12.4

Values used for the plot in Fig 6 B

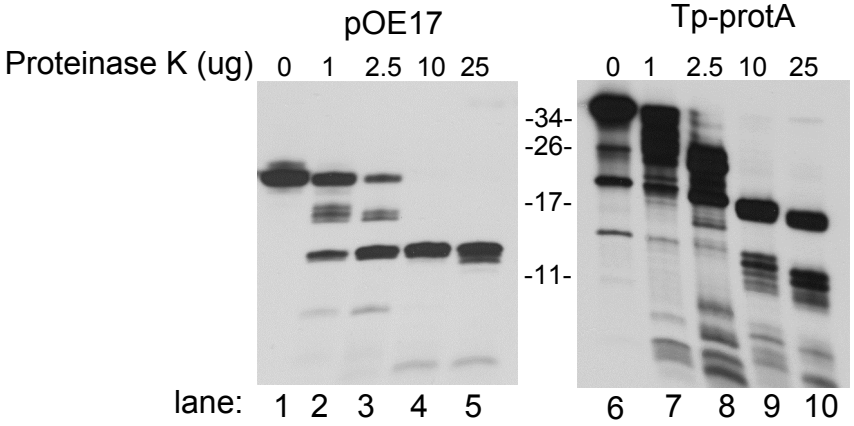
Supplemental Table II

precursor	est area $(nm)^2$	competed transport (%)
pOE17-GS3-mOE17	45.5	84
pOE17-GS9-mOE17	51.8	79
pOE17-GS15-mOE17	58.1	69
pOE17-GS15-$(mOE17)_2$	69.8	8
pOE17-GS3-protA	77.14	0
pOE17-GS9-protA	83.4	0
pOE17-GS15-protA	89.7	0

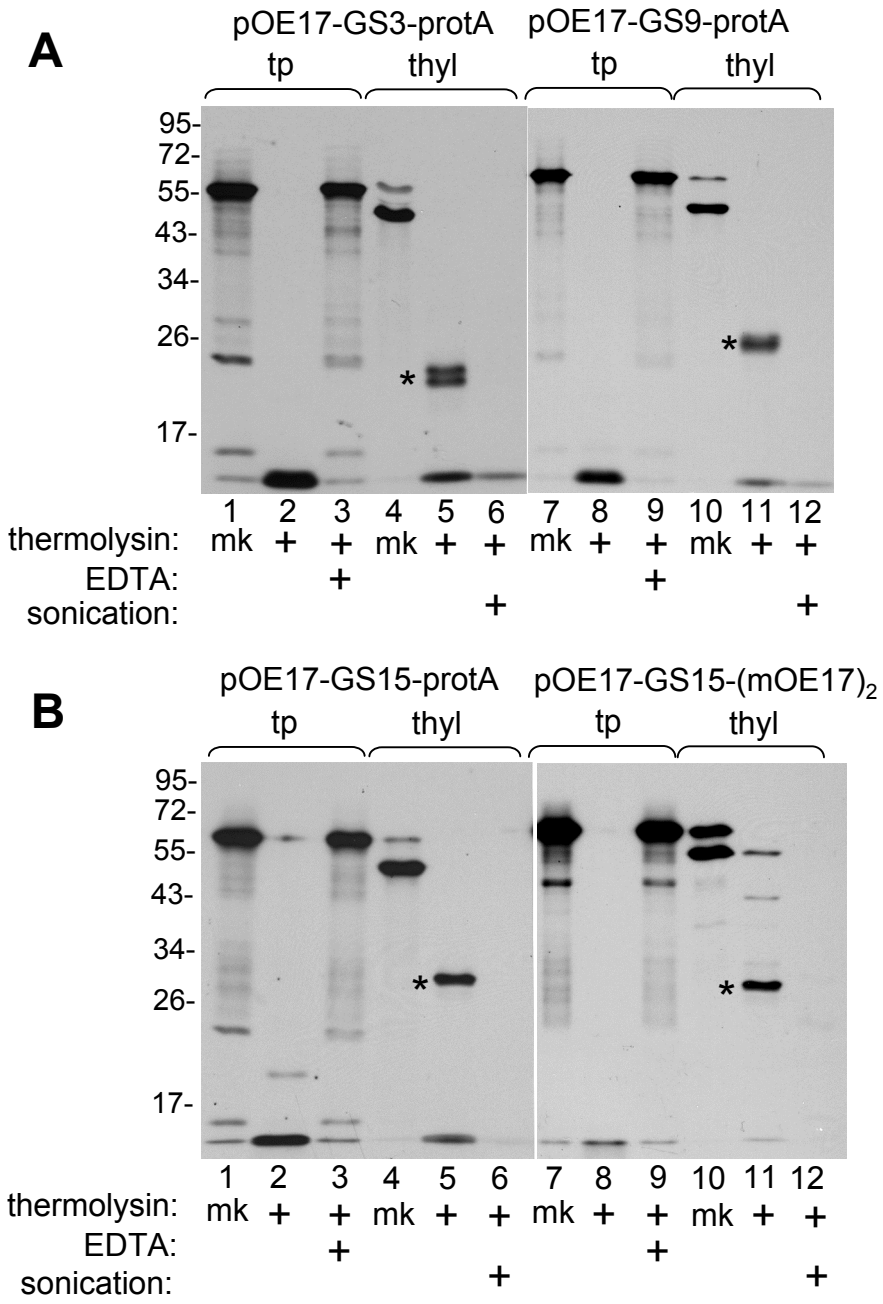
Values used for the plot in Fig 6 C

Supplemental References

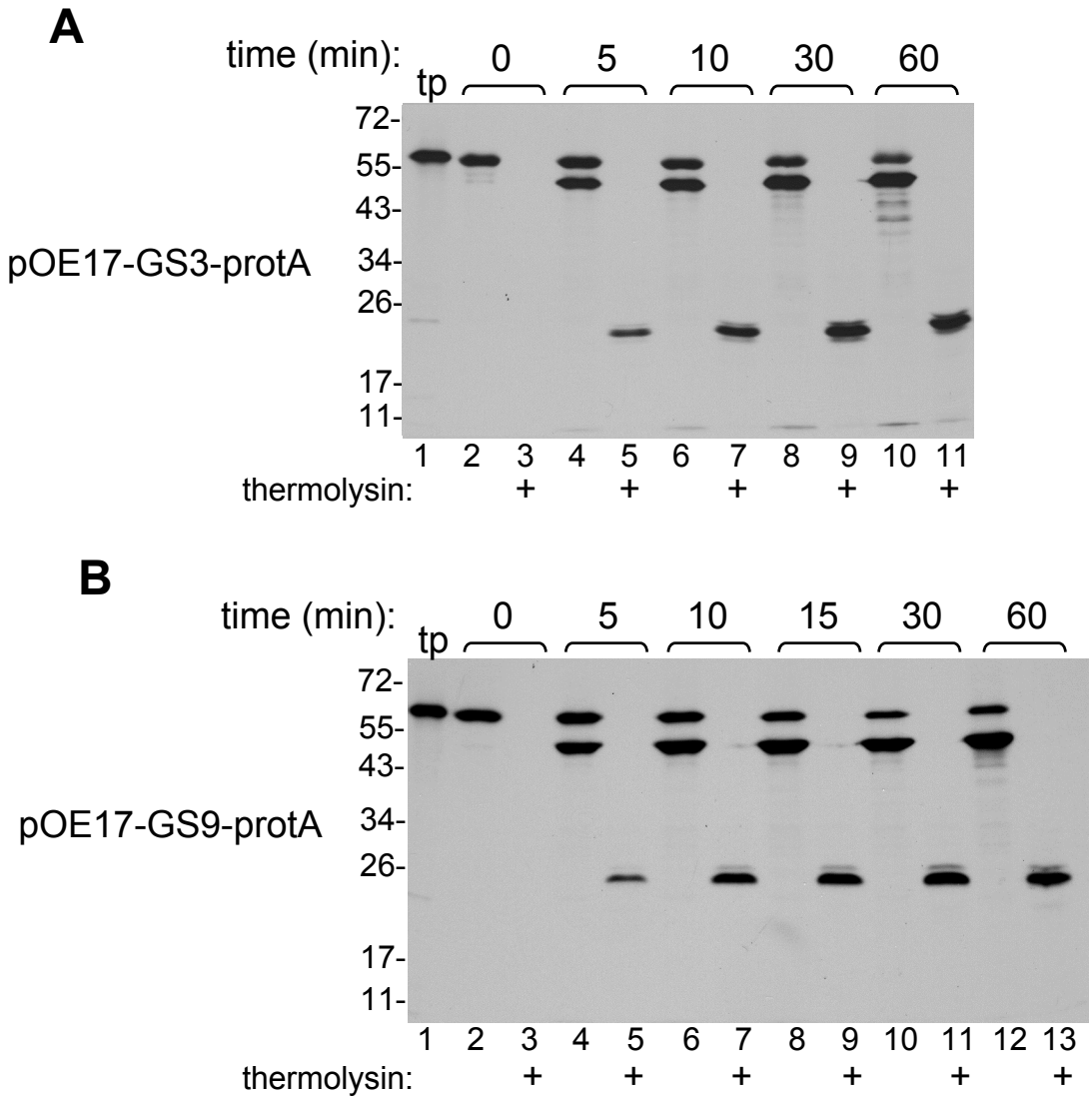
Dabney-Smith, C., Mori, H. and Cline, K. (2003) Requirement of a Tha4-conserved transmembrane glutamate in thylakoid Tat translocase assembly revealed by biochemical complementation. *J Biol Chem*, **278**, 43027-43033.



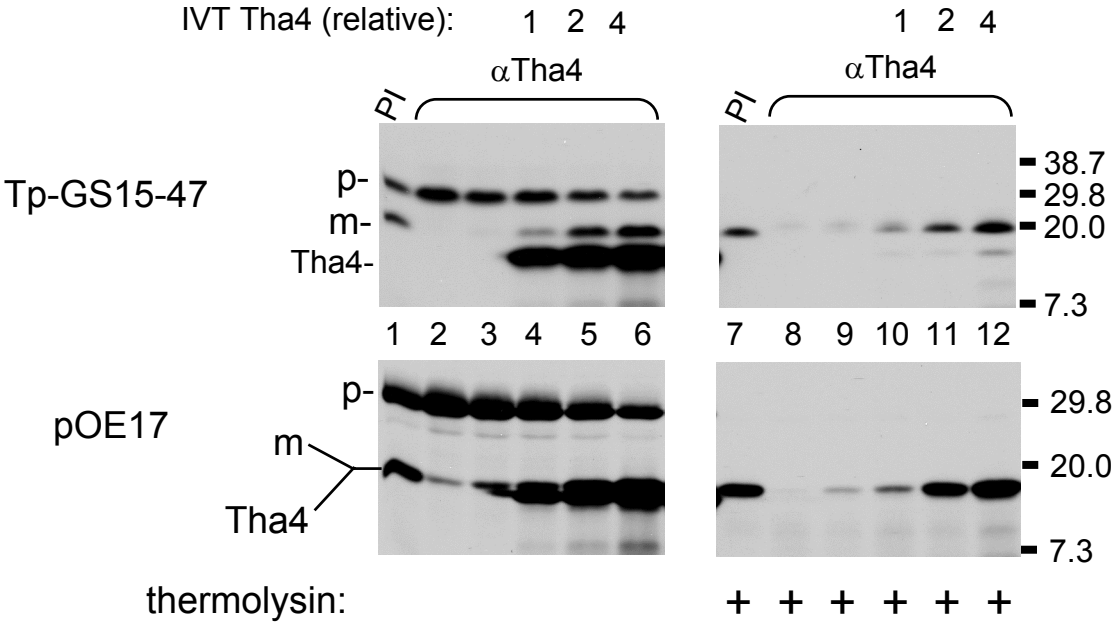
Supplemental Fig 1. **In vitro translated pOE17 and Tp-protA are folded as assessed by partial resistance to proteinase K.** pOE17 and Tp-protA were translated with a wheat germ TnT system in the presence of [^{3}H] leucine and were subsequently diluted with one volume of 60 mM unlabeled leucine in 2X IB and 2 volumes of IB. Twenty µl aliquots were treated with varying amounts of proteinase K, dispensed from a 2 mg/ml stock solution in IB as shown above the panels. Reactions were for 15 min at 25 ^{0}C and were terminated with 1 volume of freshly prepared 4 mM PMSF. After 10 min on ice, one volume of 95 ^{0}C 2X SDS sample buffer was added and the samples immediately heated for 4 min at 95 ^{0}C. Samples were analyzed on a 16 % Tris-Tricine SDS polyacrylamide gel.



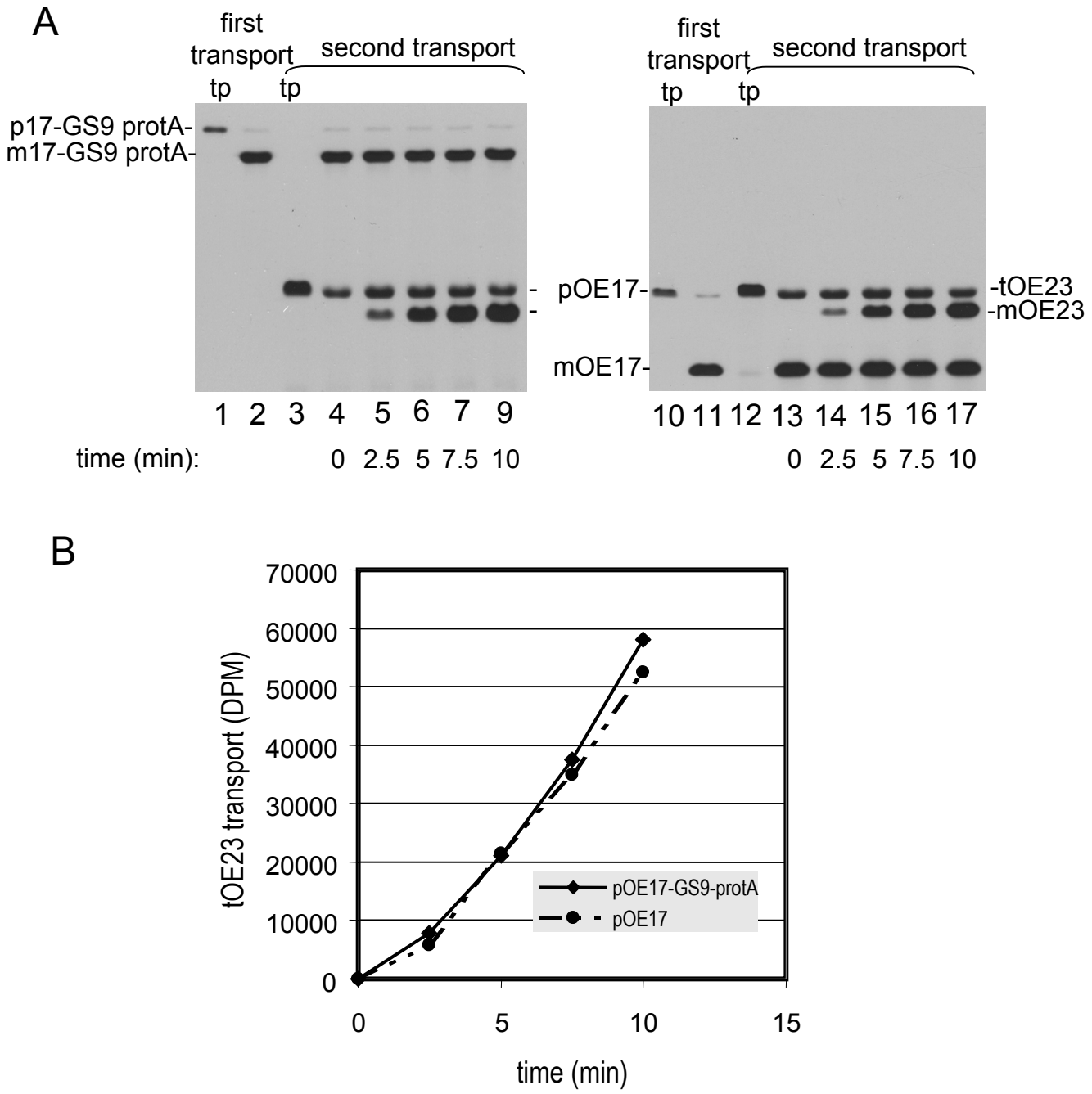
Supplemental Fig 2. **p- or m-OE17-GS-protA and p- or m-OE17-GS15-$(mOE17)_2$ are completely degraded by thermolysin without protection by the thylakoid bilayer.** In vitro translated precursors were diluted with one volume of 60 mM leucine 2X IB. An aliquot of each precursor was used for a 110 µl thylakoid transport assay with chloroplast lysate (Materials and Methods). The remaining precursor was diluted 3-fold with IB and 25 µl aliquots were incubated at 4 ^{0}C without (lanes 1, 7) or with 20 µg of thermolysin for 50 min (lanes 2, 8). Proteolysis was terminated with 5 µl of 500 mM EDTA and samples denatured with an equal volume of 100 ^{0}C 2X SDS sample buffer for 4 min. As a control for potential proteolysis during denaturation, one untreatreated aliquot received 20 µg of EDTA-inhibited thermolysin immediately before SDS-denaturation (lanes 3, 9). Thylakoids from the transport assay were resuspended in three 25 µl aliquots in IB and either mock treated (lanes 4, 10), treated with 20 µg of thermolysin (lanes 5, 11), or treated with thermolysin in a bath sonicator for 50 min at 4 ^{0}C (lanes 6, 12). Proteolysis was terminated and samples denatured as above.



Supplemental Fig 3. **Partially transported mOE17-GS3-protA and mOE17-GS9-protA are not intermediate stages but are end products of the transport reaction.** In vitro translated precursors were mixed with washed thylakoids, divided into 150 µl aliquots and warmed to 25 ^{0}C. Transport reactions were initiated with light and terminated at the times indicated above the panels with 700 µl of cold IB containing 0.75 µM nigericin and 1.5 µM valinomycin. Recovered thylakoids were divided into two aliquots. One aliquot was washed with IB and the other treated with thermolysin as shown below the panels.



Supplemental Fig 4. **Tp-GS15-47 requires Tha4 for transport similar to the natural Tat substrate pOE17**. Thylakoids at 0.33 mg chorophyll per ml were preincubated either with 0.5 mg/ml of either preimmune IgG (PI; lane 1, 7) or anti-Tha4 IgG (α-Tha4; lanes 2-6, 8-12) at 0^0 C for 45 min exactly as described (Dabney-Smith et al., 2003) and shown above the panels. After washing, thylakoids received protein A and were then incubated with *in vitro* translated [^{3}H]-Tha4 (lanes 4-6, 10-12), with buffer (lanes 2, 8), or with 8 μM Tha4 antigen (lanes 3, 9) which serves as a control for competitive release of antiTha4 (Dabney-Smith et al., 2003). Recovered thylakoids were then assayed for transport of Tp-GS15-47 (upper panels) or pOE17 (lower panels) for 15 min at 15 ^{0}C. Thylakoids recovered from the transport assays were either analyzed directly (left panels) or treated with thermolysin before analysis by SDS-PAGE and fluorography. Transport is indicated by the appearance of the mature form of each substrate (left panels), which is protected from thermolysin treatment (right panels). Designations: p, precursor; m, mature form; Tha4, the integrated in vitro translated Tha4, which co-migrates with mOE17, but which is completely degraded by thermolysin. The relative amounts of Tha4 of 1, 2, and 4 shown above the panels correspond to dilution of the in vitro Tha4 translation mixture used for integration 16, 8 and 4-fold, respectively.



Supplemental Fig 5. **Time course of tOE23 transport by membranes from transport assays with pOE17-GS9-protA or pOE17.** A. Chloroplast lysate was incubated with in vitro translated pOE17-GS9-protA (lane 1, left panel) or pOE17 (lane 10, right panel). After 20 min in the light, each assay was supplemented with additional precursor and incubation continued for 15 min. Thylakoids were recovered by centrifugation and washed. Aliquots of thylakoids were analyzed directly (lanes 2, 11) or incubated with tOE23 in vitro translated in homemade wheat germ (lanes 3, 12) in a second transport reaction. Aliquots were removed at the times shown and immediately quenched with ice-cold import buffer containing 0.75 μM nigericin and 1.5 μM valinomycin. The amounts transported in the first transport incubation was determined by scintillation counting of extracted gel bands and quantitative immunoblotting to be ~5,0000 molecules of mOE17-GS9-protA per chloroplast equivalent and ~19,0000 molecules of mOE17 per chloroplast equivalent. B. Quantification of the amounts of the mOE23 bands in lanes 4-9 and 13-17 in panel A.