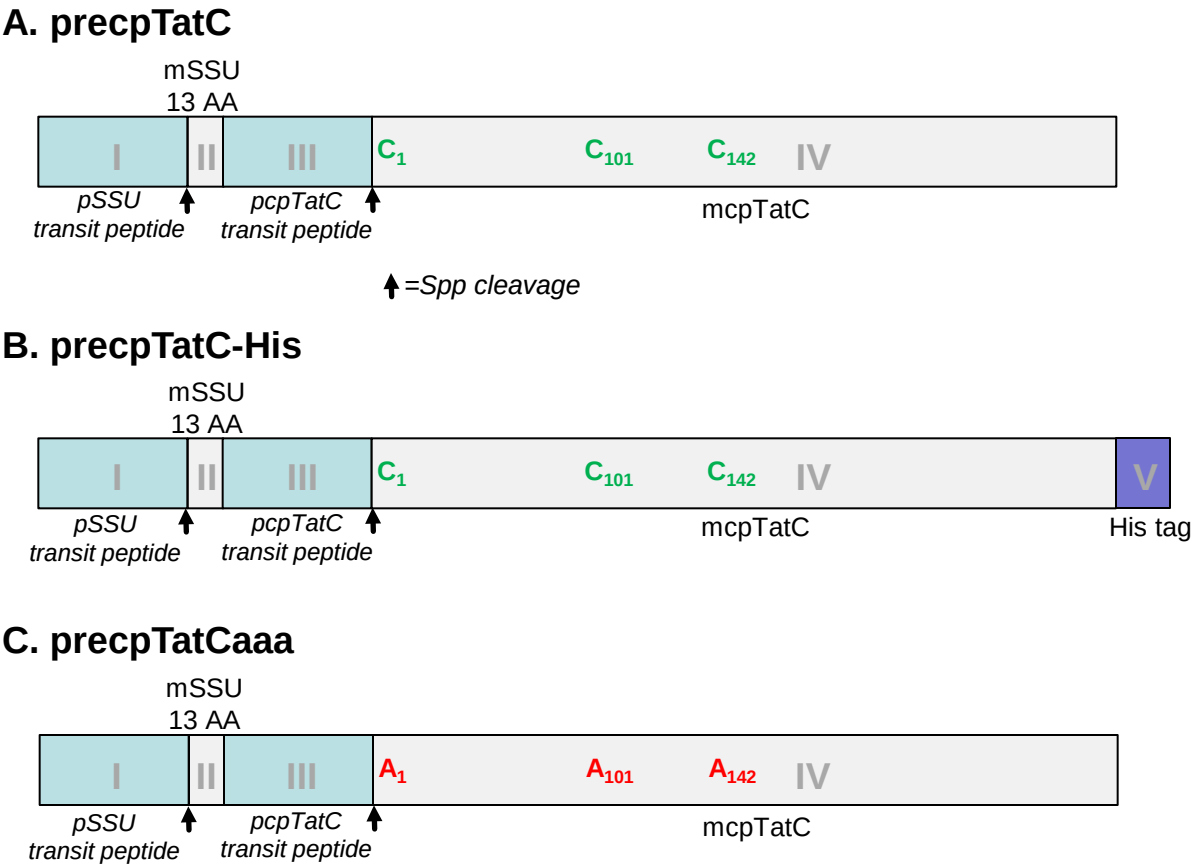
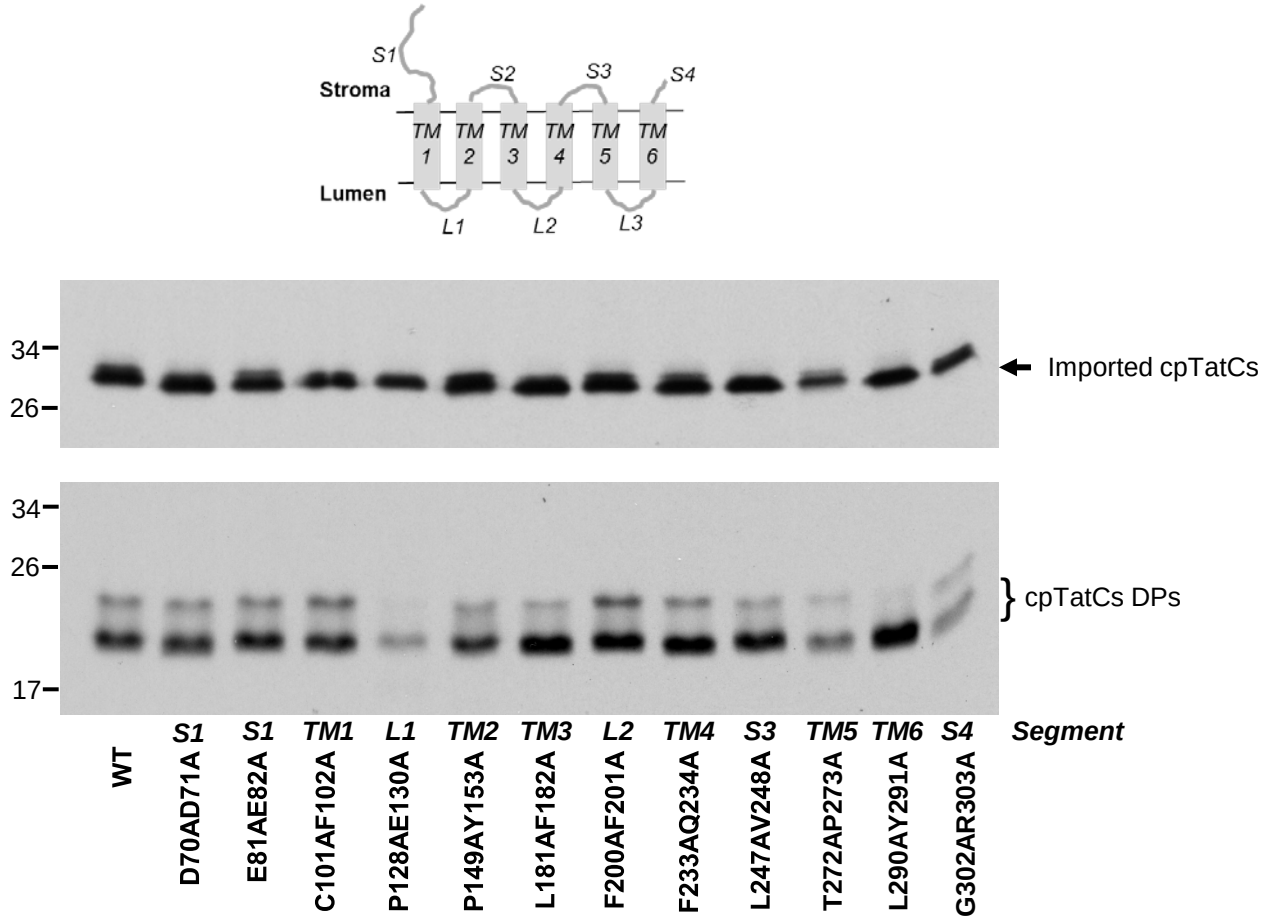


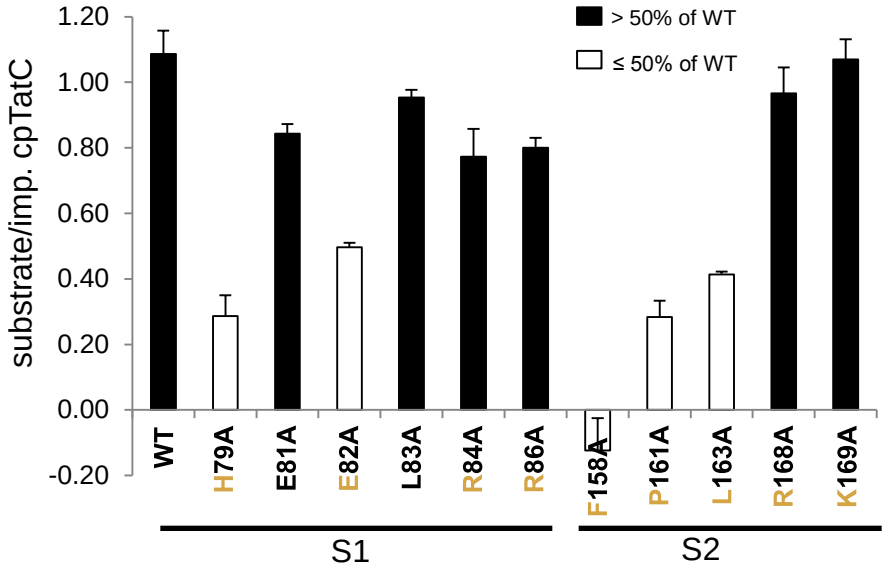
Supplemental Figure 1. The cpTatC precursors in this study contain two transit peptides that are cleaved by the stromal processing peptidase upon import into the chloroplast.

(A) precpTatC was constructed by splicing by overlap extension (SOE) and contains the transit peptide (part I) and first 13 residues of the mature domain (part II) of the pea small subunit of Rubisco fused to the amino terminus of the complete pea cpTatC precursor including its transit peptide (part III) and mature cpTatC (mcpTatC, part IV). Stromal processing peptidase (Spp) cleavage sites are depicted by arrows; cysteine residue positions are also depicted.
(**B**) precpTatC-His was constructed by ligating the His tag sequence GGGGSGGGGSGGGGSHHHHHH (part V) to the carboxyl terminus of the precpTatC.
(C) precpTatCaaa was constructed by replacing the three cysteine residues of the mature cpTatC with alanine residues.



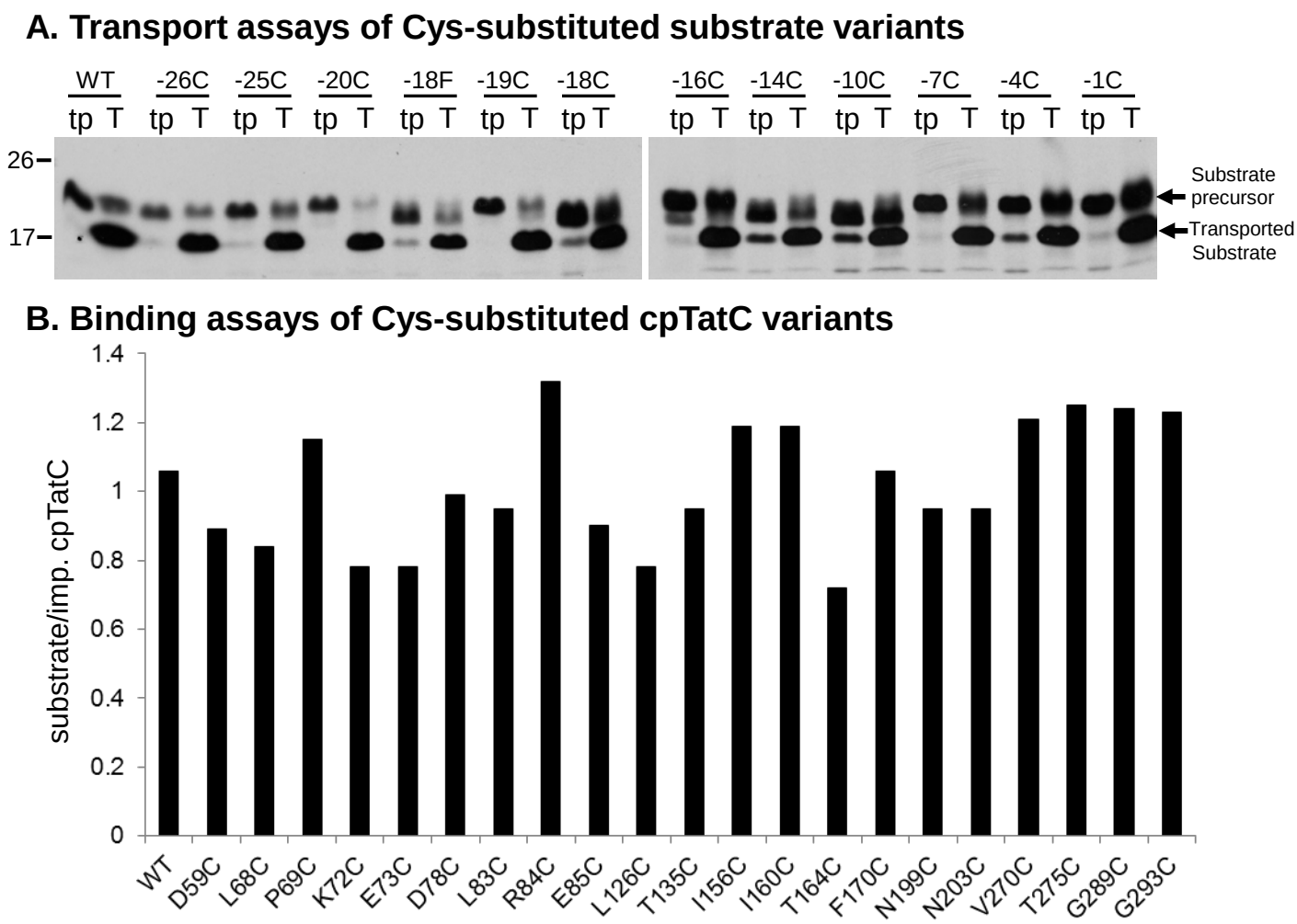
Supplemental Figure 2. cpTatC double alanine mutants are correctly integrated into thylakoids.

Radiolabeled precpTatC with alanine substitutions in different segments as depicted were each incubated with isolated chloroplasts in protein import assays. The recovered membranes were either analyzed directly (top panel) or treated with thermolysin (bottom panel) and then analyzed by SDS-PAGE and fluorography. The mutants were correctly integrated into the thylakoid membrane as they produced the characteristic thermolysin degradation products (DPs), although mutants in L1, TM3, and TM6 were deficient in the upper degradation product.



Supplemental Figure 3. Single alanine mutations in cpTatC segments S1 and S2 exhibit a range of substrate binding capability.

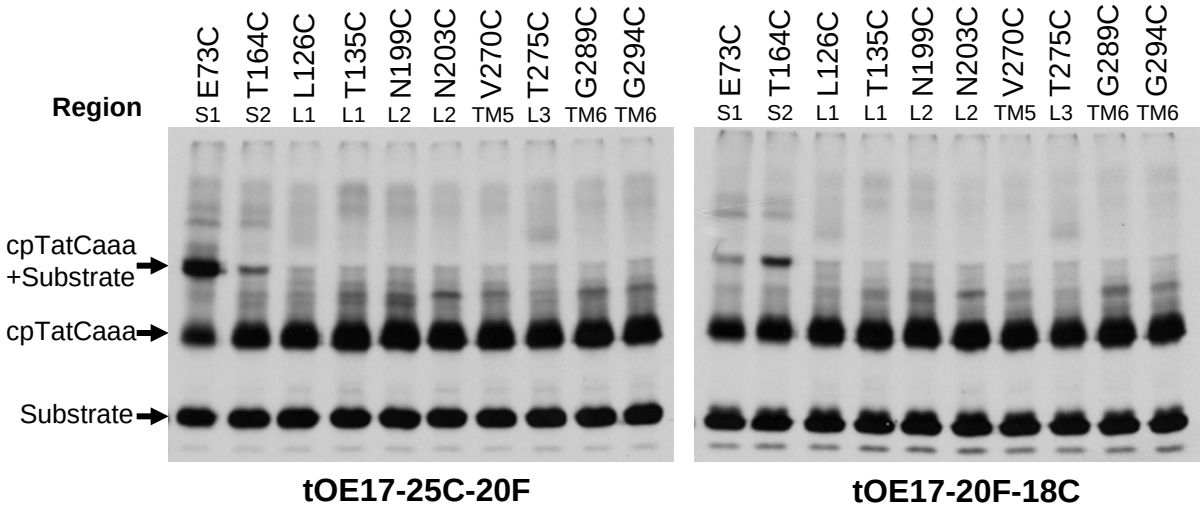
cpTatC single alanine substitutions were made to the selected residues of segments S1 and S2. The radiolabeled precursors to the cpTatC mutants were imported into chloroplasts, with mock and wild type (WT) precpTatC as import controls. Recovered thylakoids were incubated with radiolabeled tOE17-20F substrate for binding assays. Imported cpTatCs and the bound substrate were quantified. The numbers of bound substrate of each imported cpTatC (substrate/imported cpTatC), plotted as determined in Figure 3B, are the average ± SE (n =3). The amino acid position of the substitution is shown under each column; tan colored residues are conserved as determined by SIFT analysis.



Supplemental Figure 4. Cys-substituted substrate and cpTatC variants are functional for transport or binding.

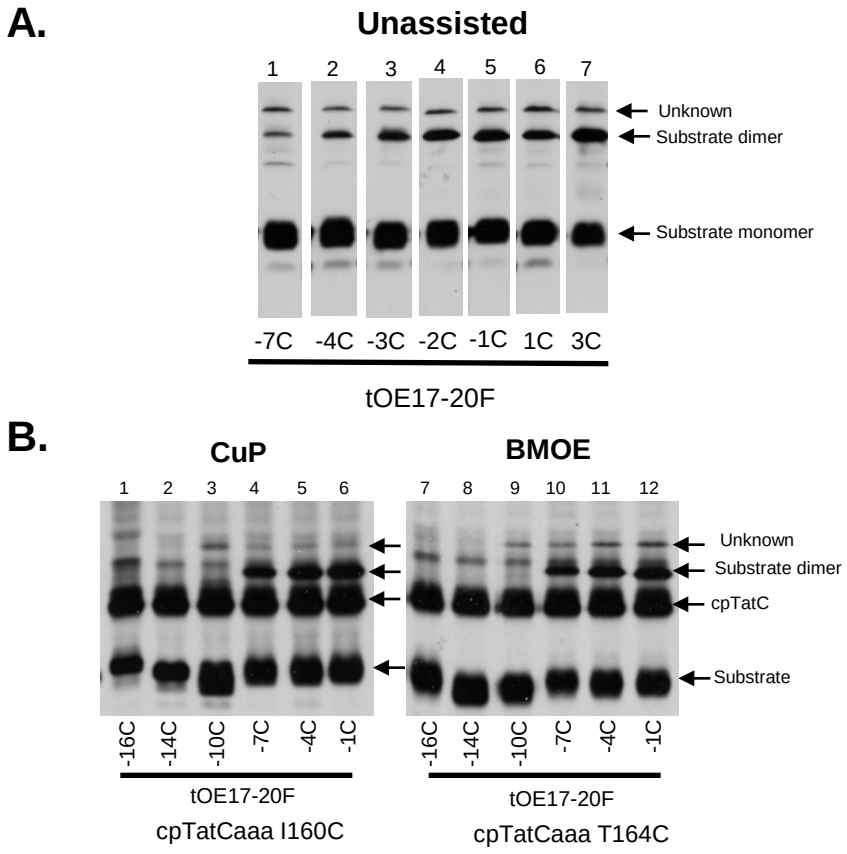
(A) Cysteine-containing variants of the substrate tOE17-20F are functional for protein transport. The tOE17-20F variants are named according to the position of the Cys residue as in Figure 7A and are depicted above the panel. Variant "-18F" has a cysteine substitution at the -20 position and a phenylalanine substitution at the -18 position. Radiolabeled tOE17-20F (WT) and its cysteine-containing variants were each incubated with chloroplast lysates in import buffer with 5 mM Mg-ATP. After incubation in a 25ºC illuminated water bath for 15 min, the thylakoid membranes were recovered by centrifugation and washed with import buffer, and analyzed by SDS-PAGE and fluorography. Lanes tp = *in vitro* translated precursor proteins; lanes T = thylakoids recovered after the transport assay. The upper bands of lanes are the substrate precursors; the lower bands are the transported mature substrates.

(B) All cysteine-containing cpTatC variants used for cross-linking experiments are functional for substrate binding. Single cysteine substitutions were made to non-conserved cpTatC residues. Radiolabeled precpTatC variants were imported into chloroplasts, with mock and wild type (WT) cpTatC serving as import assay controls. Recovered thylakoids were incubated with radiolabeled tOE17-20F substrate for binding assays. Imported cpTatCs and the bound substrate were quantified. The numbers of bound substrate per imported cpTatC (substrate/imported cpTatC) were plotted. The amino acid position of each substitution is shown under each column.



Supplemental Figure 5. No interactions were detected between the signal peptide RR domain and cpTatC segments ,other than S1 and S2, that exhibited substrate binding defects.

The mutations to the conserved residues on the cpTatC regions S1, L1, S2, L2, TM5, L3 and TM6 significantly reduced the binding ability of cpTatC to the substrate (Figure 4B). cpTatC variants with a single cysteine substitution to the non-conserved residues on these regions were each imported into chloroplasts. Recovered thylakoids were incubated with radiolabeled tOE17-25C-20F (left panel) or tOE17-20F-18C (right panel) in binding assays. Washed thylakoids were subjected to cross-linking with BMH. Samples were analyzed by SDS-PAGE and fluorography. The Cys-substituted residues and their cpTatC segment positions are depicted above each lane. The different protein species are indicated to the left of the panels. The results shown that the RR domain of substrate signal peptide only interacted with the amino-terminus (S1) and the first stromal loop (S2) of cpTatC.



Supplemental Figure 6. Bound substrate proteins are in close contact throughout downstream residues of the signal peptide.

(A) tOE17-20F substrate containing a unique cysteine at residue -7, -4, -3, -2, -1, 1, or 3 forms disulfide linked dimers upon membrane binding. *In vitro* translated [^{3}H] substrate was incubated with washed thylakoids in a binding reaction. The membrane samples were obtained by centrifugation and washed with DTT-free buffer. Samples were analyzed by non reducing SDS-PAGE and fluorography. Lanes were from two parallel gels from the same experiment but are cropped for presentation purposes.
(B) Substrate dimer formation with oxidant CuP or the bis-maleimide cross-linker BMOE. The thylakoids with integrated cpTatCaaa I160C or cpTatCaaa T164C were incubated with substrates as shown below the panels for binding assays. Recovered thylakoids were treated with the oxidant CuP to promote disulfide bond formation (left panel) or subjected to cross-linking with BMOE (right panel), and then analyzed by non-reducing SDS-PAGE and fluorography. The different protein species are indicated to the right of the panels.