ABSTRACT

Over 95% of plastid proteins are nuclear-encoded as their precursors containing an N-terminal extension known as the transit peptide (TP). Although highly variable, TPs direct the precursors through a conserved, posttranslational mechanism involving translocons in the outer (TOC) and inner envelope (TOC). The organelle import specificity is mediated by one or more components of the Toc complex. However, the high TP diversity creates a paradox on how the sequences can be specifically recognized. An emerging model of TP design is that they contain multiple loosely conserved motifs that are recognized at different steps in the targeting and transport process. Bioinformatics has demonstrated that many TPs contain semi-conserved physicochemical motifs, termed FGLK. In order to characterize FGLK motifs in TP recognition and import, we have analyzed two well-studied TPs from the precursor of RuBisCO small subunit (SStp) and ferredoxin (Fdtp). Both SStp and Fdtp contain two FGLK motifs. Analysis of large set mutations in these two motifs using in vitro, in organello, and in vivo approaches support a model in which the FGLK domains mediate interaction with TOC34 and possibly other TOC components. In vivo import analysis suggests that multiple FGLK motifs are functionally redundant. Furthermore, we discuss how FGLK motifs are required for efficient precursor protein import and how these elements may permit a convergent function of this highly variable class of targeting sequences.

Key words: chloroplast biology, protein translocation, Toc34, transit peptide, cell biology

INTRODUCTION

Eukaryotic organisms are defined by their compartmentalization and various organelles. The membranes that define these organelles present a barrier to the selective translocation of proteins from the cytosol into their functional location in the organelle. In plant cells, the plastid represents one of the most complex systems of protein sorting, requiring several translocons located in the three membranes found in this organelle (Schnell et al., 1990; Bruce, 2000; Schleiff et al., 2003; Li et al., 2007; Jarvis, 2008; Andres et al., 2010). The vast majority of plastid-localized proteins are nuclear encoded and must be post-translationally...
Functional Analysis of Transit Peptide Motifs

Molecular Plant

imported from the cytosol. These proteins are encoded as precursors with an N-terminal sequence of approximately 55 amino acids, called a transit peptide (TP), which is required for efficient recognition and import of target proteins into the chloroplast (Andres et al., 2010; Bruce, 2000, 2001; Chotewutmontri et al., 2012; Dobberstein et al., 1977; Li et al., 2007; Schmidt et al., 1979).

Although it has been well established that the TP is required for recognition and import of precursor proteins by the Translocon of Outer Chloroplast (TOC) membrane and Translocon of the Inner Chloroplast (TIC) membrane, neither the mechanistic details of this interaction nor how this interaction promotes organelle-specific recognition are well understood. The mechanism by which proteins are targeted to the chloroplast has been largely determined by careful analysis of \textit{in vitro} import assays using purified chloroplasts (Keegstra, 1989; Keegstra and Cline, 1999). Various models have shown that translocation can happen coordinately using ATP hydrolysis or in distinct steps across each envelope membrane (Scott and Theg, 1996; Liu et al., 2014). It is widely hypothesized that the primary targeting step providing organelle specificity is via interactions between the TP and the TOC receptors, TOC34 and TOC159 (Sveshnikova et al., 2000; Jelic et al., 2002; Reddick et al., 2007; Lee et al., 2009; Chotewutmontri et al., 2012). Although it is clear that TPs are highly divergent in sequence and length, a universal model of chloroplast protein import will need to account for a common mechanism(s) allowing highly divergent TPs to be recognized (Bruce, 2000). Previous work from multiple groups has focused on a combination of mutagenesis (Pilon et al., 1995; Lee et al., 2002), deletion (Kindle, 1998; Kindle and Lawrence, 1998; Rensink et al., 1998, 2000), and domain swapping experiments (de Castro Silva Filho et al., 1996) to identify TP functional domains (von Heijne et al., 1989); however, based on TP sequence variation, these results are difficult to extend to TPs in general. Recent work has focused on physicochemical motifs, rather than identification of primary sequence similarity, to identify common modes of TP recognition. We have identified a specific N-terminal TP domain that is required to facilitate interaction with plastid-localized molecular chaperones (Ivey et al., 2000; Chotewutmontri et al., 2012). This motif is followed by a second physicochemical element that interacts with the TOC receptor proteins and appears to promote TP chloroplast binding, but cannot support import alone. This region is termed FGLK, which is loosely defined as (1) an aromatic amino acid, (2) a turn-inducing or helix-breaking amino acid, (3) a small nonpolar amino acid, (4) a basic amino acid, and (5) the absence of negatively charged amino acids Asp or Glu (Pilon et al., 1995; Lee et al., 2009; Chotewutmontri et al., 2012). Thus, previous work suggests that TPs do not share any consensus sequences but contain specific physicochemical elements that allow them to be recognized by a common import mechanism utilizing a discrete set of Toc GTPases, a single Toc75 complex, and a universal requirement for stromal ATP. The properties of TP physicochemical blocks are likely to be context sensitive and may behave differently as a function of pH, membrane-like conditions, or the presence of translocon components (Bruce, 1998; von Heijne and Nishikawa, 1991; Lancelin et al., 1994; Wienk et al., 1999; Chotewutmontri et al., 2012;).

To dissect the contributions of the FGLK domains individually and each of the component amino acids, we have developed a systematic approach using two well-studied TPs. We have generated a comprehensive set of deletion and substitution mutants to evaluate physicochemical elements such as hydrophobicity, flexibility, and charge within the FGLK domain. Our approach includes \textit{in vitro} analysis of the interaction of mutant peptides with the isolated cytosolic GTPase domain of TOC34, and in \textit{organello} assays to measure binding and import of mutant peptides with isolated chloroplasts. We have also developed \textit{in vivo} analyses in onion epidermal cells to assess the ability of the cell to target and import mutant peptides into the plastid under physiological conditions. Overall, our results highlight the ability of a set of loosely conserved physicochemical properties of TPs to maintain both the specificity and promiscuity of the plastid import mechanism.

\textbf{RESULTS}

\textbf{Alignment of SS\text{\textsc{tp}} Reveals Semi-conserved Domains}

Previous research has suggested that semi-conserved physicochemical motifs have an important functional role in the recognition and import of chloroplast targeted proteins (Pilon et al., 1995; Ivey et al., 2000; Lee et al., 2009; Chotewutmontri et al., 2012). To determine the extent of the FGLK domain presence and conservation, we aligned 328 full-length protein sequences of the small subunit of RuBisCO from 120 green plants, shown in Supplemental Table 1 and Supplemental Figure 1. Representative sequence alignment of SS\text{\textsc{tp}} from green algae, moss, rice, Arabidopsis, pea, and tobacco are shown in Figure 1A. Identified consensus sequences from different lineages are highlighted as a logo plot in Figure 1B; the height of the black bar denotes the abundance of the consensus residue, and the height of the letter indicates the abundance of that amino acid in the consensus motif. Our analysis identified two FGLK domains, domain I (DI) and domain II (DII), which are highlighted in gray (Figure 1A–1B). FGLK domains have been implicated in TP import (Pilon et al., 1995; Lee et al., 2009; Chotewutmontri et al., 2012), so we focused our studies on the detailed analyses of this physicochemical element.

\textbf{The Semi-conserved FGLK Domain Is Required for Chloroplast Binding and Import}

To detect the binding of SS\text{\textsc{tp}} to the chloroplast surface, we used a combination of flow cytometry, fluorescent microscopy, and far-western blotting (Subramanian et al., 2001). We used a dual epitope tagged form of SS\text{\textsc{tp}}, denoted H-S-SS\text{\textsc{tp}} (H\text{\textsc{sp}}-S-peptide–SS\text{\textsc{tp}}, expressed recombinantly in \textit{E. coli} and purified; data not shown) to directly monitor the effect of the TP without contributions from the mature domain. Although this construct is able to compete with wild-type (WT) TP for preprotein binding, it cannot be translocated into the stroma (Subramanian et al., 2001). Transit peptides with the dual epitope present still function in chloroplast binding and import completion assays with the same efficiency as unmodified peptides (Subramanian et al., 2001). The three C-terminal deletion mutants, H-S-SS\text{\textsc{tp}}\text{\textsubscript{2ab}}, H-S-SS\text{\textsc{tp}}\textsubscript{2ab}, and H-S-SS\text{\textsc{tp}}\textsubscript{4ab}, have two, one, or zero FGLK motifs remaining in their sequences, respectively (Figure 2A).

We used a previously established flow cytometric analysis (Subramanian et al., 2001) to measure the amount of H-S-SS\text{\textsc{tp}} bound to chloroplast. This assay allows direct fluorescent...
measurement of the amount of H-S-SStp bound to individual chloroplasts by detection of the S-tag of H-S-SStp and using FITC-S protein (Kim and Raines, 1993). One advantage of fluorescence-activated cell sorting (FACS) is that each organelle is individually measured, instead of measuring an average signal that results from a population of organelles. Thus, this assay permits high-throughput detection, allowing a large number of organelles (>10,000) to be analyzed for statistically significant results. In our previous study, we established controls to specifically identify intact chloroplasts and controls for chlorophyll autofluorescence (Subramanian et al., 2001).

Although all three mutants have the H-S motif at the N terminus, only H-S-SStp<sub>D5</sub> binds to chloroplasts similar to the WT construct; the other mutants, H-S-SStp<sub>D25</sub> and H-S-SStp<sub>D36</sub>, are negative for fluorescein isothiocyanate (FITC) staining (Figure 2B). Comparison of the H-S-SStp<sub>D5</sub> and the H-S-SStp<sub>D25</sub> would suggest a key determinant resides between residues G35 and R54; this region contains all of domain II.

We tested the spatial distribution of the bound H-S-SStp on the chloroplast surface using laser scanning confocal microscopy (LCSM) and an S protein FITC conjugate to label intact pea chloroplasts after a binding reaction (30 μM ATP) with H-S-SStp constructs (Figure 2C). Chloroplasts are depicted in red, and green labeling indicates regions of H-S-SStp interaction on the chloroplast surface. We find that labeling of both H-S-SStp<sub>WT</sub> and H-S-SStp<sub>D5</sub> reveals punctate, patch-like labeling. However, H-S-SStp<sub>D25</sub> and H-S-SStp<sub>D36</sub> show no labeling, supporting our FACS analysis.

Chloroplast interaction was further verified using far western blotting of total chloroplast proteins using the S protein alkaline phosphatase conjugates following binding and re-isolation over a Percoll gradient (Figure 2D). Both WT and H-S-SStp<sub>D5</sub> were easily detected, and as expected H-S-SStp<sub>D25</sub> and H-S-SStp<sub>D36</sub> do not show chemiluminescent signal (Figure 2B and 2C). Importantly, these results suggest that removal of TP length and/or removal of the domain II motif prevent productive interaction with the chloroplast outer membrane.

In Vitro Import Competitions Confirm the Involvement of Both FGLK Domains

To specifically test the relative importance of the two FGLK domains in protein import, we have used in organello chloroplast import competition assays. We generated mutants of full-length tobacco RuBisCO precursor (prSSU) where we fully or partially deleted each of the two FGLK regions (denoted DI and DII). We constructed six deletion variants: D<sub>FTGLK</sub> (DI-D5), D<sub>FTG</sub> (DI-N3), D<sub>GLK</sub> (DI-C3), D<sub>FPVSR</sub> (DII-D5), D<sub>FPV</sub> (DII-N3), and D<sub>VSR</sub> (DII-C3). The radiolabeled mutant precursor proteins, WT preprotein (prSSU), and mature protein (mSSU) were recombinantly expressed in E. coli and purified (data not shown). They were then tested in chloroplast import competition assays. The concentration of 35S-labeled prSSU was held constant at 100 nM, while the concentration of the competitor protein was increased from 0 to 600 nM. Import of the labeled prSSU can be observed by quantifying accumulation of the radiolabeled mature domain in re-isolated chloroplasts. To quantify this...
Functional Analysis of Transit Peptide Motifs

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Figure 2. Truncation of the Transit Peptide C Terminus Abrogates Import and Chloroplast Interaction.

(A) Schematic showing the sequence and domains of WT P. Sativum His-S-SStp and deletion mutants. FGLK domains are highlighted in the shaded boxes.

(B) Graphical analysis of the FACS study of chloroplast binding of H-S-SStp constructs tagged with S protein FITC and incubated with chloroplasts. Binding reactions were performed in the presence of 30 μM ATP. The number of chloroplasts counted is shown on the y axis and counts of S protein FITC labeling is shown on the x axis.

(C) LSCM analysis of chloroplast binding of H-S-SStp constructs tagged with S protein FITC. Chloroplasts are depicted in red and the FITC signal is shown in green.

(D) Chemiluminescent detection of a western blot of a binding reaction showing interaction between H-S-SStpWT and H-S-SStpΔ5 and chloroplasts. Fields show conditions in each lane. Binding reactions were performed in the presence of 30 μM ATP as described previously (Subramanian et al., 2001). After the binding reaction, intact chloroplasts were subjected to SDS-PAGE. S protein alkaline phosphatase was used for detection.

In Vivo Analysis Suggests that Multiple FGLK Domains Are Redundant in Function

Although our in organello data suggest a clear importance for each of the two FGLK motifs found in prSSU, the specific role of the FGLK motif in vivo remains poorly studied. In vivo analysis allows us to capture a long timescale, physiological response to compare with our short timescale, reductionist in organello biochemical assays. We have developed an in vivo assay, using an SStp-20 mature domain residues-YFP chimeric construct to track the localization of mutant TPs in onion cells (Chotewutmontri et al., 2012; Chotewutmontri and Bruce, 2015). Although onion leucoplasts are non-green plastids, there are several recent in vitro and in vivo studies that show that import to non-green plastids is similar to differentiated chloroplasts (Hirohashi et al., 2001; Primavesi et al., 2007; Chu and Li, 2015). Collectively, these works show that although there may be different expression levels of certain Toc GTPases and even possibly different import activities of these different plastid sub-types, there is a common mechanism of transit peptide recognition and translocation.

We generated a series of internal deletion constructs, removing either part or all of each FGLK motif. As a control against TP length-dependent effects (Bionda et al., 2010), we also constructed a series of Ser substitution mutants, shown as green bars (Figure 3A). We selected Ser substitution because it is the most abundant amino acid in TP sequences (von Heijne, 1986), suggesting a relatively neutral impact on TP structure and/or function. Transient expression of TP-YFP constructs and calculating a fluorescence ratio (plastid:cytosol) allows us to indirectly determine levels of YFP-protein import to different plastid sub-types, there is a common mechanism of transit peptide recognition and translocation.

The ratio of plastid:cytosol YFP signal intensity is reduced by 5% for the DIΔ5 construct and 29% for the DIIΔ5 construct, which was found to be not statistically significant (quantification shown in Figure 3A; representative images shown in Figure 3B). However, deletion of both motifs in the same construct (construct DI/DIIΔ10) results in abrogated import (Figure 3A and 3B). Furthermore, Ser substitution of both of DI and DII motifs (construct DI/DII 10S) cannot restore plastid localization to WT levels (Figure 3A and 3B), yet provides an increase to 23% of WT plastid:cytosol signal intensity. Interestingly, removal of only the C-terminal elements of both of the motifs (construct
DI/C/DII-C Δ6) reduces import to 34% of WT levels; even when the region is substituted with Ser residues, import is only 43% of WT (construct DI/C/DII-C Δ6) (Figure 3A and 3B). These data are in agreement with our import competition results shown in Supplemental Figure 2B. Deletion of the N-terminal elements (construct DI-N/DII-N Δ6) also results in cytosolic mislocalization of the protein; however, substitution with Ser (construct DI/N/DII-N Δ6) restores to 73% of WT import levels. In addition, in every case, Ser replacement of all of the DI and DII double mutants either increased the import activity (DI/DII 10S, DI-N/DII-N 6S, and DI-C/DII-C 6S) or did not change it significantly from the deletions (DI-N/DII-C 6S, DI-C/DII-N 6S), implying that spacing may contribute function of these two domains in vivo.

**In Vitro Analysis of the Roles of Aromatic Residues in the FGLK Domain**

The first component of this motif is the inclusion of an aromatic amino acid such as Phe/Trp. We tested the role of the two Phe residues in domain I/I motifs using in organello assays with isolated chloroplasts as described in Supplemental Figure 2A and 2B. We focused on generating several constructs in which both of the FGLK Phe are mutated to Trp, Ser, or Ala. These were produced by a recombinant expression system in Escherichia coli via a recombinant expression system in Escherichia coli. We tested the role of the two Phe residues in domain I/I motifs using in organello assays with isolated chloroplasts as described in Supplemental Figure 2A and 2B. We focused on generating several constructs in which both of the FGLK Phe are mutated to Trp, Ser, or Ala. These were produced by a recombinant expression system in Escherichia coli. We tested the role of the two Phe residues in domain I/I motifs using in organello assays with isolated chloroplasts as described in Supplemental Figure 2A and 2B. We focused on generating several constructs in which both of the FGLK Phe are mutated to Trp, Ser, or Ala. These were produced by a recombinant expression system in Escherichia coli.

### Structural Flexibility Affects Binding, Import, and Processing of TPs

TPs have been described previously (von Heijne and Nishikawa, 1991) as the “perfect random coil”; some part of this lack of structure is due to their relatively high content of the helix-breaking residues Pro/Gly. Due to their placement within the FGLK motif, we investigated these specific residues of the FGLK motif using in organello assays with isolated chloroplasts. To test the role of structural flexibility, we mutated the Pro and Gly residues located near DI and DII (Pro25, Gly28, and Pro36) to Ala. We used an E. coli recombinant expression system to generate 35S-labeled WT and mutant proteins for import time course assays. We incubated 35S-prSSU WT and mutant proteins with isolated chloroplasts and monitored the rate of import and processing to the radiolabeled mSSU species. This assay allowed us to compare the kinetics import for mutant proteins compared with WT prSSU. We find that all three single Pro/Gly mutants (DI-P 1A, DI-G 1A, and DI-P 1A) are able to be imported at a reduced rate. The DI-G 1A mutant is only reduced by 25%, yet the double Pro mutations show a reduction of 50%–60% of WT SStp (Supplemental Figure 5A). A second confirmation of the import ability of these P/G-A mutants is their competition for import at near-WT levels (Supplemental Figure 5B). However, when we tested double and triple Pro/Gly substitution (double mutants DI-PG 2A, DI-G/DII-P 2A, DI-P/DII-P 2A, and triple mutant DI-PG/DII-P 3A), the triple mutant shows only 10% of the WT plastid localization (Supplemental Figure 5C). In import competition assays, the triple mutant DI-PG/DII-P 3A is virtually unable to compete for import, and the double mutants display an intermediate effect, with DI-PG 2A much more strongly affected (Supplemental Figure 5D; Table 1).

Because we established the importance of helix-breaking residues in in vitro analysis, we decided to test the in vivo localization of our Pro-deficient mutants. We tested double substitution mutants DI-PG 2S and DI-PG 2A, and our triple mutant DI-PG/DII-P 3A; yet only the triple mutant DI-PG/DII-P 3A shows significant 39% reduction in plastid localization (quantification is shown in Figure 4B). The loss of plastid localization in vivo of the triple mutant agrees with the effects seen in Supplemental Figure 5C and 5D, yet the observation that this in vivo effect is milder than the in vitro results is interesting.

Removing the helix-breaking residues P/G may extend the α-helical nature of the TP. We have already shown that SStp forms two helical segments when placed in a membrane mimetic environment (Bruce, 1998). Increasing the helical length or stability of the TP could lead to a higher energy requirement for protein translocation. Since it is well established that protein import is driven by stromal ATPase activity (Theg et al., 1989; Shi and...
Functional Analysis of Transit Peptide Motifs

### Loss of TP Flexibility Decreases Interaction with psTOC34 In Vitro

Both the *in vitro* and *in vivo* results suggest contributions of the complete domains I/II, as well as roles for specific residues. In light of these results, we investigated the effect of TP mutations on the interaction with the GTPase psTOC34 component of the TOC translocon, using *in vitro* assays (Sveshnikova et al., 2000; Schleiff et al., 2002; Sun et al., 2002; Reddick et al., 2007; Chotewutmontri et al., 2012). We employed an *E. coli*-based recombinant expression system to produce both TOC34 protein and full-length TPs, as previously described (Reddick et al., 2007; Chotewutmontri et al., 2012). We employed an *E. coli*-based recombinant expression system to produce both TOC34 protein and full-length TPs, as previously described (Reddick et al., 2007; Chotewutmontri et al., 2012). Two of these assays include the ability of WT SStp to induce monomerization of recombinant TOC34 and to function as GTPase activating partner (GAP)-like stimulatory activity on the GTP hydrolysis rate of psTOC34 using a sensitive phosphate release assay to monitor psTOC34 (Reddick et al., 2007; Chotewutmontri et al., 2012; Figure 5 C). PsTOC34 GTPase basal catalytic rates have been previously described (Reddick et al., 2007; Chotewutmontri et al., 2012) (Figure 5 B, lane 4).

The disruption of homodimerization affinity was confirmed with a simple SDS–PAGE assay following covalent crosslinking using a sulfhydryl-reactive, homobifunctional crosslinking agent, bismaleimidohexane (BMH). BMH reacts with the single Cys residue (Cys215) in psTOC34 that flanks the homodimeric interface observed in the crystal structure of TOC34 (Sun et al., 2002). Figure 6B summarizes our crosslinking results with SStp and psTOC34. Under control conditions, addition of BMH stabilizes approximately 66% of the protein in a dimeric species on SDS–PAGE (Figure 5B, lane 2), yet addition of WT SStp protein strongly shifts the TOC34 protein toward a monomeric species, with only 15% dimeric species (Figure 5B, lane 3). In agreement with our AUC analysis, the triple mutant DI-PG/DII-P 3A does not monomerize TOC34 to the same extent as the WT SStp protein, resulting in about 30% dimeric species (Figure 5B, lane 4).

We further tested the effect of the DI-PG/DII-P 3A substitution using a sensitive phosphate release assay to monitor psTOC34 GTPase activity (Reddick et al., 2007; Chotewutmontri et al., 2012) (Figure 5C). PsTOC34 GTPase basal catalytic rates have been previously described (Reddick et al., 2007); we find that titration of a 25-fold molar excess of WT SStp results in a maximal stimulatory effect, increasing the V\text{max} by ~1.6 fold to 69.3 nmol GTP/min/\mu mol psTOC34. However, incubation with the same concentration of DI-PG/DII-P 3 mutant maintains near basal TOC34 GTP hydrolysis levels with a V\text{max} of 32 nmol GTP/min/\mu mol psTOC34 and K\text{m} of 2.9 \mu mol. These two assays in combination indicate that the loss of flexibility of SStp directly reduces the productive interaction with Toc34 *in vitro*.

#### Basic Residues Required for *In Vivo* Import Efficiency

Since our previous assays (Supplemental Figure 2B) suggested a unique requirement for the C-terminal portion of the DI and DII residues (Pro\text{25}, Gly\text{28}, Pro\text{36}) results in a reduced monomerization effect.

The disruption of homodimerization affinity was confirmed with a simple SDS–PAGE...
Molecular Plant

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Figure 4. Analysis of Phe and Pro Mutations on TP Import Efficiency.

(A and B) Transient expression of chimeric SStpNt-YFP constructs in onion (Allium cepa) were live imaged using epifluorescence microscopy. Alignment depicts location of mutations in the SStp sequence; domains DI and DII are highlighted in gray. Bars show the ratio of plastid-localized signal intensity/cytosolic-localized signal intensity. Substitution to Ala is shown in gray bars, Ser is shown in green. N = at least 15 cells per condition. One-way ANOVA with Bonferroni post hoc analysis. No asterisks = P > 0.05, *P ≤ 0.05; **P ≤ 0.01.

(C) Graphical analysis of in vitro protein import assays using isolated chloroplasts and 35S labeled prSSU with increasing concentrations of [ATP], shown as a function of %WT mSSU import levels.

domains and since both of these domains contain at least one basic residue, we decided to more carefully test its importance by substituting the TP basic residues with either Ser or Ala (Figure 6A and 6B). We focused on in vivo analysis to test the importance of basic residues because our previous analysis established that only mutations with a strong in organello affect in vivo localization. Our previous in vivo localization analysis required substantial deletions or substitutions in DI and DII for construct mislocalization (Figure 3A–3B). Mutation of a single basic residue (K30, R39, K40) results in mildly reduced plastid localization levels (a 10% reduction for R39 and K40); only K30 mutagenesis shows a significant 26% reduction (Figure 6A). However, we find that substitution of all three basic residues in the DI and DII domains results in a strong cytosolic mislocalization (Figure 6A). Substitution of K-RK with Ala results in a 78% reduction in plastid localization; Ser substitution reduces localization by 45%. Moreover, double mutations result in intermediate phenotypes (DII-RK 2S and 2A). There are two other basic residues in the SStp sequence, Arg14 and Arg54, so we tested whether the mislocalization of DI-K/DII-RK 3A or 3S is due to loss of basic residues in the physiochemical DI/DII domains or if the effect is nonspecific to loss of positive charge. We find that substitution mutant R14R54 2A has no effect on protein localization compared with the WT control protein. Furthermore, addition of R14R54 mutation to the DI-K/DII-RK 3A construct (R14-DI-K/DII-RK-R54) does not increase mislocalization of the DI-K/DII-RK 3A construct (Figure 6A and 6B).

Importance of FGLK Domains in Other TP Sequences

Since our analyses suggest that FGLK is a required element in TP import in SStp, we decided to extend our analysis to a second model TP. The ferredoxin TP (FdTP) is probably the second most well-characterized transit peptide (Pilon et al., 1992a, 1992b, 1995; Chotewutmontri et al., 2012). These are highly abundant preproteins targeted to the stoma of chloroplasts. Much of the early work focused on the FdTP from Silene latifolia (S. pratenis) (Smeeckens et al., 1985). Alignment of ~40 FdTPs from angiosperms (shown in Supplemental Figure 2) indicate that these TPs also contain two FGLK domains (labeled as domain I and domain II) with a consensus sequence FLRKQP and FGLK, respectively.

Based on this alignment, we identified two regions in the Silene precursor that contain the basic elements of the FGLK motif, denoted as DI-DII (Supplemental Figure 6 and Figure 7). We systematically mutated these regions to Ser and used our in vivo YFP chimera assay to test the localization of the WT versus mutant constructs. We find that mutation of DI alone (FGLK; DI-4S) has no effect on the plastid localization of Fdtp; however, mutation of DI (PKQQPMPM) in combination with the DI domain (DI-6S/DII-4S/RSS) abrogates chloroplast localization, similar to what we observed with SStp. To test the relative contributions of the amino acids in DI, we performed a similar Ser replacement and tested the effect of substitution of the N (PKQ) and C (QPM) regions. We find that DI-N substitution (DI-N 3S) results in a 29% reduction in import; however, mutagenesis of DI-C (DI-C 3S) results in a 41% reduction from WT localization. However, when DI-N/DI-C is mutated in combination with DII, the construct is significantly mislocalized, indicating the additive effect of DI and DII domains and suggesting that these two FGLK motifs are redundant in function.

TP Phosphorylation Is Not Required for Plastid Protein Import

Earlier work has shown that chloroplast precursors can be phosphorylated at specific Ser (Waegemann and Soll, 1996; Martin et al., 2006). These Ser residues have been partially mapped in prSSU to a motif that falls between the two FGLK motifs. The result of the phosphorylation has been proposed to enhance the affinity of the TP for the 14:3:3 proteins found within a guidance complex (May and Soll, 2000). Because of this activity and the proximal location of this phosphorylation site, we decided to directly test the role of phosphorylation in...
precursor recognition and import into leucoplasts using a series of non-phosphorylatable or phosphomimetic mutations in FDtp and SStp (Figure 8A and 8B). Analysis of the ∼40 angiosperm ferredoxin transit peptides indicate that there is a variable number (from 2 to 5) of phosphorylatable residues between domain I and domain II (Supplemental Figure 6). In the Silene Fdtp sequence, we selected three possible phosphorylation sites in the FDtp sequence, S24, S25, and T28 (denoted SST). We then made pairwise comparisons of the non-phosphorylatable S-to-A mutants with the WT, as well as a pairwise comparison between non-phosphorylatable and the phosphomimetic mutants in FDtp (Figure 8A). In SStp, we selected residues that have been previously implicated as phosphorylation sites (S31/S34, T27/S38) (Figure 8B). Interestingly, in each of the non-phosphorylatable mutations (S/T to A), the import was actually slightly higher than the WT FdTP. This ranges from ∼6% in ASA to a high of 33% in SAA, and overall these averaged about 22% above the WT FdTP; in no case was the introduction of a non-phosphorylatable Ala residue statistically lower than the WT FdTP. We observed a similar slight stimulation with the two Ser residues in SStp when they were changed to Ala.

However, upon mutation of the Ser to a phosphomimetic residue (S/T to D/E), we observed a general decrease in import. This ranged from a minor but not statistically significant change in T28 (SSA versus SSE) to an S24-to-D (SDT) compared with a very strong effect for S28 (DST) that resulted in a 30% reduction in correct localization (Figure 8A). The change in T28 was intermediate with about a 20% reduction in plastid accumulation. This effect was somewhat additive since we find that the double substitution SDE and DDT mutants show about 35% lower plastid import versus the correlating single Ala mutants (SAA and AAT, respectively). Similar to FDtp, SStp S31/S34A also shows an increase in plastid localization, whereas T27/S37 results in about a 30% reduction in protein localization (Figure 8B). Overall, we find that the replacement of S/T with a non-phosphorylatable alanine consistently results in plastid localization equal to or greater than the WT counterparts. However, these results indicate that the introduction of the phosphomimetic residue, Asp, consistently lowers the targeting activity in vivo in both SStp and Fdtp.

**DISCUSSION**

Despite the wide divergence of TP sequence and structure (Bruce, 2000), previous studies implicate two physicochemical motifs in precursor protein targeting and import (Pilon et al., 1995; Ivey et al., 2000; Lee et al., 2006, 2009; Chotewutmontri et al., 2012). One motif is an uncharged N-terminal HSP70 interaction domain that functions to engage stromal ATP-dependent chaperones driving translocation (Chotewutmontri and Bruce, 2015). Considerable evidence now suggests that HSP70 functions as this translocation motor for a subset of precursors (Shi and Theg, 2010; Chotewutmontri et al., 2012; concentration of SStpNT DI-PG/DII-P 3A mutant cannot. The γ32P-GTP was held constant at 10 nM throughout the experiment. Counts/min of TOC34 hydrolysis at increasing GTP concentration were used to calculate a slope that was mathematically transformed into a rate expressed as nanomoles of GTP hydrolyzed per minute per micromole TOC34, and graphed to yield a Michaelis-Menten substrate velocity plot. Experiments were carried out essentially as described in Reddick et al. (2007).
Molecular Plant

Functional Analysis of Transit Peptide Motifs

A Construct

<table>
<thead>
<tr>
<th>WT</th>
<th>DI</th>
<th>DII</th>
</tr>
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<tbody>
<tr>
<td>R14...R34</td>
<td>PFTGLKSAASFVPVSRK...R34</td>
<td></td>
</tr>
<tr>
<td>DI-C Δ3 / DII-C Δ3</td>
<td>R14...PFT---SAASF---K...R34</td>
<td></td>
</tr>
<tr>
<td>DI-C 3S / DII-C 3S</td>
<td>R14...PFTSSSAAASFPSSSS...R34</td>
<td></td>
</tr>
<tr>
<td>DI-K30A</td>
<td>R14...PFTGLASAAASFVPVRK...R34</td>
<td></td>
</tr>
<tr>
<td>DII-R35S</td>
<td>R14...PFTGLKASAAASFVPSSS...R34</td>
<td></td>
</tr>
<tr>
<td>DII-RK 2S</td>
<td>R14...PFTGLKASAAASFVPSA...R34</td>
<td></td>
</tr>
<tr>
<td>DII-RK 2A</td>
<td>R14...PFTGLKASAAASFVPSA...R34</td>
<td></td>
</tr>
<tr>
<td>DI-K /DII RK 3S</td>
<td>R14...PFTGLASAAASFVPSSS...R34</td>
<td></td>
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<tr>
<td>DI-K /DII RK 3A</td>
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<td></td>
</tr>
<tr>
<td>R14R34 2A</td>
<td>A14...PFTGLKASAAASFVPSR...A34</td>
<td></td>
</tr>
<tr>
<td>R14-DI-K/DII RK-R34 5A</td>
<td>A14...PFTGLASAAASFVPSSA...A34</td>
<td></td>
</tr>
</tbody>
</table>

Liu et al., 2014). We have previously shown that this motif is followed by one or more FGLK motifs, which were initially reported many years ago, yet detailed description of their function is still lacking (Pilon et al., 1995). Importantly, FGLK motifs represent a physicochemical element versus a sequence consensus. In this work, we greatly extend the analysis by combining in vitro, in organello, and in vivo assays. This provides a highly quantitative and resolved biochemical insight while confirming a clear physiological role in living cells.

FGLK Domains Are Required for In Organello Binding

In prior work, chimeric constructs placed the reporter protein C-terminal to the TP sequence, analogous to a native TP domain structure. These studies showed robust activity of the chloroplast translocator, resulting in complete translocation of the chimeric fusion protein into the stroma (America et al., 1994). However, our previous work (Subramanian et al., 2001) characterized H-S-SStp, which could not be translocated into the chloroplast stroma yet was able to compete with TP for preprotein binding to intact chloroplasts. The addition of the S-tag provides a sensitive and quantitative reporter via its interaction with S protein (Kim and Raines, 1993). The chimeric construct places 21 charged residues at the N terminus of SStp, which conflicts with a widely regarded property of TPs (Chotewutmontri and Bruce, 2015; Chotewutmontri et al., 2012; Pilon et al., 1995; Subramanian et al., 2001; von Heijne and Nishikawa, 1991). Despite the inability of this construct to translocate across the chloroplast envelope (Subramanian et al., 2001; Chotewutmontri et al., 2012), it is an ideal substrate to explore binding to the chloroplast surface.

Previous transmission electron microscopy showed that the inner and outer chloroplast membranes come together at punctate contact sites (Cline et al., 1985), and biochemical analysis of the TOC-TIC core complex suggests that membrane contact sites represent the locations of active protein transport (Schleiff et al., 2003). Thus, we conclude that the punctate fluorescent pattern observed for the WT and Δ5 constructs represents H-S-SStp bound to the TOC-TIC supramolecular complexes (Figure 2C). Prior work has also demonstrated a punctate TP immunofluorescent imaging pattern (Schnell and Blobel, 1993; Subramanian et al., 2001); however, unlike the divalent antibody-based studies, our labeling approach uses a monovalent S protein FITC. This assay obviates the possibility that the punctate labeling arises from capping or clustering due to the multivalent nature of antibodies. It is not clear why they are punctate in nature but suggests an additional level of supramolecular organization. Blue native–PAGE has observed at least three sizes of the TOC complex so this punctate fluorescent labeling may be detecting these larger complexes (Kikuchi et al., 2006; Chen and Li, 2007).

The C-terminal TP truncations clearly disrupt H-S-SStp interaction with isolated chloroplasts (Figure 2A–2D). It is clear from the differential effect of Δ5 versus Δ25 that the critical residues are between G26 and R52. This region contains all of the second, C-terminal FGLK motif, which in pea is FPVKK. Interestingly, when we test the deletion of either the full domain I or II in import competition, both are nearly equal in their ability to compete with prSSU. This confirms that it is FGLK domains themselves that are key for recognition and binding to chloroplasts. However, it is not clear why the two assays show somewhat different importance of the two domains. The charged N terminus of His-S-SStp possibly counteracts or reduces the efficacy of the single N-terminal FGLK domain to support binding.

Differential Role of the Individual Amino Acids

We further resolve this interaction by comparing the deletion of either the first three residues of either DI (FTG) or DII (FPV) versus...
the last three residues of DI (GLK) or DII (VSR), revealing a more severe result when the C-terminal residues were removed (Supplemental Figure 2B). However, both the N- or C-terminal mutants were more active than the complete removal of either DI or DII, suggesting that productive import involves all of the physicochemical elements in the FGLK domain. Since the Ser substitution partially restores import of the DI-N/DII-N D6, it suggests spacing may be critical but could also suggest that simply removing the N-terminal residues may eliminate some secondary structural elements that affect TP recognition. These results indicate that element(s) in the C-terminal regions of the FGLK motifs, such as positively charged residues, may play a critical role in binding and/or import efficiency (Figure 6A and 6B). Interestingly, a basic residue doublet (RxRy) located within an FGLK domain has been implicated as a recognition sequence element necessary for TP recognition/import in older chloroplasts (Ling et al., 2012). Mutating this basic residue doublet in the Tic40 precursor abrogated import of the precursor into older chloroplasts but not younger chloroplasts (Yi-Shan et al., 2012). Furthermore, import analysis of Arabidopsis E1ζtp (the TP of E1ζ subunit of pyruvate dehydrogenase) found that both block Ala mutagenesis of an FGLK domain or a region containing three basic residues resulted in significant equivalent reduction in import (Lee et al., 2009). In agreement, our data suggest a dose-dependent threshold for basic residues in import efficiency; loss of two or more basic residues within the FGLK domains abrogates import (Figure 6A). The functional contribution of the positive charge requires their centralized placement proximal to the FGLK recognition elements, since removal of flanking residues at the N and C terminus (R14 and R54, respectively) had no effect on import efficiency in SStp (Figure 6A). Thus, it appears that a direct correlation exists between the basic residues in the FGLK domain and TP recognition/import. Future work will explore the structural basis of this interaction.

The effects of mutation on FGLK components such as Phe (Supplemental Figure 4A–4D; Figure 4A and 4C) are milder in vivo experiments versus in vitro. Collectively, our data suggest that there may be factors present in the cytosol and absent in our

Figure 7. In Vivo Localization Reveals FGLK Domains in FdTP Are Required for Efficient Plastid Localization. (A) Transient expression of chimeric FDF-YFP constructs in onion (Allium cepa) were live imaged using epifluorescence microscopy. Alignment depicts location of mutations in the FDF sequence; domains DI and DII are highlighted in gray. Bars show the ratio of plastid-localized signal intensity/cytosolic-localized signal intensity. N = at least 15 cells per condition. One-way ANOVA with Bonferroni post hoc analysis. DNI = Does not import; No asterisks = P > 0.05; * P < 0.05; **** P < 0.0001. (B) Representative epifluorescence microscopy images of the quantified data shown in Figure 7A.
in organello assays that contribute to the import process, which negate the effect of Phe or Pro/Gly substitution on TP secondary structure. Furthermore, this implies that the basic residues have a greater contribution to TP recognition/import since their mutagenesis abrogates import \textit{in vivo} (Figure 6A and 6B).

We further verified the redundancy of multiple FGLK domains using \textit{in vivo} localization analysis of FdTP, which also contains two FGLK physicochemical motifs. Similar to SStp, mutagenesis of only one FGLK domain resulted in WT plastid localization; whereas mutagenesis of both domains abrogated import (Figure 7). Collectively, these results suggest that the \textit{in vivo} import can utilize either DI or DII with some redundancy. It is also interesting that partial deletions of FGLK domains in SStp still show some ability to compete for preprotein recognition (Supplemental Figure 2A and 2B), suggesting that the two domains may be somewhat redundant in promoting recognition. How these two domains are recognized is not known in detail, but it is possible each element is recognized by adjacent monomers in either a hetero- or homo-dimeric form of the TOC receptors, TOC34 and/or TOC159 (Lumme et al., 2014).

**Figure 8. Phosphorylation of FDtp and SStp Is Not Required for Plastid Import.**
(A and B) Transient expression of chimeric FDF-YFP constructs (A) or SStpNt-YFP constructs (B) in onion (Allium cepa) were live imaged using epifluorescence microscopy. Alignment depicts the location of mutations in the FDF sequence. Bars show the ratio of plastid-localized signal intensity/cytoplasmic-localized signal intensity. N = at least 15 cells per condition. One-way ANOVA with Bonferroni post hoc analysis. No asterisks = P > 0.05; ** P ≤ 0.01; ****P ≤ 0.0001.

Direct Effects on TOC34 Structure and Activity
The effects of TP-TOC34 interaction on the Toc34 homodimeric state and basal GTP hydrolysis activity have potential...
implications in the regulation of chloroplast protein import. Previously, the GTPase cycle of Toc34 has been hypothesized to function as a major regulatory switch in protein import. There is considerable evidence that the homo- and possibly heterodimerization of Toc34 contribute to its GTPase cycle (Jelic et al., 2002; Reddick et al., 2008; Aronsson et al., 2010; Lumme et al., 2014). Previous experimental data support that the FGLK motif functions as a TOC34-binding element (Sun et al., 2002; Reddick et al., 2007; Chotewutmontri et al., 2012; Lee et al., 2009). In the future, it would be interesting to see if the FGLK sequence can interact directly with TOC34 and either disrupt the stability of the dimer or stimulate GTPase activity. We find that removal of Pro residues from the vicinity of the FGLK sequence can interact directly with TOC34 and either disrupt the stability of the dimer or stimulate GTPase activity. In support of our data, previously, SStp S31/34A and S31/34D prSSU-GFP mutants were transiently expressed in Arabidopsis protoplasts (Lamberti et al., 2011). These experiments suggested that removal of phosphorylation sites (S31/S34A) results in plastid localization; whereas phosphomimetic S31/34D mutations lead to mislocalization of the construct (Lamberti et al., 2011). Interestingly, western blot analysis of protoplast import suggests that the S31/34A construct shows an increase in plastid binding compared with the WT construct. Collectively, our results suggest that phosphorylation is not essential for precursor import nor does a non-dephosphorylated, phosphomimetic substrate abolish precursor import or yield mislocalization. We speculate that phosphorylation may contribute to enhanced interaction with the cytosolic guidance complex (May and Soll, 2000), but an unidentified phosphatase removes phosphoryl groups before precursor import (Lamberti et al., 2011). Whether this effect is simply a change in the net charge of TPs or some other more specific interaction is involved, such as enhanced binding to the TOC receptors, is not known. However, we have previously shown that the phosphorylated form of an SStp-derived synthetic peptide interacts more tightly with a recombinant form of pS34TOC34 that would support this latter model (Schleiff et al., 2002).

Conclusions

We propose a new TP import model that incorporates a dynamic recognition mechanism as depicted in Figure 9. TP import is likely driven by formation of a secondary structural element, which would allow TOC34 to rapidly sample the side-chain conformation of the FGLK physicochemical domain possibly regardless of orientation (Chotewutmontri et al., 2012), as is the case for the reversed sequences. This dynamic sampling would enable rapid yet relatively promiscuous binding so that many divergent TP sequences that only contain a recognition element for Hsp70 or an alternative ATP-dependent molecular motors. Requiring two independent and spatially separate modes of recognition would allow the chloroplast to rapidly recognize a diverse set of proteins on the surface yet only import those that contain a recognition element for Hsp70 or an alternative ATP-dependent molecular motor. Future work is needed to see how these two domains must be spatially separated in the transit peptide to insure both rapid and precise client recognition. In addition, it is possible that these peptides may first encounter the nonpolar environment of the outer membrane and form helical segments that help provide some structural conformity to the TPs facilitating both recognition and lowering diffusion barriers.

EXPERIMENTAL PROCEDURES

Alignment

The full-length protein sequences of the small subunit of RuBisCO from green plants (Viridiplantae) were retrieved from the UniProt database (release 2014_05) by query search using key words “Ribulose bisphosphate carboxylase small chain”, “taxonomy:33 090,” and “fragment:no.” A total of 429 sequences matched these criteria. Sequences with 100% identity were deemed redundant and removed using
Functional Analysis of Transit Peptide Motifs

**Figure 9. Model for Chloroplast TP Import.**

We propose a dynamic recognition model, where (1) cytosolic TP recognition is driven through productive interaction between the TOC receptor components, such as TOC159 and TOC34. This is likely driven through electrostatic interactions involving Arg residues in the TP FGKL domains. Initial recognition may occur through interaction between TP hydrophobic residues and chloroplast lipids (not shown). (2) Following initial recognition, the TP is captured, and the TOC translocon is made accessible, possibly through structural changes driven by GTP hydrolysis. (3) Cytosolic capture is followed by recognition of sequence elements in the N terminus of the TP and stromal chaperones. (4) “Pulling” of the TP into the chloroplast by stromal chaperones such as chloroplast HSP70 and HSP93.

**CD-HIT** (Li and Godzik, 2006). The remaining 384 sequences were aligned using MAFFT (Katoh et al., 2005), which was shown to outperform other programs in aligning the benchmark short-linear motif datasets (Perrodou et al., 2008). The aligned sequences were submitted to MaxAlign (Gouveia-Oliveira et al., 2007) to remove poorly aligned sequences and to reduce gap positions. Four additional poorly aligned sequences were removed via visual inspection resulting in the final 328 sequences. These sequences were realigned with MAFFT. The consensus and logo plot of the alignment were generated using the Jalview program (Waterhouse et al., 2009). Only TP sequences were shown, identified based on the cleavage site of the tobacco sequence (Mueller et al., 1983).

**Production of TP Constructs**

Tobacco (Nt) and pea (Ps) SStp TP constructs for *in vitro* and *in organello* assays were generated in the pTYB2 vector; *in vivo* assay constructs were generated in pAN187 vector (Nelson et al., 2007) with a C-terminal YFP tag as previously described (Chotewutmontri et al., 2012). All *in vivo* SStp domain deletion constructs also have a domain substitution counterpart to control for length-dependent effects. Dual-tag constructs (H-S-SStp) were expressed in the pET30a vector with an N-terminal 6XHis-S protein tag. PCR amplification using TaKara high-fidelity polymerase and site-directed mutagenesis was performed based on Quik-Change instructions provided by the manufacturer (Stratagene). All PCR-generated constructs were sequenced to ensure fidelity of amplification.

**Protein Expression and Purification**

The soluble, truncated Δ-transmembrane C-terminally His-tagged psTOC34 was prepared as previously described (Reddick et al., 2007) as a recombinant *E. coli* BL-21 (DE3) expressed protein. TPs for *in vitro* import analyses were expressed in *E. coli* ER2566 from pTYB2 constructs and purified as described previously (Reddick et al., 2008). Proteins were separated by 15% acrylamide SDS–PAGE (psTOC34) or 19.2% Tris–Tricine gels (TPs) and visualized using Coomassie brilliant blue. Protein identities were verified using MALDI-TOF MS as described in Chotewutmontri et al. (2012). PrSSU and mSSU proteins were expressed in *E. coli* BL-21 (DE3) and purified essentially as published in Reddick et al. (2008) and Chotewutmontri et al. (2012). For import assays, prSSU and mSSU proteins were solubilized in a buffer containing 8 M urea, 50 mM DTT, and 20 mM Tris (pH 8.0). Radiolabeling of prSSU was performed as described in Reddick et al. (2008). Dual-tag constructs (H-S-SStp) were recombinantly expressed in *E. coli* BL-21 (DE3) using the pET30a vector. Cells were lysed in buffer A (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 8.0] containing protease inhibitors), centrifuged at 50,000 g to remove insoluble protein, and loaded on Ni²⁺-Sepharose columns. Columns were washed with buffer B (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 8.0], 6 M urea), and eluted with elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 8.0], 6 M urea). Samples were dialyzed against import buffer (50 mM HEPES-KOH [pH 8.0], 330 mM sorbitol, 6 M urea) before use in assays.

**Chloroplast Isolation and *In Vitro* Chloroplast Protein Import Assays**

*In vitro* analyses of protein import were performed essentially as previously described (Bruce et al., 1994; Reddick et al., 2008; Chotewutmontri et al., 2012). Reactions were performed in a 300-l reaction containing 0.125 mg chlorophyll/ml chloroplasts isolated from pea plants (*Pisum sativum*), 100 nM [35S]-prSSU, 1 mM BME, 2 mM Mg-ATP, 0.5% BSA, 250 mM urea, 330 mM sorbitol, 50 mM HEPES-KOH (pH 8.0) and varying amounts of competitor proteins. The reactions were stopped after incubation for 15 min at room temperature. The chloroplasts were re-isolated and SDS–PAGE was performed. Autoradiography was performed on a Storm 840 Phosphoimager (GE Healthcare) followed by quantification with ImageQuant software. At least three replicates were performed. The values were normalized to the value from the reaction with no competitor controls, and the data were fitted to the one-phase exponential decal model with non-linear regression using GraphPad Prism 5.0.

**S Protein FITC Labeling of the Chloroplast Bound H-S-SStp**

Samples were processed as previously described for *in vitro* binding assays, then centrifuged at 1500 g to remove unbound H-S-SStp, and suspended in 300 µl of blocking solution (buffer containing 1% BSA and 1% nonfat milk). S protein FITC (Novagen) was added at 1:500 concentration and incubated for 30 min on ice. Intact chloroplasts were separated over a 40% Percoll cushion and resuspended in import buffer (50 mM HEPES-KOH [pH 8.0], 330 mM sorbitol) for FACS and LCMS analysis.

**Western Analysis**

Samples were boiled in 4 x sample buffer and separated on a 15% SDS–PAGE gel. The gel was electroblotted onto a pre-wetted Immobilon-P membrane (Millipore) using transfer buffer (46 mM Tris base, 39 mM Gly, 0.04% SDS, 20% methanol). The blot was blocked for 15 min in Tris-buffered saline (TBS) containing 1% gelatin + 0.5% Tween-20.
Functional Analysis of Transit Peptide Motifs

The S peptide was detected by incubation with an S protein alkaline phosphatase conjugate (Novagen) diluted 1:100 000 in TBS, followed by extensive washing in TBS + 0.2% Tween-20. Visualization was conducted using a 1:1 dilution of LumiPhos Plus (Lumigen) and subjected to film autoradiography.

FACS Analysis

A dual-laser Becton Dickinson FACstar Plus was used for the flow cytometric analyses. Data were acquired and analyzed using Becton Dickinson CellQuest V.3.1 software. The argon ion laser in the first position was tuned for 488 nm output running on a constant output mode at 250 mW. Dot plot analysis of forward and right-angle light scatter was used to calculate the percent intact plastids (70%–80% total plastids; Kausch and Bruce, 1994). Total chloroplasts were gated to include only intact plastids for chlorophyll autofluorescence (gate 1) and S protein FITC (gate 2). Fluorescence intensities of approximately 10 000 intact chloroplasts were analyzed per sample. The extent of positive labeling was set manually with unlabeled chloroplasts for each experiment to yield a value of 2%–3% FACS positive. The flow rate was maintained at about 1200 chloroplasts/min.

Phosphate Release Assay for GTP Hydrolysis

Phosphate release analysis, scintillation counting, and data fitting to the Michaelis-Menton equation were performed as reported (Reddick et al., 2007, 2008; Chotewutmontri et al., 2012).

Sedimentation Velocity Experiments for psToc34 and SSTp

Sedimentation velocity analysis was performed as published previously (Chotewutmontri et al., 2012). Briefly, analyses were performed using a Beckman Optima XL-A analytical ultracentrifuge using the interface mode. The psToc34 and TPs were dialyzed into GBS buffer (20 mM Tricine-KOH [pH 7.65], 1 mM MgCl2, 50 mM NaCl, and 1 mM β-mercaptoethanol) with 10 000 and 3500 molecular weight cutoffs, respectively. Samples were prepared by gentle mixing of psToc34, TPs, GTP, and dialysis buffer to a final concentration of 13.5 μM psToc34, 135 μM TP, and 2 mM GTP. Analytical ultracentrifugation sample cells with sapphire windows and Epon charcoal-filled two-sector 12-mm centriplates were loaded with 400 μl of sample using the final dialysis buffer as reference. Interference scans were performed at 50 000 rpm (~200 000 g) after temperature equilibration of an An-50 Ti rotor and centriplates for at least 1 h at 25°C. A differential distribution of the sedimentation coefficients, c(s), of the samples was fit to the experimental data using the program SEDFIT (Schuck, 2000). The best fit c(s) analysis was distributed as described previously (Dam and Schuck, 2004; Lebowitz et al., 2002). The c(s) distribution was then transformed to a distribution of molecular mass. The fractions of psToc34 monomer and dimer were calculated by integrating the area under the sedimentation distributions from 2.6 to 3.3 S and 3.3 to 4.4 S for monomer and dimer, respectively. For presentation, the distributions were exported and graphed using GraphPad Prism 5.0.

Biollistics Transformation

All assays were performed essentially as described in Chotewutmontri et al. (2012) and Nelson et al. (2007). Briefly, transient expression in onion (Allium cepa) epidermis peel using particle bombardment was performed using 1 μg of plasmid DNA to coat the tungsten particles. Microscopy was performed at 16 h post-bombardment unless otherwise noted.

Microscopy

Epifluorescence imaging was performed as described in Chotewutmontri et al. (2012) using an Axiovert 200 M microscope (Zeiss) fitted with YFP/cyan fluorescent protein filters (filter set 52017; Chroma). Image capture was performed with a digital camera (Orca ER; Hamamatsu Photonics) using OPENLAB software (Improvision). All images were captured 16 h after transformation unless noted. LCMS of S protein FITC labeled samples were analyzed using a Leica TCS-NT laser confocal microscope. FITC and chlorophyll autofluorescence were simultaneously visualized using 488 nm excitation into a 530/30 BP filter (FITC) and a 660 LP filter (chlorophyll autofluorescence). A 100×, 1.4 NA oil immersion lens at an Airy disc setting of 0.91 was used. Resizing and cropping for presentation was performed using Photoshop (Adobe).

Relative Intensity Ratio Measurements

Measurements of camera-noise-subtracted epifluorescence images were taken using ImageJ software (Abramoff et al., 2004) essentially as described in Chotewutmontri et al. (2012). Briefly, the intensity per pixel values from different areas in the images were calculated from the summation of intensity signals in the area divided by the total number of pixels in the area. A circle was drawn to fit around individual plastids to measure the plastid intensity per pixel. The same circle was enlarged to threefold diameter, so that the area shared the same center, but integrated signals from the cytosol. The cytosol intensity was calculated from the ring area between the former and enlarged circles. A rectangular area outside the fluorescing cell in the same image was used to calculate the background intensity per pixel. The background intensity value was subtracted from the plastid and cytosol intensity per pixel values. For each plastid, a ratio between the background-removed plastid and cytosol intensity per pixel values were calculated. The ratio of intensity of each cell is the average of all plastid ratio values.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at Molecular Plant Online.

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AUTHOR CONTRIBUTIONS


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Functional Analysis of Transit Peptide Motifs


