Mitochondrial Targeting of the *Arabidopsis* F1-ATPase γ-Subunit via Multiple Compensatory and Synergistic Presequence Motifs

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The majority of mitochondrial proteins are encoded in the nuclear genome and imported into mitochondria posttranslationally from the cytosol. An N-terminal presequence functions as the signal for the import of mitochondrial proteins. However, the functional information in the presequence remains elusive. This study reports the identification of critical sequence motifs from the presequence of *Arabidopsis thaliana* F1-ATPase γ-subunit (pFAγ). pFAγ was divided into six 10–amino acid segments, designated P1 to P6 from the N to the C terminus, each of which was further divided into two 5–amino acid subdivisions. These P segments and their subdivisions were substituted with Ala residues and fused to green fluorescent protein (GFP). Protoplast targeting experiments using these GFP constructs revealed that pFAγ contains several functional sequence motifs that are dispersed throughout the presequence. The sequence motifs DQEEG (P4a) and VVRNR (P5b) were involved in translocation across the mitochondrial membranes. The sequence motifs IAARP (P2b) and IAAIL (P3a) participated in binding to mitochondria. The sequence motifs RLLPS (P2a) and SISTQ (P5a) assisted in pulling proteins into the matrix, and the sequence motif IAARP (P2b) functioned in Tom20-dependent import. In addition, these sequence motifs exhibit complex relationships, including synergistic functions. Thus, multiple sequence motifs dispersed throughout the presequence are proposed to function cooperatively during protein import into mitochondria.

INTRODUCTION

Mitochondria (MT) play essential metabolic and signaling roles in many processes of eukaryotic cells (Balsera et al., 2009) and contain up to 1500 unique proteins (Heazlewood et al., 2004). Despite harboring its own genome, the majority of mitochondrial proteins are encoded by the nuclear genome and imported into MT after translation in the cytosol (Dolezal et al., 2006; Neupert and Herrmann, 2007; Chacinska et al., 2009; Endo et al., 2011). For targeting from the cytosol to the MT, mitochondrial proteins contain a targeting sequence. Depending on the individual protein, mitochondrial targeting sequences have been identified at three characteristic positions in the protein: the N terminus, internal sequences, and the C terminus (Brix et al., 1999). Of these three subtypes, targeting sequences located at the N terminus (Brix et al., 1999). Of these three subtypes, targeting sequences located at the N terminus of the protein have been best characterized to date. The N-terminal presequences (PS) range in size from 15– to 70–amino acid residues and have been demonstrated to be both necessary and sufficient for protein import into MT (von Heijne, 1986; Rapaport, 2003). Although no primary sequence consensus for targeting to the MT has been derived, detailed analyses have revealed several characteristic features, including the enrichment of hydroxylated and hydrophobic amino acids, an abundance of Arg residues, a lack of acidic amino acids, and a propensity to form an α-helix (Glaser et al., 1998; Huang et al., 2009). The configuration of positively charged and hydrophobic amino acids has been demonstrated to form an amphipathic α-helix in solution, and NMR analysis of the central mitochondrial outer membrane (MOM) import receptor, Tom20, in complex with an N-terminal PS revealed that the hydrophobic surface of the amphipathic α-helix binds to the hydrophobic groove of Tom20 (Abe et al., 2000). Recent studies revealed that Tom20 binding sites contain a consensus sequence of ϕxxϕ (where ϕ indicates a hydrophobic residue and x indicates any amino acid residue) (Muto et al., 2001; Obita et al., 2003; Zhang et al., 2010; Yamamoto et al., 2011). The PS is recognized by the import machinery located in the mitochondrial membranes (Schatz and Dobberstein, 1996; Neupert and Herrmann, 2007; Chacinska et al., 2009; Endo and Yamano, 2009; van der Laan et al., 2010). Translocase of the outer membrane of MT (TOM) in yeast is composed of the import channel Tom40, outer membrane receptors (Tom20, Tom22, and Tom70), and small accessory proteins (Tom5, Tom6, and Tom7) and is responsible for translocating precursors through the outer membrane. At the inner membrane,
two TIMs (translocase of the inner membrane of MT) exist for the translocation of precursors through the inner membrane or insertion of proteins into the inner membrane (Bauer et al., 2000). The TIM23 complex functions in translocation through the inner membrane and is composed of the import channel Tim23, inner membrane–localized accessory proteins (Tim17 and Tim50), and a matrix component (Tim44). In the matrix, mitochondrial Hsp70 contributes to the unidirectional translocation of proteins in an ATP-dependent manner (Matouschek et al., 2000; Voos and Röttgers, 2002). After translocation into the matrix, mitochondrial processing peptidase (MPP) removes the PS from the precursors (Ito, 1999).

In an attempt to characterize the mechanism responsible for the specific protein targeting to these organelles in plant cells, critical sequence motifs involved in protein import into the MT were identified from the PS of F1-ATPase γ-subunit (pFAγ) in protoplasts. This report presents evidence that pFAγ contains multiple motifs that are crucial for protein import into MT. Many of these motifs exhibit overlapping functions, so that synergistic and compensatory relationships are observed at various steps during mitochondrial protein import, despite differences in the amino acid sequences of the various motifs.

RESULTS

The PS of pFAγ Contains Multiple Sequence Motifs for Protein Import into MT

To characterize the molecular mechanism underlying protein import into MT in plant cells, sequence motifs were identified in pFAγ. pFAγ contains the N-terminal 77 amino acids of Arabidopsis thaliana pFAγ with a putative processing site after the 41st amino acid (see Supplemental Figure 1A online). When pFAγ:GFP, a fusion construct between pFAγ and green fluorescent protein (GFP), was transiently expressed in protoplasts from leaf tissues of Arabidopsis, a punctate staining pattern was observed that colocalized with the mitochondrial marker MitoTracker Red (see Supplemental Figure 1B online). In addition, when a lysate of these cells was analyzed by immunoblotting using an anti-GFP antibody, two protein bands at 32 and 38 kD were detected (see Supplemental Figure 1C online). The 32-kD band was the expected size of the processed form of pFAγ:GFP, generated by cleavage at the processing site following its import into MT. Indeed, the size of the processed form was nearly identical to that of an N-terminal deletion mutant, pFAγN280:GFP, generated by cleavage at the processing site following its import into MT. The size of the processed form was 58, 87, and 96% at 8, 16, and 24 HAT, respectively (Figure 1C). The Ala substitution mutants displayed various import efficiencies, contrasting with their localization patterns (Figure 1B). The import efficiency of pFAγ[P1A]:GFP and pFAγ[P6A]:GFP, with an Ala substitution in P3 and P6, respectively, was similar to that of pFAγ:GFP at 24 HAT. However, pFAγ[P3A]:GFP exhibited a slight decrease in import efficiency at early time points. This raised the possibility that P3 contains a sequence motif that functions in an early step of the import process. By contrast, pFAγ[P2A]:GFP, pFAγ[P4A]:GFP, and pFAγ[P5A]:GFP, which had Ala substitutions in P2, P4, and P5, respectively, displayed import efficiencies of 28, 18, and 12% at 8 HAT and 56, 51, and 41% at 24 HAT, respectively. These efficiencies were significantly lower than that of pFAγ:GFP (Figure 1C), indicating that P2, P4, and P5 contain sequence motifs that are important for protein import into MT.

To examine the import of the mutant and the full-length pFAγ:GFP constructs at the biochemical level, protein extracts were prepared from protoplasts at 8, 16, and 24 HAT and analyzed by immunoblotting using anti-GFP antibody. The import efficiency of pFAγ:GFP was 58, 87, and 96% at 8, 16, and 24 HAT, respectively (Figure 1C). The Ala substitution mutants displayed various import efficiencies, contrasting with their localization patterns (Figure 1B). The import efficiency of pFAγ[P1A]:GFP and pFAγ[P6A]:GFP, with an Ala substitution in P3 and P6, respectively, was similar to that of pFAγ:GFP at 24 HAT. However, pFAγ[P3A]:GFP exhibited a slight decrease in import efficiency at early time points. This raised the possibility that P3 contains a sequence motif that functions in an early step of the import process. By contrast, pFAγ[P2A]:GFP, pFAγ[P4A]:GFP, and pFAγ[P5A]:GFP, which had Ala substitutions in P2, P4, and P5, respectively, displayed import efficiencies of 28, 18, and 12% at 8 HAT and 56, 51, and 41% at 24 HAT, respectively. These efficiencies were significantly lower than that of pFAγ:GFP (Figure 1C), indicating that P2, P4, and P5 contain sequence motifs that are important for protein import into MT.

To characterize the molecular mechanism underlying protein import into MT, critical sequence motifs involved in protein import into the MT were identified from the PS of F1-ATPase γ-subunit (pFAγ) in protoplasts. This report presents evidence that pFAγ contains multiple motifs that are crucial for protein import into MT. Many of these motifs exhibit overlapping functions, so that synergistic and compensatory relationships are observed at various steps during mitochondrial protein import, despite differences in the amino acid sequences of the various motifs.
Figure 1. P2, P4, and P5 Segments Contain the Critical Motif for Mitochondrial Protein Import.

(A) Sequences of the Ala substitution mutants. The mutants were fused to GFP.

(B) Localization of Ala substitution mutants. The indicated constructs were introduced to protoplasts, and their localization was observed at 24 HAT. Protoplasts were stained with MitoTracker Red. Green, GFP; red, MitoTracker Red Bar = 20 μm.

(D) Western blot analysis showing the import efficiency of the Ala substitution mutants. The blots were probed with anti-GFP, anti-AALP, and anti-Porin antibodies.

(E) The effects of thermolysin and 0.5% Triton X-100 on the import efficiency of the Ala substitution mutants. The blots were probed with anti-GFP, anti-AALP, and anti-Porin antibodies.

(F) Summary table showing the import efficiency of the Ala substitution mutants under different conditions.
gentle protoplast lysates were treated with thermolysin in the presence and absence of Triton X-100 and analyzed by immunoblotting using anti-GFP antibody. In these conditions, cytosolic proteins or MOM proteins exposed to the cytosol are sensitive to thermolysin. By contrast, proteins within MT are protected from degradation by thermolysin but become sensitive to thermolysin in the presence of Triton X-100. The tightly folded core domain of GFP is known to be resistant to proteolytic degradation by thermolysin. Thus, only the N-terminally exposed regions are degraded by thermolysin, and the product is a smaller protein species (Hyunjong et al., 2004; Lee, D.W. et al., 2009). The processed forms of all the Ala substitution mutants pFAγ[P2A]:GFP, pFAγ[P3A]:GFP, pFAγ[P4A]:GFP, and pFAγ[P5A]:GFP were protected from thermolysin and became sensitive to thermolysin in the presence of Triton X-100, indicating that they are imported into MT (Figure 1E). However, the precursors of all these reporter constructs with exception of pFAγ[P5A]:GFP were largely sensitive to thermolysin, indicating that the precursors were associated with the surface of MT. In the case of pFAγ[P5A]:GFP, the thermolysin treatment also increased the amount of the degradation band at 27 kD, indicating that a proportion of the precursors were associated with the surface of MT. At the same time, a proportion of the precursors was resistant to thermolysin but became sensitive to thermolysin in the presence of Triton X-100, raising the possibility that they are imported into MT but not processed into mature forms or protected by a membranous structure.

In P4A and P5A mutants, the highly conserved Arg residue (-2R) and the processing site were replaced with Ala residues, respectively. The Arg residue is thought to be critical for precursor processing (Niidome et al., 1994). Therefore, it is possible that the observed reduction in import efficiency of these PS mutants, in particular pFAγ[P5A]:GFP, was caused by defective processing of the imported precursors, rather than by defects in the import process itself. In fact, the presence of pFAγ[P5A]:GFP precursors that are resistant to thermolysin raised this possibility. To test this possibility, we determined the exact location of the thermolysin-resistant pFAγ[P5A]:GFP precursors. Transformed protoplasts were gently lysed and treated with thermolysin. After inactivation of thermolysin, the lysates were further lysed by sonication and separated into the soluble and membrane fractions by ultracentrifugation. In this experimental scheme, precursors located in the cytoplasm should be degraded by thermolysin and the unprocessed precursors in mitochondrial matrix should be protected from thermolysin and detected in the soluble fraction after separation by ultracentrifugation. Indeed, the matrix protein IDH was protected from thermolysin-mediated degradation and detected in the soluble fraction. The thermolysin-resistant pFAγ[P5A]:GFP precursors were detected in the pellet fraction (see Supplemental Figure 2 online), indicating that they are not fully imported into MT but rather associated with mitochondrial membranes. This result supports the notion that the Ala substitution at P5 may not affect the processing. Consistent with this notion, pFAγ[P5aA]:GFP, which had an identical Ala substitution to pFAγ[P5A] at the processing site, did not show any defect in protein import into MT in protoplasts (Figure 3D).

To further confirm this finding, we performed in vitro import experiments using wild-type and mutant PS constructs (FAγ, FAγ[P2A], FAγ[P3A], FAγ[P4A], and FAγ[P5A]) (Whelan et al., 1995). For in vitro experiments, the full-length FAγ constructs with these Ala substitution mutations in the PS were used instead of the GFP fusion constructs. FAγ, FAγ[P2A], FAγ[P3A], FAγ[P4A], and FAγ[P5A] gave import efficiencies of 23, 2.9, 3.4, 16.6, and 18.5%, respectively (see Supplemental Figure 3 online), indicating that Ala substitution in P2 and P3 severely affected the import efficiency, whereas Ala substitution in P4 and P5 had only minor effect on import (Figure 1C; see Supplemental Figure 3 online). Thus, the import efficiency of these mutants in vitro was different from their import efficiency in protoplasts. This difference may be caused by a difference in import conditions, such as the amount of precursors and the time course we used in these experiments. However, despite the difference in the import efficiency between the protoplast and in vitro system, the processed forms of all the Ala substitution mutants were identical in size to the processed form of the wild-type construct (Figure 1F; see Supplemental Figure 3 online). In addition, in the presence of valinomycin, an inhibitor of protein import into MT (Whelan et al., 1995), none of these constructs produced proteinase K-resistant mature proteins, confirming that the processed forms are generated by MPP after their import into mitochondrial matrix. Moreover, despite the Ala

**Figure 1.** (continued).  
(C) Import efficiency of Ala substitution mutants. Protein extracts from protoplasts at the indicated time points were analyzed by immunoblotting using anti-GFP antibody. The import efficiency was calculated from the signal intensity of the processed form over that of the total expressed proteins (processed forms plus precursors). Pre, precursor; Pro, processed form. Error bars indicate SD.  
(D) Subcellular fractionation of Ala substitution mutants. Protein extracts were separated into soluble and pellet fractions by ultracentrifugation and analyzed by immunoblotting using anti-GFP antibody. Porin and AALP that were used as markers of the membrane and soluble fractions, respectively, were detected using their corresponding antibodies. GFP alone was included as a negative control. Asterisks, the intermediate form; P, pellet (membrane) fraction; Pre, precursor; Pro, processed form; S, soluble fraction; T, total.  
(E) Thermolysin sensitivity of Ala substitution mutants. Protoplast lysates were treated with thermolysin in the presence (+) or absence (−) of 0.5% Triton X-100 for 30 min and analyzed by immunoblotting using anti-GFP antibody. Asterisk, the intermediate form; D, degradation product; Pre, precursor, Pro, processed form.  
(F) Import of Ala substitution mutants into MT in vitro. [35S]−labeled precursors were incubated with purified MT for 10 min. After proteinase K treatment, MTs were pelleted and protein extracts from MTs were separated by SDS-PAGE. Valinomycin was used to inhibit the protein import into the mitochondrial matrix. Pre, precursor; Pro, processed protein.
import efficiency of pFA\textsubscript{Y}[\Delta P4a]:GFP was similar to that of the Ala substitution mutants pFA\textsubscript{Y}[P4a]:GFP and pFA\textsubscript{Y}[P4aA]:GFP (Figures 2D and 3C). Taken together, these results support the notion that the import defects observed in Ala substitution in P2, P3, P4, and P5 segments are not directly correlated with any possible alteration of their secondary structure by Ala substitution.

### P2, P4, and P5 Segments Contain Critical Sequence Motifs RLLPSIAARP, DQEEG, and VVRNR, Respectively

To further define the critical sequence motifs in P2, P4, and P5, these segments were further divided into two subdivisions containing five residues (indicated by “a” and “b” for the first and second 5-amino acid subdivisions, respectively). Each of these subdivisions was then substituted with five Ala residues (Figure 3A). The mutants were fused to GFP, and the resulting constructs were introduced into protoplasts to examine the import efficiency, pFA\textsubscript{Y}[P2aA]:GFP and pFA\textsubscript{Y}[P2bA]:GFP, containing Ala substitutions at the first and second 5-amino acid subdivision of P2, respectively, exhibited import efficiencies of 81 and 84% at 24 HAT, respectively (Figure 3B), indicating that their import efficiency is higher than that of pFA\textsubscript{Y}[P2A]:GFP but lower than that of wild-type pFA\textsubscript{Y}:GFP. It is possible that the sequence motifs in P2a and P2b are functionally redundant and both motifs are required for full activity. Alternatively, it is possible that the sequence motifs in P2a and P2b may constitute a single motif and Ala substitution in P2a or P2b renders the motif partially defective in protein import into MT.

To define the sequence motif in P4, the import efficiency of pFA\textsubscript{Y}[P4aA]:GFP and pFA\textsubscript{Y}[P4bA]:GFP was examined. These constructs contained Ala substitutions in the first and second 5-amino acid subdivision of P4, respectively. The import efficiency of pFA\textsubscript{Y}[P4aA]:GFP was 58% at 24 HAT, similar to that of pFA\textsubscript{Y}[P4A]:GFP. By contrast, the import efficiency of pFA\textsubscript{Y}[P4bA]:GFP was similar to that of pFA\textsubscript{Y}:GFP. These results indicate that the DQEEG sequence in P4a is critical for protein import into MT (Figure 3C). In addition, in the DQEEG motif, all five amino acid residues were critical for import (see Supplemental Figure 6 online). This motif contains multiple acidic amino acids. This represents an unusual feature, as the lack of acidic residues is a characteristic property of the PS (von Heijne, 1986). Next, the import efficiency of pFA\textsubscript{Y}[P5aA]:GFP and pFA\textsubscript{Y}[P5bA]:GFP, containing Ala substitutions at the first and second 5-amino acid subdivisions of P5, respectively, was examined. pFA\textsubscript{Y}[P5aA]:GFP, but not pFA\textsubscript{Y}[P5aA]:GFP, exhibited a significant defect in mitochondrial protein import, as was observed with pFA\textsubscript{Y}[P5A]:GFP (Figure 3D). Similar to the DQEEG motif, all five amino acid residues in the VVRNR motif were important for the import process (see Supplemental Figure 6 online), suggesting that this is the combination of the 5 amino acids that is important.

pFA\textsubscript{Y}[P4a5bA]:GFP contained double mutations in DQEEG and VVRNR. In protoplasts, pFA\textsubscript{Y}[P4a5bA]:GFP produced only 38-kD precursors with a small amount of a degradation product at the 28-kD position (Figure 3E), indicating that the protein import into MT was almost completely inhibited. Thus, these two motifs are most crucial for protein import into MT, and upon mutation of these motifs, the PS loses the ability to target to MT.
Figure 2. The Internal Deletion Mutants of pFAγ Reveal Regions Critical for Protein Import into MT.

(A) Sequences of the deletion mutants. The mutant constructs were fused to GFP. Bold letter represents the highly conserved Arg (R) residue located at the -2 position from the processing site.

(B) Localization of the deletion mutants. The deletion mutants were introduced to protoplasts and their localization was observed by fluorescence microscopy at 24 HAT. Green, GFP; red, autofluorescence of chlorophyll. Bars = 20 μm.

(C) Effect of the deletions on mitochondrial import. Protoplasts were transformed with the indicated deletion mutant constructs, and protein extracts from the protoplasts were analyzed by immunoblotting using anti-GFP antibody. The import efficiency was calculated from the signal intensity of the processed form over that of the total expressed proteins (processed forms plus precursors). Pre, precursor; Pro, processed form. Error bars indicate SD.

(D) Subcellular localization and import efficiency of pFAγ[ΔP4a]:GFP. Protoplasts were transformed with pFAγ[ΔP4a]:GFP and its localization was examined. The import efficiency was calculated as described in Figure 1C. To simplify the labels, GFP was omitted from the construct names. Green, GFP; red, autofluorescence of chlorophyll. Pre, precursor; Pro, processed form. Bar = 20 μm.
Figure 3. The Sequence Motifs DQEEG and VVRNR in P4 and P5, Respectively, Are Crucial for Protein Import into MT.

(A) Sequences of Ala substitution mutants of 5-amino acid subdivisions.

(B) to (D) Localization and import efficiency of 5-amino acid subdivision mutants. Protoplasts were transformed with pFAγ[P2aA]:GFP or pFAγ[P2bA]:GFP (B), pFAγ[P4aA]:GFP or pFAγ[P4bA]:GFP (C), or pFAγ[P5aA]:GFP or pFAγ[P5bA]:GFP (D), and the localization of the GFP reporter proteins was examined. In addition, the import efficiency was determined as described in Figure 1C. The graph represents the average import efficiency obtained from three independent experiments. Green, GFP; red, autofluorescence of chlorophyll. Error bars indicate SD. Pre, precursor; Pro, processed form. Bars = 20 μm.

(E) Localization and import efficiency of pFAγ[P4a5bA]:GFP. Two subdivisions, P4a and P5b, were simultaneously substituted with Ala residues and fused to GFP. Pre, precursor. Bar = 20 μm.
The DQEEG and VVRNR Motifs in P4 and P5, Respectively, Are Crucial for Crossing the Mitochondrial Membranes

The mitochondrial import process can be divided into multiple steps. These include the cytosolic navigation to MT, binding to mitochondrial TOM receptors, and translocation through outer and inner mitochondrial membranes (Esaki et al., 2004; Chacinska et al., 2009). To define the specific protein import steps affected in the Ala substitution mutants, protein extracts from protoplasts transformed with pFAγ[P4aA]:GFP or pFAγ[P5bA]:GFP were separated into soluble and membrane fractions by ultracentrifugation and analyzed by immunoblotting using anti-GFP antibody. Anti-porin and anti-AALP antibodies were used as markers of subcellular fractionation of pFAγ.

Figure 4. Precursors of pFAγ[P4aA]:GFP and pFAγ[P5bA]:GFP Display Differential Sensitivity to Thermolysin.

(A) Subcellular fractionation of pFAγ[P4aA]:GFP and pFAγ[P5bA]:GFP proteins. Protein extracts from protoplasts were separated into soluble and pellet fractions by ultracentrifugation and analyzed by immunoblotting using anti-GFP antibody. Anti-porin and anti-AALP antibodies were used as markers of the membrane and soluble fractions, respectively. T, total; I, intermediate; P, pellet (membrane) fraction; Pre, precursor; Pro, processed form; S, soluble fraction; T, total.

(B) Thermolysin sensitivity of pFAγ[P4aA]:GFP and pFAγ[P5bA]:GFP. Thermolysin sensitivity was determined as described in Figure 1E. D, degradation product.

To further define the specific steps affected by these motifs, the thermolysin sensitivity of pFAγ[P4aA]:GFP and pFAγ[P5bA]:GFP was examined. The precursor of pFAγ[P4aA]:GFP was also largely sensitive to thermolysin. However, the pFAγ[P5bA]:GFP precursors were mainly resistant but became sensitive to thermolysin in the presence of 0.5% Triton X-100 (Figure 4B). These data indicate that although both mutant precursors were associated with membranes, this association occurs at different stages. The processed forms of both mutant proteins became sensitive to thermolysin upon treatment with Triton X-100, confirming that the processed proteins are located within MT (Figure 4B). Based on these results, the DQEEG motif is involved in an earlier step than the VVRNR motif, and both steps occur while the precursors are passing through the import channel.

VVRNR-Like Sequence Motifs Are Found in the PSs of Several Arabidopsis Mitochondrial Proteins

To assess how frequently the two critical motifs DQEEG and VVRNR are found in the PSs of mitochondrial proteins, we used an approach that has been previously used to compare the sequence motifs of transit peptides (Lee et al., 2008). A total of 198 Arabidopsis mitochondrial proteins that are located in the MT were identified using the Uniprot database. The first 50 amino acids of these proteins were used in a global alignment to identify motifs that were identical or similar to DQEEG or VVRNR. The N-terminal 50 residues were chosen because the DQEEG and VVRNR motifs of pFAγ are located within this region. Of the 198 PSs, 35 and 40 PSs were predicted to contain DQEEG-like and VVRNR-like motifs, respectively. Statistical significance of the global alignment scores was assessed by calculating P values. In both cases, we used the P value of global alignment scores lower than 0.2, and the predicted sequence motif that had the P value lower than 0.2 was defined as a -like motif (see Supplemental Data Sets 1 and 2 online). In addition, six PSs were predicted to contain both DQEEG-like and VVRNR-like motifs. The PS (N-terminal 74 amino acids) of NADH dehydrogenase (ubiquinone) iron-sulfur protein 8-B, pNDS8B, was predicted to contain DQEEG-like and VVRNR-like motifs at amino acids 44 to 49 (DDEEA, P = 0.024) and 12 to 16 (ALRAR, P = 0.064), respectively (Figure 5A). To test whether the two motifs in pNDS8B play a role in mitochondrial import, the motifs were replaced with Ala residues and the resulting mutants were fused to GFP to create pNDS8B[DDEEA/AAAAA]:GFP and pNDS8B[ALRAR/AAAAA]:GFP. These mutants or the wild-type construct, pNDS8B:GFP, were transformed into protoplasts and their localization was examined. All constructs exhibited a punctate staining pattern, indicating that they were targeted to MT (Figure 5B). To determine the import efficiency of these mutants, protein lysates from the protoplasts were analyzed by immunoblotting using an anti-GFP antibody. The import efficiency of the wild-type construct, pNDS8B:GFP, was 74% at 24 HAT (Figure 5C). Similarly, the import efficiency of pNDS8B[DDEEA/AAAAA]:GFP was 69% at 24 HAT (Figure 5D), indicating that substitution of the DDEEA motif with Ala residues had only marginal effect on the mitochondrial import of pNDS8B. Although this result suggests that the DDEEA motif is not important for mitochondrial protein import, it cannot be excluded that another sequence motif was able to compensate for the loss of the DDEEA. By contrast, the import efficiency of pNDS8B[ALRAR/AAAAA]:GFP was 43% and an intermediate...
The PS of ATP synthase subunit β-2 (pFAβ) was predicted to contain a VNRNPR motif between amino acids 23 and 28 (P = 0.070), similar to the VVRNR motif of pFAγ (Figure 5E). To examine the importance of the VNRNPR for mitochondrial import, the motif was replaced with Ala residues and the resulting mutant was fused to GFP to give pFAβ[VNRNPR/AAAAA]:GFP. This mutant or the wild-type construct pFAβ:GFP was transformed into protoplasts and their localization was examined. Both proteins exhibited punctate fluorescence, indicating they were targeted to MT (Figure 5F). Immunoblotting was used to examine the import efficiencies of these proteins. The wild-type and mutant proteins showed 67 and 44% of the import efficiency, respectively (Figures 5G and 5H), indicating that the VNRNPR motif plays an important role in protein translocation across the mitochondrial membranes. Together, these results suggest that VNRNPR-like motifs present in PSs of other mitochondrial proteins play an important role in mitochondrial protein import.
import. Since we found no evidence that DEQEG-like motifs contribute to mitochondrial import of other proteins, it is possible that this motif is unique to pFAγ.

pFAγ Contains Additional Sequence Motifs That Function Compensatorily or Synergistically in Various Steps of Mitochondrial Protein Import

The PS was further analyzed to investigate the presence of additional sequence motifs that had not been identified during the first round of Ala substitution mutations. It was reasoned that functionally compensatory motifs might not be identified by Ala substitution of a single motif. Indeed, PSs are known to contain multiple Tom20 binding sites (Rimmer et al., 2011; Yamamoto et al., 2011). In addition, functionally compensatory sequence motifs present in the transit peptide of the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (RbcS) (Lee et al., 2006). Thus, a new set of Ala substitution mutants was generated into two subdivisions in a combinatorial manner (Figure 6A). These mutants were fused to GFP, and the resulting constructs were introduced into protoplasts. The import efficiency was evaluated by immunoblot analysis using an anti-GFP antibody (Table 1). Among the double mutant constructs, a large number of mutants exhibited significantly lower import efficiencies compared with their single mutants. These results indicate that pFAγ contains numerous additional motifs. The double Ala substitution mutants were grouped into two different categories. In one group, the substitution of either single motif caused negligible inhibitory effects (<10%) on import efficiency, but simultaneous substitution of both motifs caused an import efficiency reduction >50%. In the second group, Ala substitution of single subdivisions did not have any effect on import efficiency, but their Ala substitutions greatly aggravated the import efficiency of other mutants that had Ala substitution on critical motifs such as those in P2a, P2b, P4a, and P5b. The motifs in the first and second groups were defined as functionally compensatory and synergistic, respectively.

Among the double substitution mutants (Figure 6A), pFAγ[P3a5aA]:GFP, pFAγ[P3a5aA]:GFP, pFAγ[P3b5aA]:GFP, and pFAγ[P4b5aA]:GFP belonged to the first group. These mutants exhibited significant defects in mitochondrial protein import, although their single Ala substitution mutants did not significantly affect protein import efficiency. pFAγ[P3a4bA]:GFP, pFAγ[P3a5aA]:GFP, pFAγ[P3b5aA]:GFP, and pFAγ[P4b5aA]:GFP, which had Ala substitutions in a combination of two subdivisions (P3a/P4b, P3a/P5a, P3b/P5a, and P4b/P5a, respectively) primarily produced precursors with only a small portion of processed forms. Thus, these mutants yielded import efficiencies of 33, 32, 45, and 24% at 24 HAT, respectively (Figure 6B, Table 1). In addition, pFAγ[P3a5aA]:GFP, pFAγ[P3b5aA]:GFP, and pFAγ[P4b5aA]:GFP produced a small portion of intermediate forms. However, despite the lower import efficiency, all of these mutants produced the typical mitochondrial pattern (Figure 6C), indicating that they are targeted to MT but not imported into MT. Consistent with this hypothesis, precursors of these constructs were detected in the membrane fraction following ultracentrifugation (Figure 6D). The behavior of the processed forms differed slightly among these constructs. The processed forms of pFAγ[P3a5aA]:GFP, pFAγ[P3b5aA]:GFP, and pFAγ[P4b5aA]:GFP were detected in the soluble fraction, but the processed form of pFAγ[P3a4bA]:GFP was detected in the pellet fraction. To further define the steps affected by mutations of these motifs, the thermolysin sensitivity of these constructs was examined. All the mutants were resistant to thermolysin (Figure 6E). However, when pretreated with Triton X-100, the mutants became sensitive to thermolysin and produced the 27-kD form, with the exception of pFAγ[P3a4bA]:GFP. This mutant appeared completely degraded. It is possible that when these constructs were trapped at the import channel, the GFP moiety of pFAγ[P3a4bA]:GFP was unfolded so that it became sensitive to thermolysin, whereas the GFP moiety of pFAγ[P3a5aA]:GFP, pFAγ[P3b5aA]:GFP, and pFAγ[P4b5aA]:GFP exists as a folded form. Mitochondrial proteins are unfolded during transport through the import channel (Matouschek et al., 2000; Voos and Röttgers, 2002).

The second group contained six constructs: pFAγ[P2a3aA]:GFP, pFAγ[P2a5aA]:GFP, pFAγ[P2b3aA]:GFP, pFAγ[P2b4aA]:GFP, pFAγ[P2b5aA]:GFP, and pFAγ[P3b4aA]:GFP (Figure 7A). These constructs exhibited a great reduction in protein import efficiency compared with their corresponding single Ala substitution mutants. pFAγ[P2a3aA]:GFP contained Ala substitutions in both P2a and P3a. The single substitution mutations in P2a and P3a had a moderate effect and almost no effect on protein import, yielding 81 and 95% import efficiency at 24 HAT, respectively (Table 1, Figure 5B). However, pFAγ[P2a3aA]:GFP yielded an import efficiency of only 32% (Figure 7B), indicating that simultaneous mutations in both P2a and P3a greatly hamper protein import into MT. pFAγ[P2a3aA]:GFP produced the typical mitochondrial pattern, indicating that it is targeted to MT but not imported into MT (Figure 7C). Consistent with the images, both precursor and processed forms were detected in the membrane fraction following ultracentrifugation (Figure 7D). In addition, the processed form was resistant but became sensitive to thermolysin when pretreated with Triton X-100, indicating its tight association with mitochondrial membranes. These results suggest that the sequence motif in P3a functions cooperatively with P2a for translocation through the membrane.

pFAγ[P2a5aA]:GFP, containing Ala substitutions in both P2a and P5a, showed very little processed form at 24 HAT (Figure 7B), indicating that the P5aA mutation alone does not have much inhibitory effect on protein import efficiency but has a strong inhibitory effect when combined with the P2aA mutation. When examined under a fluorescence microscope, no GFP signal was detected. When GFP is trapped at the chloroplast envelope membrane, it does not fold properly, and as a result, does not produce GFP fluorescence (Lee et al., 2008). Thus, pFAγ[P2a5aA]:GFP may have been trapped in the membrane in an unfolded form. To test this hypothesis, protoplasts were immunostained with anti-GFP antibody and the localization of GFP was examined. A punctate staining pattern was evident (Figure 7F), indicating that it was targeted at the mitochondrial membrane. Consistent with this localization pattern, the precursor of pFAγ[P2a5aA]:GFP was detected in the membrane fraction (Figure 7D). Moreover, the protein was resistant but became sensitive to thermolysin when pretreated with Triton.
Similar to pFAγ[P3a4bA]:GFP (Figure 6E), the protein was completely degraded by thermolysin following Triton X-100 treatment (Figure 7E). These results suggest that the motif in P5a functions together with the motif in P2a for translocation through the import channel.

pFAγ[P2b3aA]:GFP, containing Ala substitutions in P2b and P3a, produced only small amounts of intermediate forms but no processed forms (Figure 7B). Mutation of P2b alone resulted in 84% import efficiency, but mutation of P3aA alone did not affect import efficiency, indicating that the P3aA mutation greatly aggravates the moderate inhibitory effect of the P2bA mutation. When examined with a fluorescence microscope, pFAγ[P2b3aA]:GFP produced a diffuse cytosolic pattern (Figure 7C). When separated by ultracentrifugation, significant amounts of the protein were detected in both the soluble and the pellet fractions (Figure 7D). However, the majority of the protein was sensitive to thermolysin even in the absence of Triton X-100 treatment (Figure 7E). These results suggest that the two motifs in P2b and P3a are crucial for binding to MT. Additionally, the motif in P2a functions cooperatively with that in P3a.

pFAγ[P2b4aA]:GFP contained Ala substitutions in both P2b and P3a. This mutant exhibited <10% import efficiency at 24 HAT, but pFAγ[P2bA]:GFP and pFAγ[P4aA]:GFP exhibited 84 and 58% import efficiencies, respectively (Figure 7B, Table 1).

Figure 6. pFAγ Contains Multiple Functionally Compensatory Sequence Motifs.

(A) Sequences of 5–amino acid double substitution mutants. Amino acid sequences of pFAγ[P3a4bA], pFAγ[P3a5aA], pFAγ[P3b5aA], and pFAγ[P4b5aA] are shown.

(B) Import of 5–amino acid double substitution mutants into MT. Protein extracts from transformed protoplasts at the indicated time points after transformation were analyzed by immunoblotting using anti-GFP antibody. I, intermediate; Pre, precursor; Pro, processed form.

(C) Localization of a subset of 5–amino acid double substitution mutants. Protoplasts were transformed with the indicated constructs, and the localization of reporter proteins was examined at 24 HAT. Green, GFP; red, autofluorescence of chlorophyll. Bars = 20 μm.

(D) Subcellular fractionation. Protein extracts from protoplasts were separated into soluble and membrane fractions by ultracentrifugation and analyzed by immunoblotting using anti-GFP, anti-porin, and anti-AALP antibodies. P, pellet (membrane) fraction; S, soluble fraction; T, total.

(E) Thermolysin sensitivity. Thermolysin sensitivity was determined as described in Figure 1E. D, degradation product.
Image analysis revealed an aggregate pattern in the cytosol (Figure 7C). When fractionated by ultracentrifugation, the precursors were detected in the pellet fraction. This is likely due to aggregation of the protein. In addition, the protein was sensitive to thermolysin (Figure 7E), similar to pFA$_g$[P4aA]:GFP. This is consistent with a cytosolic localization. These results suggest that the combined mutations in the sequence motifs of P2b and P4a affect an early transport step, such as the binding of proteins to MT, during protein import into MT.

pFA$_g$[P2b5aA]:GFP contained Ala substitutions in both P2b and P5a and was localized to MT. However, this mutant displayed an import efficiency of 49% at 24 HAT (Figures 7B and 7C, Table 1). When separated by ultracentrifugation, the precursors were primarily detected in the membrane fraction, while processed and intermediate forms were detected in the soluble fraction (Figure 7D). This confirms that the precursors were trapped at the import channel. In addition, the protein was resistant to thermolysin but became sensitive when pretreated with Triton X-100 (Figure 7E). These results suggest that the motif in P5a may function together with that in P2b for translocation across the membranes.

pFA$_g$[P3b4aA]:GFP contained Ala substitutions in both P3b and P4a. This mutant exhibited an import efficiency of <5% and primarily produced the precursor form. In comparison, the import efficiency of pFA$_g$[P3bA]:GFP and pFA$_g$[P4aA]:GFP was 93 and 58%, respectively (Figure 7B). However, pFA$_g$[P3b4aA]:GFP showed a mitochondrial pattern, indicating mitochondrial targeting (Figure 7C). Consistent with this result, the precursors were primarily detected in the pellet fraction (Figure 7D) and sensitive to thermolysin even in the absence of Triton X-100, suggesting that pFA$_g$[P3b4aA]:GFP is loosely associated with MT (Figure 7E). Thus, the motif in P3b may cooperatively function with the DQEEG in P4a at an earlier translocation step.

### Table 1. Import Efficiency of Double Substitution Mutants of pFA$_g$

<table>
<thead>
<tr>
<th>Mutant Name</th>
<th>Import Efficiency</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2a3aA</td>
<td>0.20</td>
<td>Mito</td>
</tr>
<tr>
<td>P2a3bA</td>
<td>0.67</td>
<td>Mito</td>
</tr>
<tr>
<td>P2a4aA</td>
<td>0.49</td>
<td>Mito</td>
</tr>
<tr>
<td>P2a4bA</td>
<td>0.23</td>
<td>Mito</td>
</tr>
<tr>
<td>P2a5aA</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>P2a5bA</td>
<td>0.31</td>
<td>Mito</td>
</tr>
<tr>
<td>P2b3aA</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>P2b3bA</td>
<td>0.49</td>
<td>Mito</td>
</tr>
<tr>
<td>P2b5aA</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>P2b5bA</td>
<td>0.33</td>
<td>Mito</td>
</tr>
<tr>
<td>P3a4aA</td>
<td>0.68</td>
<td>Mito</td>
</tr>
<tr>
<td>P3a4bA</td>
<td>0.22</td>
<td>Mito</td>
</tr>
<tr>
<td>P3a5aA</td>
<td>0.16</td>
<td>Mito</td>
</tr>
<tr>
<td>P3a5bA</td>
<td>0.57</td>
<td>Mito/Cyto</td>
</tr>
<tr>
<td>P3b4aA</td>
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<td>No</td>
</tr>
<tr>
<td>P3b4bA</td>
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<td>Mito</td>
</tr>
<tr>
<td>P3b5aA</td>
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<td>Mito</td>
</tr>
<tr>
<td>P3b5bA</td>
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<td>Mito</td>
</tr>
<tr>
<td>P4a4aA</td>
<td>0.12</td>
<td>Mito</td>
</tr>
<tr>
<td>P4a4bA</td>
<td>0.58</td>
<td>Mito</td>
</tr>
<tr>
<td>P4a5aA</td>
<td>0.31</td>
<td>Mito</td>
</tr>
<tr>
<td>P4a5bA</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>P4b5aA</td>
<td>0.12</td>
<td>Mito</td>
</tr>
<tr>
<td>P4b5bA</td>
<td>0.58</td>
<td>Mito</td>
</tr>
</tbody>
</table>

Protoplasts were transformed with the indicated constructs, and the localization of reporter proteins was observed at 24 HAT. Protein extracts were prepared from the transformed protoplasts at each time point and analyzed by immunoblotting using anti-GFP antibody. Import efficiency of various double substitution mutants was determined by protein gel blot analysis using anti-GFP antibody at the indicate time points. Asterisk indicates that localization was detected by immunohistochemistry using anti-GFP antibody; no, no import efficiency.

**IAARP in P2b Exhibits Tom20 Dependency in Protein Import into MT**

To better understand the mechanism of mitochondrial protein import, the role of the MOM receptors was examined. Plant cells contain multiple types of import receptors at the MOM. These include primary import receptors Tom20 and OM64, which are likely to contain secondary binding sites that function in passing the precursor protein through the MOM as described by the binding chain hypothesis, and METAXIN, Tom40, and small Toms that are also likely to contain secondary binding sites (Lister et al., 2007). For Tom20, four isoforms (TOM20-1 to TOM20-4) exist in the Arabidopsis genome. Three of these isoforms, TOM20-2 to TOM20-4, are known to be expressed and functional (Lister et al., 2007). To examine the role of these import receptors, protoplasts were prepared from *tom20-2*...
Figure 7. pFAγ Contains Multiple Functionally Synergistic Sequence Motifs.

(A) Sequences of 5-amino acid double substitution mutants. Amino acid sequences of pFAγ[P2a3aA], pFAγ[P2a5aA], pFAγ[P2b3aA], pFAγ[P2b4aA], pFAγ[P2b5aA], and pFAγ[P3b4aA] are shown.

(B) Import of 5-amino acid double substitution mutants into MT. Protein extracts from protoplasts at the indicated time points after transformation were analyzed by immunoblotting using anti-GFP antibody. I, intermediate; Pre, precursor; Pro, processed form.

(C) Localization of a subset of 5-amino acid double substitution mutants. Protoplasts were transformed with the indicated constructs and the localization of reporter proteins was examined at 24 HAT. Green, GFP; red, autofluorescence of chlorophyll. Bars = 20 μm.

(D) Subcellular fractionation. Protein extracts from protoplasts were separated by ultracentrifugation and analyzed by immunoblotting using anti-GFP, anti-porin, and anti-AALP antibodies. I, intermediate; Pre, precursor; Pro, processed form; S, soluble fraction; T, total.
tom20-3 tom20-4 triple mutant plants. These protoplasts were transformed with pFA\textgamma:GFP, and its localization was examined. In the tom20 triple mutant protoplasts, pFA\textgamma:GFP produced primarily the punctate staining pattern with a minor diffuse cytosolic pattern (Figure 8A), suggesting that the import efficiency was slightly affected in the mutants. To determine its import efficiency in the mutants, protein extracts from the transformed protoplasts were analyzed by immunoblotting using anti-GFP antibody. The amount of the 31-kD processed form at 24 HAT was decreased by 20% in tom20 triple mutant protoplasts compared with the wild-type protoplasts (Figure 8B). This result is consistent with the image analysis results. The import efficiency of pFA\textgamma:GFP in tom20-2 or tom20-4 protoplasts decreased to <10% at 24 HAT compared with the wild-type protoplasts (see Supplemental Figure 7A online). Additionally, the tom20-3 mutation did not significantly affect the import efficiency of pFA\textgamma:GFP, indicating that the Tom20s are functionally redundant. This is consistent with results obtained from in vitro import experiments (Lister et al., 2007). The protein import of pFA\textgamma:GFP was also examined in mtom64 mutant protoplasts, and the import efficiency was identical to that of wild-type protoplasts (see Supplemental Figure 7B online).

Next, the sequence motif involved in Tom20 binding was identified. It was reasoned that the protein import efficiency of the Ala substitution mutants of Tom20 binding sites in tom20 triple mutant protoplasts should be identical to the wild type. Thus, the protein import efficiencies of various Ala substitution mutants in wild-type and tom20 triple mutant protoplasts were compared. From the various Ala substitution mutants, pFA\textgamma[P2aA]:GFP, pFA\textgamma[P2bA]:GFP, pFA\textgamma[P4aA]:GFP, or pFA\textgamma[P5bA]:GFP were selected for transformation into tom20 triple mutants or wild-type protoplasts. The import efficiency was determined by immunoblot analysis using an anti-GFP antibody. Among the four mutants, pFA\textgamma[P2aA]:GFP, pFA\textgamma[P4aA]:GFP, and pFA\textgamma[P5bA]:GFP had a 20% or higher decrease in import efficiency in the tom20 triple mutant protoplasts compared with that of pFA\textgamma:GFP in the wild-type protoplasts (Figure 8C), indicating that these three mutants still exhibit Tom20-dependent protein import into MT. By contrast, the import efficiency of pFA\textgamma[P2bA]:GFP in tom20 triple mutant protoplasts was similar to that of the wild-type protoplasts, indicating that pFA\textgamma[P2bA]:GFP no longer exhibits Tom20-dependent protein import into MT. It is possible that IAARP in P2b is involved in Tom20-dependent protein import into MT. To further confirm this hypothesis, we generated a new mutant pFA\textgamma[ΔP2b]:GFP that contained a deletion of the P2b region. The import efficiency of this mutation was examined in tom20 triple mutant and wild-type protoplasts. The import efficiency was identical in both tom20 triple mutant and the wild-type protoplasts (Figure 8D), supporting the hypothesis that the IAARP motif is involved in Tom20-dependent protein import into MT. Indeed, IAARP in P2b together with the neighboring amino acid Ile in P3a (IAARP) conforms to I\textalpha\textbeta\textbeta\textalpha\textbeta\textbeta\textalpha\textgamma, the consensus sequence of Tom20 binding sites (Okamoto et al., 2002; Rimmer et al., 2011; Yamamoto et al., 2011).

**DISCUSSION**

To date, most studies of mitochondrial import have employed in vitro import experiments using purified MT and in vitro–translated precursors. In this study, transient expression in protoplasts was used to dissect the sequence motifs responsible for protein import into MT. Transient expression in protoplasts has been successfully used to dissect the sequence motifs in transit peptides (Lee et al., 2002; Lee et al., 2006, 2008). Various mutants of pFA\textgamma were fused to GFP and introduced into protoplasts. The localization of GFP with various mutant PSs was examined by fluorescence microscopy, subcellular fractionation by ultracentrifugation, and thermolysin sensitivity. From these analyses, we provide evidence that multiple motifs are involved in protein import into MT. These motifs are dispersed throughout the entire PS, including the region downstream of the processing site. In particular, Ala substitutions of single 10– or 5– amino acid blocks in pFA\textgamma revealed two critical motifs, DQEEG and VVRNR, located in segments P4a and P5b, respectively. Interestingly, VVRNR-like motifs are found in other PSs and appear to play an important role in mitochondrial import; however, it appears that DQEEG is unique to FA\textgamma. In fact, the DQEEG motif is unusual in its composition with three acidic amino acid residues that are rare in PSs (von Heijne, 1986; Huang et al., 2009). These results suggest that PSs of mitochondrial proteins can contain a specific motif as well as a conserved motif. Similar observation has been made with transit peptides of chloroplast proteins (Lee et al., 2008). Additionally, two moderately important motifs were identified in P2a and P2b. The result showing that pFA\textgamma contains only few motifs is rather surprising as many motifs are expected to participate in various steps of the protein import process. Previously, specific motifs were identified, including Tom20 binding sites and mThsp70 binding sites (Okamoto et al., 2002; Obita et al., 2003; Zhang et al., 2010; Rimmer et al., 2011; Yamamoto et al., 2011). Moreover, the hydrophobic amino acid residues in the N-terminal region are important for efficient protein import into plant MT (Duby et al., 2001; Ambard-Bretteville et al., 2003; (Lee and Whelan, 2004).

Based on the identification of only four sequence motifs, two moderate and two critical, in the FA\textgamma PS, it was hypothesized that the motifs in pFA\textgamma may be functionally compensatory, as was observed in the transit peptide of RbcS (Lee et al., 2006). Consistent with this hypothesis, a large number of motifs were

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**Figure 7.** (continued).

(E) Thermolysin sensitivity was determined as described in Figure 1E. D, degradation product.
(F) Immunolocalization of reporter proteins. Protoplasts transformed with pFA\textgamma[P2aA]\textgamma:GFP were stained with MitoTracker Red, fixed with paraformaldehyde on glass slides, and immunostained with anti-GFP antibody, followed by staining with fluorescein isothiocyanate–labeled anti-mouse IgG as the secondary antibody. Bar = 20 μm.
identified using double substitution mutants of 5–amino acid subdivisions that were generated in a combinatorial manner. For example, the motif in P3a was functionally compensatory to those in P4b or P5a, and the motif in P4b was functionally compensatory with that of P5a. However, these functionally compensatory motifs do not exhibit sequence similarity. This phenomenon occurs with the sequence motifs found in transit peptides (Lee et al., 2006, 2008).

In addition to the functionally compensatory sequence motifs, another type of motif was identified. In this class, substitution of these motifs alone did not affect protein import efficiency. However, strong detrimental effects were noticed when the initial substitutions were combined with the substitution mutations of the two critical and two moderate motifs. For example, the motifs IAAIR and SISTQ in P3a and P5a, respectively, are synergistic to RLLPS in P2a; IAAIR and SISTQ in P3a and P5a, respectively, are synergistic to IAARP in P2b; and SPLSS in P3b acts in synergy to DQEEG in P4a. Thus, this study revealed that these motifs possess complex relationships with each other. For example, the motif in P5a has functional redundancy with those in P3a, P3b, and P4b. At the same time, the motif in P5a acts in synergy to that in P2a. Previous studies demonstrated that the PS interacts with various components of TOM/TIM complexes, such as Tom40, Tim23, and Tim50 during translocation through these channels (Pfanner and Geissler, 2001). Accordingly, one possible scenario is that each component of the translocators at the outer and inner membranes may contain multiple sites for the binding of the motifs. Additionally, each single motif may interact with more than one component of the translocators. These interactions may occur sequentially while the PS is passing through the import channel. Moreover, the multiple interactions may occur simultaneously between the multiple sequence motifs in the PS and multiple components of the translocators during translocation through the import channel. All of these interactions may contribute to increasing import efficiency. Accordingly, loss of a single interaction between the components of translocation and sequence motifs, in particular, the functionally compensatory or supportive motifs, may not significantly affect the import process.

Figure 8. The P2b Segment Contains a Sequence Motif for Tom20-Dependent Import into MT.
(A) Localization of pFAγ:GFP in tom20 triple mutant protoplasts. Protoplasts from tom20 triple mutants were transformed with pFAγ:GFP, and the localization of the reporter proteins was examined at 24 HAT. Green, GFP; red, autofluorescence of chlorophyll. Bar = 20 μm.
(B) Import efficiency of pFAγ:GFP in the wild type and tom20-2/20-3/20-4 mutants. Protoplasts were transformed with pFAγ:GFP, and protein extracts were analyzed by immunoblotting using anti-GFP antibody. The import efficiency was determined at 8, 16, and 24 HAT. Error bars indicate SD.
(C) and (D) Import efficiency of various Ala substitution or deletion mutants in the wild-type and tom20-2/20-3/20-4 mutant protoplasts. Protoplasts were transformed with the indicated constructs. The import efficiency was determined as described in Figure 1C. Error bars indicate SD.
To characterize the exact manner in which these motifs function during the import process, it is necessary to identify the factors that recognize these motifs and study this interaction in detail. Various biochemical approaches have been used to study the interaction of PSs with translocator complexes (Zhang et al., 2010; Rimmer et al., 2011; Saitoh et al., 2011). In this study, the localization of these mutants provided insight into the specific steps in which these motifs may be involved (Figure 9). The double mutants P2b3aA and P2b4aA showed a cytosolic pattern of localization, indicating that the motifs in these subdivisions are involved in an early step, such as the binding of precursors to the Tom receptors at the surface of MT. Indeed, IAARP in P2b together with a neighboring Ile in P3a conforms to the consensus sequence \( \phi xx\phi \) of the Tom20 binding site (Okamoto et al., 2002; Obita et al., 2003; Rimmer et al., 2011; Yamamoto et al., 2011). The second group of mutants, P2a3aA and P3b4aA, caused reporter proteins to localize to the mitochondrial membrane and be sensitive to thermolysin, suggesting that the motifs in these mutants are necessary for translocation through the import channel. However, at this stage, the GFP moiety is still accessible to thermolysin. This raises the possibility that these motifs are necessary for the initial stage of translocation, possibly for the binding of the TOM components. The third group of mutants, P2b5aA, P3a4bA, P3a5aA, P3b5aA, and P4b5aA, localized to the mitochondrial membrane but were resistant to thermolysin, suggesting that the proteins are trapped in the import channel. Since the GFP moiety was no longer accessible to thermolysin, this implies that the reporter proteins are translocated further into the channel than the second group. It is possible that the sequence motifs in this group are necessary for binding to the TIM components. A final group containing mutant P2a5aA also localized to the mitochondrial membrane with the GFP moiety unfolded. The phenotype of this mutant raised the possibility that the mutant was defective in exiting into the matrix. A previous study showed that pSu9-DHFR, a chimeric protein consisting of pSu9 and DHFR, undergoes unfolding at stage B during import into MT (Kanamori et al., 1999). The N terminus of the mature region is involved in pulling the protein into the mitochondrial matrix via mTHsp70 (Yamano et al., 2008). Interestingly, RLLPS in P2a exhibits a certain degree of sequence homology to mTHsp70 binding sites such as NRLLLTG and CALLLSAPRR (Okamoto et al., 2002). In addition, P5a belongs to the N-terminal region of the mature portion of reporter proteins. Thus, it is possible that the motifs in the P2a and P5a subdivisions may be involved in pulling the protein into the matrix by serving as the binding site of mTHsp70.

Among the identified sequence motifs, IAARP in P2b was ascertained to be the Tom20 binding site. This conclusion was based on the fact that the protein import efficiency of substitution or deletion mutants in tom20 triple mutants was identical to that of the wild-type plants. Previous studies identified several Tom20 binding motifs, such as LSRL, WKRCM, and LRRAY in the PSs of yeast ALDH, F1-ATPase \( \beta \)-subunit, and Hsp60, respectively (Muto et al., 2001; Saitoh et al., 2007). From these sequences, Tom20 was deduced to bind the consensus sequence \( \phi xx\phi \) (where \( \phi \) indicates a hydrophobic residue and \( x \) indicates any residue) (Okamoto et al., 2002; Obita et al., 2003; Rimmer et al., 2011; Yamamoto et al., 2011). In addition, NMR studies have demonstrated that Tom20 interacts with LRLTA and LRRHF in Oryza sativa threonyl tRNA synthetase, respectively (Zhang et al., 2010). These plant motifs conform to the Tom20 consensus sequence derived from yeast PSs. This suggests that Tom20 recognizes the same \( \phi xx\phi \) motif in plants and yeast, despite the differences in the sequence and topology of Tom20 in these organisms. IAARP (IAARP in P2b and I in P3a) of pFA\( \gamma \) conforms to the consensus sequence \( \phi xx\phi \) and may interact with Tom20. However, the deletion of an Ala substitution of IAARP caused only a 20% decrease in import efficiency in the wild-type protoplasts. This is consistent with previous studies showing that Tom20 is not essential for protein import into MT (Mukhopadhyay et al., 2006; Lister et al., 2007).

Ala is a small hydrophobic amino acid found abundantly in the PSs of mitochondrial proteins. To identify pFA\( \gamma \) sequence motifs, we generated a range of mutants, in which 10–5 amino acid blocks of the PS were replaced with Ala residues. The abilities of these mutants to be efficiently delivered to the MT were tested using in vivo or in vitro import assays. A caveat of this approach is that these mutations may alter the secondary structures and/or hydrophobicity of pFA\( \gamma \). A prediction tool revealed that some of these mutants had secondary structure alterations (see Supplemental Figure 4 online). Some of these mutants with structural changes showed a defect in mitochondrial import efficiencies, supporting the hypothesis that the secondary structure is important for protein import into MT. However, some mutants with modified secondary structures were imported normally, suggesting that secondary structure alterations did not necessarily affect import efficiency. Another group of mutants showed a defect in protein import into MT, despite the absence of predicted alterations in their secondary structures, the mitochondrial import efficiency of some mutants was defective. Moreover, despite the fact that pFA\( \gamma \)[P3b4aA] and pFA\( \gamma \)[P3b4bA] had identical secondary structural changes, pFA\( \gamma \)[P3b4aA] exhibited a severe defect in mitochondrial protein import, whereas pFA\( \gamma \)[P3b4bA] was imported into MT as efficiently as the wild-type construct. Thus, although we cannot exclude definitively the possibility that the secondary structure of proteins affects their mitochondrial import, the results we obtained with pFA\( \gamma \) did not show any clear correlation between secondary structure alterations and import efficiencies. In addition, changes in the hydrophobicity of various segments of pFA\( \gamma \) did not clearly correlate with the defect in mitochondrial import (see Supplemental Data Set 3 online). In fact, the import efficiencies of the internal deletion mutants of each segment were similar to those of the Ala substitution mutants.

Import assays with various mutant PSs were performed using both in vitro and in vivo assays. In some cases, similar results were found, but in some cases, differences were observed (see Supplemental Figure 2 online). This may be due to a variety of reasons; first, in vitro assays are performed using a rabbit reticulocyte lysate, as a plant-based translation system, the wheat germ lysate, does not support mitochondrial import (Dessi et al., 2003). This has been shown to be due to the characteristics of the mature protein; thus, chaperone and folding status may differ in vitro and in vivo (Dessi et al., 2003). Second, the in vitro
Figure 9. Model for Proposed Roles of Sequence Motifs in Translocation of pFAγ into MT.

(A) Functional relationship of sequence motifs in FAγ PS. Segments P1 to P6 represent 10-amino acid block of pFAγ, and “a” and “b” indicate the subdivisions of each P segment. The box below the subdivisions contains the proposed function of each subdivision. Lines above and below the sequence indicate the functionally compensatory and synergistic motifs, respectively. The sequence motifs involved in specific stages of import processes are indicated. Tom20, Trans-OM, Binding, and Trans-IM indicate sequence motifs involved in Tom20 binding, translocation of outer envelope membrane, mitochondrial binding, and translocation of inner envelope membrane, respectively.

(B) A model of pFAγ:GFP import to the mitochondrial matrix. Figures on the left side show the sequence motifs that are functionally related to each other. They can be divided to four major groups depending on the localization of precursors during the import. Figures on the right side represent the localization of proteins when these motifs are mutated singly (indicated by single lines) or simultaneously (indicated by brackets). Black and gray lines represent pFAγ and the GFP moiety, respectively. Boxes in the outer or inner membranes represent the translocon components. IM, inner envelope membrane; IMS, intermembrane space; OM, outer envelope membrane.
assays are performed with radiolabeled proteins, which are in relatively large amounts or concentrated, with often only 25% of the added precursor protein being imported. By contrast, in vivo it is envisaged that all precursor proteins are imported, as shown with the wild-type precursor in these studies. Finally, mitochondrial protein import can take place posttranslationally, the basis of the in vitro assay, but in vivo there may be closer links among translation, binding, and import (Verner, 1983). In fact, binding of mRNA-encoding mitochondrial proteins to MT is proposed to be a mechanism to increase protein targeting specificity and kinetics (Marc et al., 2002; Eliyahu et al., 2010). Thus, it is not unexpected that some differences may be observed between the two systems. The in vitro assays can be widely used to define the components involved, while the in vivo assays give greater insight into the complexity of the process, where a variety of motifs, playing roles in binding, translocation, and processing, with some motifs playing overlapping roles, can be dissected.

The analysis of sequence motifs in PSs is essential to the characterization of the dynamic interaction of the PS with various translocator components during protein import into MT. This study demonstrates that pFAγ contains multiple motifs that are crucial for various steps of the import process. However, the binding proteins for these motifs have not been addressed in this study. To fully understand the exact role of these motifs in the protein import process, further study should be directed toward the identification of the binding proteins and the interaction between these proteins and the motifs.

**METHODS**

**Growth of Plants**

*Arabidopsis thaliana* (Columbia-0) was grown on Gamborg B5 plates (Duchefa) at 40% relative humidity and 22°C with a 16-h/8-h light/dark cycle. Leaf tissues were harvested from 18-d-old plants and used immediately for protoplast isolation. Tom20 single-gene knockout mutants (tom20-2, tom20-3, and tom20-4), Tom20 triple knockout mutant (tom20-2 tom20-3 tom20-4), and mtOM64 knockout mutant (mtom64) were isolated from the T-DNA insertion lines (Lister et al., 2007).

**PCR-Based Mutagenesis and Plasmid DNA Construction**

Construction of F1-ATPase-γ-ps:GFP (pFAγ:GFP) was described previously (Lee, S. et al., 2009). PSs of pNDS88 and pFAβ were isolated by PCR using a forward primer containing the first 20 bases of the PS and a reverse primer containing last 20 bases of the PS and 15 bases corresponding to the N-terminal five amino acids of GFP. The PCR product containing the PS was fused to GFP by PCR using the forward and nos-t primers. The PCR-based mutagenesis approach was used to generate various substitution or deletion mutations in the PS of FAγ as described previously (Lee et al., 2006). Two complementary forward and reverse primers were designed for each mutant (see Supplemental Table 1 online). Using these primers, the first round of PCR was performed to generate 5’ and 3’ fragments, using pFAγ:GFP as a template. The 5’ fragments were amplified using the reverse primers and the common cauliflower mosaic virus 5’ primer. The 3’ fragments were amplified using the forward primers and the common nos terminator (nos-t) primer. The second round of PCR was performed using the primers cauliflower mosaic virus 5’ and nos-t with the 5’ and 3’ PCR products from the first round as templates. For Ala substitution mutations in two 5–amino acid subdivisions, the appropriate individual 5–amino acid subdivision mutants were used as templates. The mutations were introduced as described above for the individual 5–amino acid subdivision mutants. The PCR products were subcloned into a pUC-based expression vector (Jin et al., 2001), and the sequences were confirmed by nucleotide sequencing. Full-length sequence of pFAγ was amplified by PCR using primers FAγ-F1 and FAγ-R1. The PCR product was subcloned into a pUC-based expression vector containing T7 promoter and nos terminator, and the sequences were confirmed by sequencing.

**Transient Expression and in Vivo Targeting of Reporter Proteins**

All plasmid constructs were purified using a plasmid purification kit (Qiagen) according to the manufacturer’s protocol. Polyethylene glycol-mediated transformation was used to introduce the plasmids into protoplasts prepared from *Arabidopsis* leaf tissues (Jin et al., 2001; Bae et al., 2008). Images were taken using a cooled charge-coupled device camera and a Zeiss Axioskup fluorescence microscope and presented in pseudocolor format (Kim et al., 2001). For Mitotracker staining, the transformed protoplasts were incubated in W5 media (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 5 mM Gic, and 1.5 mM MES, pH 5.6) containing 100 nM MitoTracker Red (Molecular Probes) in the dark for 10 min. After staining, the protoplasts were washed twice with fresh W5 media and observed with a fluorescence microscope after 2 h of incubation.

**Immunohistochemistry**

Immunohistochemistry was performed as described previously (Lee et al., 2006). Transformed protoplasts stained with MitoTracker Red were placed on poly-L-Lys-coated glass slides and fixed by 3% paraformaldehyde. The fixed protoplasts were washed with TSW buffer (10 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.25% gelatin, 0.02% SDS, and 0.1% Triton X-100) three times and incubated with an anti-GFP antibody (Calbiochem) for 12 h at 4°C. The protoplasts were then washed and incubated with fluorescein isothiocyanate–conjugated goat anti-mouse IgG (Sigma-Aldrich) for 2 h at room temperature.

**Immunoblot Analysis of Protoplast Extracts**

Protein extracts were prepared from transformed protoplasts as described previously (Park et al., 1997). Monoclonal anti-GFP (Calbiochem) or rabbit polyclonal anti-GFP antibodies (Bio-App) were used as the primary antibody to detect GFP proteins. The amount of proteins on the immunoblots was quantified by measuring the band intensity using Multi-gauge (LAS 3000) and ImageJ.

**Subcellular Fractionation**

Transformed protoplasts were harvested and suspended in sonication buffer (20 mM Tris-HCL, 2.5 mM MgCl₂, 2 mM EGTA, 1 mM EDTA, and 160 mM NaCl). Cellular membranes were disrupted by sonication. After discarding debris, the soluble fractions were subjected to ultracentrifugation at 100,000g for 1 h. Soluble and pellet fractions were collected separately and placed in denaturation buffer (2.5% SDS and 2% β-mercaptoethanol), Anti-porin (Calbiochem), anti-AALP (Song et al., 2006), and anti-IDH antibodies (Agrisera) were used to confirm the separation of the membrane, soluble, and mitochondrial matrix proteins, respectively.

**Thermolysin Treatment**

Protoplasts were collected in microtubes and gently lysed in HMS buffer (330 mM sorbitol, 50 mM HEPES-KOH, pH 7.8, and 3 mM MgCl₂) by pipetting 15 times. Thermolysin (0.5 μM) (Sigma-Aldrich) was added to
each sample as described previously (Forke et al., 2003; Lee et al., 2006), and the samples were incubated for 30 min on ice. In addition, samples were treated with 0.5% Triton X-100 to solubilize the mitochondrial membranes. Subsequently, the protein extracts were analyzed by immunoblotting using the anti-GFP antibody.

Global Sequence Alignment to Identify Motifs in PSs of Arabidopsis Mitochondrial Proteins

The sequences of nuclear-encoded mitochondrial proteins from Arabidopsis were obtained from the SWISS-PROT database (2011-06 release, UniProt Consortium). From these sequences, we selected 198 mitochondrial proteins (see Supplemental Data Sets 1 and 2 online) that had been confirmed to be located in the mitochondrial matrix or inner membrane. A global sequence alignment between the PSs of these proteins and two sequence motifs was performed as described previously (Lee et al., 2006).

In Vitro Import of Ala Substitution Mutants

Met-labeled precursors were synthesized using rabbit reticulocyte in vitro import of Ala substitution mutants (Lee et al., 2008). Proteins and two sequence motifs was performed as described previously (Murcha et al., 1999; Lister et al., 2007). Protein samples separated by SDS-PAGE were detected as described previously (Whelan et al., 1995; Lister et al., 2007). Protein samples detected by SDS-PAGE were described as described previously (Murcha et al., 1999; Lister et al., 2007). The radiolabeled proteins were detected by a BAS 2500 and imaging plate (FUJIFILM).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: pFAY, At2g33040; pNDS8B, At1g16700; and pFAY, At5g08690.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. The N-Terminal 57 Amino Acids Are Sufficient to Target GFP to the Mitochondrial Matrix.

Supplemental Figure 2. Subcellular Fractionation of pFAY[PSA]:GFP after Thermolysin Treatment.

Supplemental Figure 3. In Vitro Import Efficiency of Precursors into Mitochondria.

Supplemental Figure 4. Secondary Structure Prediction Using Psi-pred.

Supplemental Figure 5. Localization of pFAY[ΔP4]:GFP in Protoplasts.

Supplemental Figure 6. Import Efficiency of Partial Substitution Mutants of DQEEG or VVRNR Motifs.

Supplemental Figure 7. Protein Import of pFAY:GFP in tom20 Single Knockout and mtom64 Knockout Plants.

Supplemental Table 1. Primers Used for the PCR-Based Mutagenesis.

Supplemental Data Set 1. Global Alignment of the DQEEG Motif with 173 Arabidopsis Mitochondrial PSs.

Supplemental Data Set 2. Global Alignment of the VVRNR Motif with 167 Arabidopsis Mitochondrial PSs.

Supplemental Data Set 3. Hydrophobicity of Residues in pFAY and Its Ala Substitution Mutant.

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AUTHOR CONTRIBUTIONS

S.L., D.W.L., and I.H. designed the experiments. S.L., D.W.L., Y.J.O., Y.J.L., and G.L. performed the experimental procedures and carried out the data analysis. Y.-J.Y. predicted sequence motifs in PSs and analyzed the secondary structure and the hydrophobicity of mutant PSs. O.D. and J.W. designed and performed in vitro import experiments. S.L., J.W., and I.H. wrote the article.

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