Phosphorylation and Dephosphorylation of the Presequence of Precursor MULTIPLE ORGANELLAR RNA EDITING FACTOR3 during Import into Mitochondria from Arabidopsis 1

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The nucleus-encoded mitochondria-targeted proteins, multiple organellar RNA editing factors (MORF3, MORF5, and MORF6), interact with Arabidopsis (Arabidopsis thaliana) PURPLE ACID PHOSPHATASE2 (AtPAP2) located on the chloroplast and mitochondrial outer membranes in a presequence-dependent manner. Phosphorylation of the presequence of the precursor MORF3 (pMORF3) by endogenous kinases in wheat germ translation lysate, leaf extracts, or STY kinases, but not in rabbit reticulocyte translation lysate, resulted in the inhibition of protein import into mitochondria. This inhibition of import could be overcome by altering threonine/serine residues to alanine on the presequence, thus preventing phosphorylation. Phosphorylated pMORF3, but not the phosphorylation-deficient pMORF3, can form a complex with 14-3-3 proteins and HEAT SHOCK PROTEIN70. The phosphorylation-deficient mutant of pMORF3 also displayed faster rates of import when translated in wheat germ lysates. Mitochondria isolated from plants with altered amounts of AtPAP2 displayed altered protein import kinetics. The import rate of pMORF3 synthesized in wheat germ translation lysate into pap2 mitochondria was slower than that into wild-type mitochondria, and this rate disparity was not seen for pMORF3 synthesized in rabbit reticulocyte translation lysate, the latter translation lysate largely deficient in kinase activity. Taken together, these results support a role for the phosphorylation and dephosphorylation of pMORF3 during the import into plant mitochondria. These results suggest that kinases, possibly STY kinases, and AtPAP2 are involved in the import of protein into both mitochondria and chloroplasts and provide a mechanism by which the import of proteins into both organelles may be coordinated.

Chloroplasts and mitochondria are endosymbiotic organelles that are intimately involved in energy metabolism in plants (Araújo et al., 2014). The majority of proteins located in chloroplasts and mitochondria are encoded in the nucleus, translated in the cytosol, and imported into the organelles (Murcha et al., 2014). For both chloroplasts and mitochondria, over 1,000 different proteins are required to be specifically imported into each organelle. Furthermore, while most proteins are imported specifically into one organelle, a significant number of proteins are dually targeted to both via ambiguous targeting signals (Carrie et al., 2009a; Ye et al., 2012, 2015). The sorting of proteins to chloroplast and mitochondria is achieved through the inclusion of targeting signals in newly synthesized proteins that act in combination with receptor domains present in outer membrane multisubunit protein complexes to specifically direct proteins to their destination compartments (Jarvis, 2008; Shi and
Fluorescence136 (pHCF136), but not the presequence as the precursor for the small subunit of Rubisco peptides of several chloroplast precursor proteins, such as kinases are also involved in phosphorylating the transit peptides of several chloroplast precursor proteins, such as the precursor for the small subunit of chloroplasts (Jarvis, 2008; Fellerer et al., 2011; Flores-Pérez and Jarvis, 2013; Lee et al., 2013; Schweiger et al., 2013) but not into mitochondria.

In addition to cytosolic chaperone factors, three STY kinases are also involved in phosphorylating the transit peptides of several chloroplast precursor proteins, such as the precursor for the small subunit of Rubisco (pSSU) and the precursor for HIGH CHLOROPHYLL FLUORESCENCE136 (pHCF136), but not the presequence of the tobacco (Nicotiana tabacum) precursor for the β-subunit of the mitochondria ATP synthase (pF1β; Martin et al., 2006; Lamberti et al., 2011a, 2011b). In addition, a pea (Pisum sativum) 14-3-3 protein has also been reported to bind to the phosphorylated Ser on the transit peptide of pSSU but not to an S→A mutant. The formation of the pSSU/14-3-3/HEAT SHOCK PROTEIN70 (HSP70) complex enhances the kinetics of the import of pSSU into chloroplasts, as free pSSU is imported relatively slowly (May and Soll, 2000). Phosphatase inhibitors, NaF and NaMoO₄, inhibit pSSU import into plastids in a reversible manner, suggesting that the dephosphorylation of the phosphorylated transit peptide of pSSU is required for import (Flügge and Hinz, 1986; Waegemann and Soll, 1996). A model for pSSU recognition and TOC translocation has been proposed in which the transit peptide of pSSU is phosphorylated at Ser-34 and the transit peptide binds to Toc33 and Toc159 to form a trimeric complex (Becker et al., 2004; Oreb et al., 2011). Hydrolysis of GTP at Toc33 dissociates it from the complex, and an as yet unknown phosphatase dephosphorylates pSer-34 on pSSU, allowing import to proceed through the combined action of Toc159 and Toc75 (Becker et al., 2004; Oreb et al., 2011). Phosphorylation seems to affect import, as phosphomimicking transit peptides of pSSU and pHCF136 reduced their import rates into chloroplasts (Lamberti et al., 2011a; Nickel et al., 2015).

Unlike chloroplast import, there is no evidence that the phosphorylation and dephosphorylation of plant mitochondrial presequences are required for efficient protein import. In yeast (Saccharomyces cerevisiae), Tom22 functions as a cytosolic facing receptor and transfers precursor proteins to the Tom40 channel. It can be phosphorylated by Casein Kinase2 (CK2) and the mitochondria-bound CK1 to stimulate the activity and assembly of the TOM complex (Harbauer et al., 2014). Protein Kinase A (PKA) can phosphorylate Tom22, impairing its import rate. Thus, PKA, CK1, and CK2 act antagonistically. It has also been demonstrated that the cyclin-dependent kinase CDK1 stimulated assembly of the TOM complex by the phosphorylation of Tom6, an accessory subunit of the TOM complex, enhancing its import into mitochondria (Gerbeth et al., 2013). Thus, in yeast mitochondria, the phosphorylation of the protein import machinery itself appears to play a role in import.

While the overall theme of mitochondrial protein targeting, machinery, and pathways utilized is conserved between different systems, significant variations have been observed in plants. First, the plant outer mitochondrial protein receptor Tom20 is not an ortholog to the yeast or mammalian receptors (Perry et al., 2006). Structural studies on the plant Tom20 import receptor suggest a discontinuous bidentate hydrophobic binding mechanism, somewhat different from that observed in other systems (Rimmer et al., 2011). Furthermore, plant mitochondria are required to distinguish between chloroplast and mitochondrial proteins. Transit peptides and presequences display some similarities in that both are enriched in positively charged residues. However, while mitochondrial presequences are proposed to form α-amphiphilic structures, chloroplast transit peptides do not and, instead, are predicted to form β-sheet secondary structures (Zhang and Glaser, 2002). The identification of a plant-specific outer membrane receptor, Toc64, involved in the import of specific precursor proteins (Chew et al., 2004) highlights the possibility of additional and varied mechanisms between plant mitochondrial protein targeting and other systems.

Previously, we identified an outer mitochondrial membrane protein, called PURPLE ACID PHOSPHATASE2 (AtPAP2), that is also dually targeted to the outer envelope in chloroplasts (Sun et al., 2012a, 2012b). Similar to Toc33/34 and Tom20, AtPAP2 is anchored on the outer membranes by a C-terminal transmembrane motif. Overexpression of AtPAP2 resulted in an altered growth phenotype with elevated ATP levels (Sun et al., 2012b); thus, the function of this protein was investigated. Here, we show that the phosphorylation and dephosphorylation of the presequence of the precursor MULTIPLE ORGANELLAR RNA EDITING FACTOR3 (pMORF3) alter the kinetics of its import in Arabidopsis (Arabidopsis thaliana). Furthermore, it was shown that the phosphorylation of pMORF3, when translated in wheat germ lysate (WGL), resulted in relatively slow import kinetics, and the inhibition of phosphorylation by site-directed mutagenesis resulted in faster import kinetics. Together, these results show that the phosphorylation and dephosphorylation of a mitochondrial precursor protein play a role in its rate of import. The roles of the outer membrane AtPAP2 protein and STY kinases were also investigated, supporting a role for phosphorylation and dephosphorylation in the import of some precursor proteins into plant mitochondria.

**RESULTS**

**AtPAP2 Interacts with the Presequences of MORF Proteins**

Using AtPAP2 as the bait in a yeast two-hybrid screen, four MORF family proteins, MORF2/3/5/6, were
identified (Fig. 1A). When the predicted mitochondrial targeting presequences of MORF2 (Δ40aaMORF2) and MORF3/5/6 (Δ50aaMORF3/5/6) were removed, their interactions with AtPAP2 were completely abolished (Fig. 1B). Previously, a large-scale protein phosphorylation study in Arabidopsis revealed three experimentally identified phosphorylation sites (Thr-17, Ser-20, and Thr-35) on the presequence of MORF3 (Wang et al., 2013). Mutations were introduced within the predicted phosphorylation sites of the MORF3 presequence, pMORF3-T17/S20/T35A, and it was found that the mutated presequence did not interact with AtPAP2 (Fig. 1B).

To confirm these interaction assays, the localization of MORF2/3/5/6 was tested by biolistic transformation with GFP fusion constructs in combination with the specific organelle markers alternative oxidase-red fluorescent protein (RFP; mitochondria) and SSU-RFP (chloroplasts). The MORF2-GFP fusion protein was observed to be targeted to plastids, the MORF3-GFP and MORF5-GFP fusion proteins were observed to be targeted to mitochondria, and the MORF6-GFP fusion protein was observed to be targeted to both plastids and mitochondria (Fig. 2; Supplemental Fig. S1). A BiFC assay was used to verify in vivo interactions between AtPAP2 and MORF2/3/5/6. In the BiFC assay, the coexpression of NYFP-AtPAP2 and MORF2-CYFP reconstituted a functional YFP signal in the chloroplasts, the coexpression of NYFP-AtPAP2 and MORF3/5-CYFP reconstituted a functional YFP signal in the mitochondria, and the coexpression of NYFP-AtPAP2 and MORF6-CYFP generated functional YFP signals in both the chloroplasts and mitochondria (Fig. 1C).

**STY8 and STY17 Phosphorylate the Presequences of Nucleus-Encoded Mitochondrial Proteins**

It was found that rabbit reticulocyte lysate (RRL), WGL, and leaf extract (LE) contain kinases that phosphorylate pMORF3, but the pMORF3-phosphorylating kinase activity of RRL is much weaker than that of WGL and LE (Fig. 3A). The specific kinase inhibitor, JNJ-101198409 (JNJ), inhibits STY kinase autophosphorylation and chloroplast substrate phosphorylation, and the nonspecific phosphatase inhibitor, NaF, inhibits chloroplast substrate dephosphorylation (Waegemann and Soll, 1996; Lamberti et al., 2011b). An in vitro phosphorylation assay indicated that JNJ can specifically inhibit the phosphorylation of pMORF3 by an intrinsic kinase in WGL, but the phosphorylation of intrinsic proteins in WGL was not affected (Fig. 3A, lane 3 versus lane 4). However, the phosphorylation of pMORF3 by an intrinsic kinase in LE was not inhibited by JNJ (Fig. 3A, lane 12 versus lane 11), suggesting that the pMORF3-phosphorylating kinases in WGL and LE have different properties. The weak pMORF3-phosphorylating activity in RRL was not affected by JNJ (Fig. 3A, lane 7). Compared with WGL (Fig. 3A, lane 1) and LE (Fig. 3A, lane 9), RRL had very weak kinase activity, not only to pMORF3 (Fig. 3A, lane 7) but also toward intrinsic proteins (Fig. 3A, lane 5). The results of the in vitro kinase assay reflect a balance of intrinsic kinase and phosphatase activities. Therefore, the activities of phosphatases in WGL, RRL, and LE were determined (Fig. 3B) by including a phosphatase inhibitor (NaF) in the kinase assays. Our results show that WGL not only has rich

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**Figure 1.** Interactions of AtPAP2 with MORF proteins as demonstrated by yeast two-hybrid and bimolecular fluorescence complementation (BiFC) assays. A. Yeast two-hybrid assay using MORF2/3/5/6 proteins and AtPAP2 in the double dropout medium without Trp and Leu (-TL) and in the triple dropout medium without Trp, Leu, and His (-TLH). MORF2 also shows interactions with quadruple dropout medium without Trp, Leu, His, or adenine (-TLHA). AD, Activation domain; BD, binding domain. B. The interaction is abolished when the presequences were removed (Δ40aaMORF2 and Δ50aaMORF3/5/6) or when three Thr residues on the presequence were changed to Ala (AD-pMORF3-T17/S20/T35A mutant). The pG8KT7 and pGADT7 vectors were used as negative controls. C. BiFC visualization of interactions between AtPAP2 and MORFs in *Agrobacterium tumefaciens*-infiltrated *Nicotiana benthamiana* leaves. The coexpression of NYFP-AtPAP2 and MORF2-CYFP reconstituted a functional YFP signal in the chloroplasts. The coexpression of NYFP-AtPAP2 and MORF2-CYFP reconstituted a functional YFP signal in the mitochondria. The coexpression of NYFP-AtPAP2 and MORF6-CYFP reconstituted functional YFP signals in both mitochondria and chloroplasts. The yellow arrows represent chloroplasts, and the white arrows represent mitochondria. Bars = 10 μm.
kinase activities but is also rich in phosphatase activities that could counteract the kinase activities in the WGL (Fig. 3B, lane 6 versus lane 5). LE also contained substantial phosphatase activities (Fig. 3B, lane 2 versus lane 1). For RRL, NaF had little effect on its kinase activities, suggesting that the lack of phosphorylation of pMORF3 is because RRL does not have rich intrinsic kinase activities and is not due to the presence of high intrinsic phosphatase activities.

The STY8 and STY17 kinases phosphorylate transit peptides for some nucleus-encoded chloroplast proteins (Martin et al., 2006; Lamberti et al., 2011b). The precursor protein (pMORF3) but not Δ50aaMORF3 was phosphorylated in the in vitro phosphorylation assay with STY8 or STY17 (Fig. 3C). The phosphorylation-deficient mutant of pMORF3-T17/S20/T35A was not phosphorylated by STY8 or STY17, indicating that one or all of Thr-17, Ser-20, and Thr-35 are the target phosphorylation sites of STY8 and STY17 (Fig. 3C). Additionally, pMORF2/5/6 were all phosphorylated by STY8 and STY17, but upon removal of the presequences (Δ40aaMORF2 and Δ50aaMORF3/5/6), no phosphorylation by STY8 or STY17 could be observed (Fig. 3C).
Figure 4. Mitochondrial import efficiency of RRL-pMORF3 and WGL-pMORF3 under various conditions. A. RRL-pMORF3 (lanes 2 and 3) and WGL-pMORF3 (lanes 7 and 8) were successfully imported into mitochondria. In the presence of valinomycin, the import of RRL-pMORF3 (lanes 4 and 5) and WGL-pMORF3 (lanes 9 and 10) was inhibited. Proteinase K (PK) was used to digest nonimported pMORF3 (lanes 3, 5, 8, and 10). B, Preincubation of RRL-pMORF3 with WGL (lanes 4 and 5) and LE (lane 7)
Dephosphorylation of the Presequence of pMORF3 Is Required for Import into Mitochondria

RRL and WGL have both been used to transcribe and translate precursor proteins for in vitro chloroplast and mitochondria protein import studies, although it has been observed that WGL-synthesized precursors are generally import incompetent into mitochondria (Dessi et al., 2003; Biswas and Getz, 2004). Here, we examined whether pMORF3 synthesized in RRL (RRL-pMORF3) and WGL (WGL-pMORF3) are import competent into isolated Arabidopsis mitochondria. In the mitochondrial import assay, RRL-pMORF3 was imported and protease protected when incubated with mitochondria, as evidenced by the generation of the mature form of MORF3 (mMORF3; Fig. 4A, lanes 2 and 3). As the generation of mMORF3 was sensitive to the addition of valinomycin, an ionophore that dissipates the membrane potential (Fig. 4A, lanes 4 and 5), the generation of mMORF3 is thus a direct result of correct import and processing. Interestingly, the transcription and translation of WGL-pMORF3 results in a mature-like product of the same apparent M₆ as mMORF3 prior to the addition of mitochondria (Fig. 4A, lane 6). This has been reported previously for other mitochondrial precursor proteins synthesized in WGL and is likely generated by a mitochondrial peptidase/protease within the WGL, as wheat germ is a rich source of mitochondrial proteins (Peiffer et al., 1990). Nevertheless, the import and processing of WGL-pMORF3, as determined by the generation of a protease-protected band (Fig. 4A, lane 8), were very weak compared with RRL-pMORF3 (Fig. 4A, lane 3). Furthermore, the truncated precursor protein of pMORF3 translated in RRL (RRL-Δ50aaMORF3) could not be imported into mitochondria, indicating that the presequence of pMORF3 is essential for import (Supplemental Fig. S2).

We have shown that WGL and LE contain kinases that could phosphorylate pMORF3 in the in vitro kinase assay (Fig. 3A), and as it had been shown previously that the addition of WGL to RRL-synthesized precursor proteins reduces the rate of import into mitochondria (Dessi et al., 2003), we further tested if this was also the case for pMORF3. The import rates of both LE-treated and WGL-treated RRL-pMORF3 were reduced when treated prior to import into isolated mitochondria, suggesting that LE and WGL both contain unknown inhibitor(s) that can reduce the import ability of RRL-pMORF3 into mitochondria (Fig. 4B). As pMORF3 can be phosphorylated by intrinsic kinases in WGL and LE, but not RRL (Fig. 3A), it was tested if the phosphorylation of pMORF3 in WGL and LE affected the rate of import uptake into isolated mitochondria. Although RRL contains an unknown inhibitor of the STY-dependent phosphorylation of pMORF3 (Supplemental Fig. S3), the treatment of RRL-pMORF3 by recombinant STY8 could increase the amount of phosphorylated RRL-pMORF3 (Supplemental Fig. S3). RRL-pMORF3 preincubated with STY8 kinase to increase the amount of the phosphorylated pMORF3 prior to mitochondrial import displayed reduced amounts of protein import into mitochondria compared with untreated RRL-pMORF3 (Fig. 4C). In the presence of the phosphatase inhibitor NaF, the import rate of STY8-treated RRL-pMORF3 exhibited additive inhibitory effect (Fig. 4C, lane 5). These results suggest that STY8 phosphorylation of the presequence of pMORF3 impedes its import into mitochondria and that its dephosphorylation is required prior to translocation.

Next, we examined whether the phosphorylation of the presequence is necessary for the import of pMORF3 into mitochondria. As shown in Figure 4D, the RRL-synthesized phosphorylation-deficient mutant pMORF3-T17/S20/T35A was imported normally, similar to wild-type RRL-pMORF3, indicating that the phosphorylation of the presequence of pMORF3 is not required for import. Taken together, unlike in the import of pSSU into chloroplasts, the phosphorylation of pMORF3 by STY kinases does not enhance, but rather impedes, import into mitochondria. Furthermore, the addition of NaF reduced the import rate of RRL-pMORF3-T17/S20/T35A. Therefore, the slower import rates of pMORF3 (Fig. 4D, lane 3) and pMORF3-T17/S20/T35A (Fig. 4D, lane 6) in the presence of the phosphatase inhibitor NaF may be due to its effects on the phosphorylation status of the TOM and TIM complexes, in addition to the phosphorylation status of the presequence of pMORF3 (Sugiyama et al., 2008; Nakagami et al., 2010; Schmidt et al., 2011; Havelund et al., 2013).

HSP70 and 14-3-3 Interact with pMORF3 But Not with pMORF3-T17/S20/T35A

HSP70 interacts with precursor proteins during translocation into mitochondria, chloroplasts, and endoplasmic reticulum (Deshaies et al., 1988; Zimmermann et al., 1988; Beckmann et al., 1990), whereas 14-3-3 proteins...
Figure 5. Comparative coimmunoprecipitation and mitochondrial import assays between pMORF3 and pMORF3-T17/S20/T35A. A and B, RRL-pMORF3 and pMORF3-T17/S20/T35A, with or without 15 min of treatment with STY8 kinase, were immunoprecipitated with anti-HSP70 (A) and anti-14-3-3 (B) antibodies. Without STY8 treatment, pMORF3 showed a weak interaction with HSP70. In contrast, STY8 treatment of RRL-pMORF3 enhanced pMORF3’s interaction with 14-3-3 and HSP70 but had no effect on pMORF3-T17/S20/T35A. The asterisk represents an additional 10 min of incubation in kinase assay buffer.
recognize transit peptides of chloroplast precursors containing a phosphoepitope-binding motif and then form a guidance complex together with HSP70 (Muslin et al., 1996). It was investigated if pMORF3 also has the ability to interact with 14-3-3 and HSP70 using a communoprecipitation assay. STY8-treated RRL-pMORF3 interacted with HSP70 and 14-3-3, whereas the STY8-treated RRL-pMORF3-T17/S20/T35A mutant could not interact with HSP70 or 14-3-3 (Fig. 5, A and B). This result suggests that the phosphorylation of the presequence of pMORF3 by STY8 kinase enables it to bind to both HSP70 and 14-3-3 proteins in vitro.

The Import Rate of WGL-pMORF3 into pap2 Transfer DNA Mitochondria Is Slower

The C-terminal transmembrane domain of Pap2 anchors AtPAP2 to the outer membrane of chloroplasts and mitochondria (Sun et al., 2012a). AtPAP2 was highly expressed in mitochondria of an Arabidopsis overexpressing AtPAP2 line (OE7) but was not expressed in the pap2 transfer DNA (T-DNA) line (Supplemental Fig. S4). We compared the import rates of RRL-pMORF3 and WGL-pMORF3 into wild-type (Columbia-0 [Col-0]), OE7, and pap2 mitochondria. The import rates of largely nonphosphorylated RRL-pMORF3 were significantly faster than the import rates of more extensively phosphorylated WGL-pMORF3 into the mitochondria of all three lines (Fig. 5, C and D). As the presequence of pMORF3 translated in WGL is phosphorylated (Fig. 3A), the presequence must be dephosphorylated before import. By contrast, the import rate of WGL-pMORF3 into mitochondria isolated from pap2 was lower when compared with the wild type (Col-0; Fig. 5D, lanes 5–7), and this decrease in import was not observed for mitochondria isolated from OE7. The import rate of WGL-pMORF3-T17/S20/T35A was also tested into mitochondria isolated from the wild type (Col-0), OE7, and pap2, and no disparity in import ability was observed (Fig. 5E). In addition, the import rate of WGL-pMORF3 and WGL-pMORF3-T17/S20/T35A was tested into mitochondria isolated from the wild type (Col-0), and it was determined that the import of WGL-pMORF3-T17/S20/T35A reached maximum ability within the first 2 min of the import assay, unlike WGL-pMORF3, which reached maximum import uptake at 15 min, suggesting that the import kinetics between the two precursors are altered (Fig. 5F).

DISCUSSION

The outer mitochondrial membrane preprotein receptors of plants are not orthologous to those in fungal or mammalian systems (Chew et al., 2004; Perry et al., 2006). This is proposed to be due to the unique cellular environments of plant cells, which contain plastids, and thus the requirement to sort proteins between these organelles, a situation that does not exist in animals or fungi. The in vivo and in vitro import of precursor proteins into mitochondria from plants (Boutry et al., 1987; Whelan et al., 1988) has been known for some time. While in vivo methods are generally considered to reflect the in planta situation with respect to targeting specificity, in vitro approaches allow the dissection of the molecular mechanisms of protein import, from synthesis of the precursor protein in the cytosol to assembly into a functional unit in mitochondria (Murca et al., 2014). For in vitro studies, precursor proteins are synthesized in cell-free translation lysates, most typically the RRL. These approaches have been very successful in elucidating the molecular mechanisms required to achieve mitochondrial protein import.

A noticeable and puzzling feature of in vitro protein import into plant mitochondria is that while it is readily supported by import from RRLs, wheat germ translation lysates do not generally support mitochondrial protein import (Dessi et al., 2003). This is puzzling given the efficient import of chloroplast precursor protein from wheat germ translation lysates (Waegemann and Soll, 1996; May and Soll, 2000), although some inhibitory effects on protein import into chloroplasts can also be attributed to proteineaceous factors in wheat germ translation lysates (Schleiff et al., 2002). Detailed studies with wheat germ translation lysates show that, not only can mitochondrial import not be supported, but, in fact, the addition of WGL to otherwise mitochondrial import-competent precursor protein inhibits import (Dessi et al., 2003). Thus, it has been proposed that there are inhibitory factors present in the WGL, although the identity of these factors remains unidentified.

Here, it is presented that the phosphorylation of at least one mitochondrial precursor protein, pMORF3, results in a large decrease in the amount of import into mitochondria. Several lines of evidence show this decrease associated with phosphorylation. First, mutation of Thr as well as Ser residues to Ala, which cannot be phosphorylated, results in much greater import from a wheat germ translation lysate. Second, the addition of WGL or LE, shown to phosphorylate pMORF3, reduces the amount

Figure 5. (Continued.) before the in vitro kinase assay. C, Import efficiencies of RRL-pMORF3 into wild-type (WT; lanes 2–4), OE7 (lanes 5–7), and pap2 (lanes 8–10) mitochondria. Import experiments were repeated three times, and the import rates were not significantly different between mitochondria. D and E, Import efficiencies of WGL-pMORF3 (D) and WGL-pMORF3-T17/S20/T35A (E) into wild-type, pap2, and OE7 mitochondria. Import experiments were repeated three times. The import rate of WGL-pMORF3 into pap2 (lanes 5–7) mitochondria was significantly reduced, with *P < 0.05 (**) and **P < 0.01 (**) using Student’s t test, but the import rates of WGL-pMORF3-T17/S20/T35A into different mitochondria were not significantly different. F, The import rate of WGL-pMORF3-T17/S20/T35A into wild-type mitochondria was significantly faster than that of WGL-pMORF3, with *P < 0.01 (**) using Student’s t test. Import experiments were repeated three times.
of import into mitochondria, and the addition of NaF, a
general inhibitor of phosphatases, results in a reduction
in the amount of protein import into mitochondria.
Third, the Arabidopsis line pap2, which has the outer
membrane phosphatase inactivated, imports WGL-
pMORF3 slower than the wild type or lines that have it
overexpressed. Finally, it can be shown that the prese-
cence was the site of action, as it was required to interact
with AtPAP2, and that purified STY7 and STY8 can
phosphorylate the presequence of pMORF3. Com-
inded, these findings show a role for phosphorylation
and dephosphorylation in the import of pMORF3 into
mitochondria on the presumption of pMORF3.
STY kinases are present in the cytosol, and AtPAP2
is present on the outer membranes of both organelles
(Martin et al., 2006; Sun et al., 2012a). In vivo BiFC assays
showed that pMORF3/5 interacted with AtPAP2 on
mitochondria but not with AtPAP2 on chloroplasts (Fig.
1C). In contrast, in vivo BiFC assays showed that the
chloroplast-targeted precursor protein pMORF2 inter-
acted with AtPAP2 on chloroplasts but not with AtPAP2
on the mitochondria (Fig. 1C). These data indicate that
the interactions of these nucleus-encoded proteins with
AtPAP2 are not the sole determining factors of their tar-
getting to these two organelles. Instead, other receptors on
TOC (Toc34, Toc64, and Toc159) and TOM (Tom20 and
OM64) complexes are responsible for cargo specificity
(Ye et al., 2012, 2015).
What is the purpose of the phosphorylation and de-
phosphorylation of transit peptides and presequences of
precursor proteins when both processes are energy con-
suming? The localizations of at least 751 mitochondrial
proteins (Duncan et al., 2011) and 1,323 chloroplastic
proteins (Smith et al., 2004) have been confirmed. Both
mitochondria- and chloroplast-targeting signals are lo-
eated at their N termini. Although these sequences are
enriched in basic and hydroxylated amino acids and are
deficient in acidic residues (Perry et al., 2008), their se-
quences are versatile. The process of STY kinase phos-
phorylation, 14-3-3 binding, and interaction with TOC/TOM
can greatly reduce the variety of sequences of transit
peptides. Complex formation also enhances the import
efficiency of the chloroplast precursor proteins into
chloroplasts and impedes the import efficiency of
mitochondrial precursor proteins into mitochondria.

This design may serve to coordinate protein import
into chloroplasts and mitochondria and may have evolved early in the green lineage. A single STY-like gene
and a single AtPAP2-like gene are present in the genome
of Ostreococcus tauri (Supplemental Fig. S5; Supplemental
Table S1), a unicellular photosynthetic alga that contains
a single chloroplast and a single mitochondrion per cell.
Both STY-like kinases and AtPAP2-like phosphatases
can be found in green plants (Supplemental Fig. S5; Super-
Table S1), suggesting that the genes may have coevolved in the plant kingdom (Martin et al., 2006; Lam-
berti et al., 2011b; Sun et al., 2012b). Conversely,
MORF family members only evolved in flowering
plants (Supplemental Fig. S5) and work together with
RNA sequence-specific pentatricopeptide repeat family
members in RNA editing in mitochondria and chloro-
plants (Takenaka et al., 2012). Bioinformatics analysis of
the first 66 amino acids of 334 MORFs from flowering
plants showed that Ser is the dominant residue at po-
sitions 17 to 29 (Supplemental Fig. S6), implying that
phosphorylation at the Ser residues at the presequences of
MORFs may be of biological significance.
Modulation of the mitochondrial import process or
import apparatus was shown to have various effects on
the physiology of plants. For example, the limiting step
for the import of precursor protein appears to be the
amount of the inner membrane translocase TIM17/23
(Wang et al., 2012). Increasing the amount of AtTim23
resulted in increased protein import but significantly re-
duced growth, while significantly reducing the rate of
protein import into mitochondria had no detectable effect
on the growth rate in Arabidopsis (Ye et al., 2012).

By contrast, Arabidopsis plants with all three Tom20 recep-
tor isofoms inactivated show only some reduced growth
rate (Lister et al., 2007). In the case of AtPAP2, while
overexpression of AtPAP2 resulted in enhanced growth
and higher seed yield (Sun et al., 2012a, 2012b), sole tar-
getting of AtPAP2 to mitochondria results in early senes-
cence and lower seed yield (Supplemental Fig. S7). This
shows the in vivo biological effects of the overexpression
of AtPAP2 in mitochondria on the physiology of plants.
In conclusion the observation that at least some mi-
 tochondrial precursor protein can be phosphorylated,
and that this impedes import into mitochondria, un-
covers a novel step in the import of precursor protein
into mitochondria and may have implications for pro-
tein sorting and the specificity of import.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Wild-type Arabidopsis (Arabidopsis thaliana) ecotype Col-0, Arabidopsis
overexpressing AtPAP2 (OE7), and a T-DNA insertion mutant (pap2 [Salk_013567];
Sun et al., 2012b) were used in this study. All of the plants were grown under
long-day conditions (16 h of light/8 h of dark) in a controlled-environ-
ment chamber. Arabidopsis seeds were sterilized with 20% (v/v) bleach and
grown for 14 d on Murashige and Skoog (MS) medium that was supplemented
with 3% (w/v) Suc.

Plasmid Construction

First-strand complementary DNA (cDNA) was reverse transcribed by the
Reverse Transcription System (Promega) according to the manufacturer’s in-
suctions. Full-length coding sequences of MORF2/3/5/6, AtPAP2, pF1β,
STY8, and STY17, coding sequence of MORF2 with deletion of the first 120
nucleotides following the start codon (Δ40aaMORF2), and coding sequences
of MORF3/5/6 with deletion of the first 150 nucleotides following the start
codon (Δ50aaMORF3/5/6) were amplified by Platinum Pfx DNA Polymerase (Life
Technologies) using the primers listed in Supplemental Table S2. For site-
directed mutagenesis, the phosphorylation-deficient mutant MORF3-T17/
S20/T35A was amplified by primers containing mutated bases as described
by Heckman and Pease (2007).

To produce transgenic lines that only overexpressed AtPAP2 on mito-
ochondria, cDNA encoding amino acids 1 to 588 of AtPAP2 and amino acids
163 to 202 of Tom20-3 was amplified separately, and then both purified PCR
products were combined and a chimeric PCR product was amplified by a forward primer
of AtPAP2 and a reverse primer of Tom20-3. The PCR product was cloned into
the pCXSN vector (Chen et al., 2009) to generate pCXSN-P2Tom.
For the yeast two-hybrid study, the PCR product of AtPAP2 was digested with NdeI and SalI and cloned into pGBK7 vector (Clontech). The PCR products of MORF2/3/5/6, Δ40aaMORF2, Δ50aaMORF3/5/6, and MORF3-T17/S20/T35A were digested with NdeI and XhoI, and the PCR product of STY8 was digested with NdeI and SacI and ligated into the pGADT7 vector (Clontech). For the BiFC assay, the cDNA of AtPAP2 was amplified and digested with SpeI and cloned into the pSPYNE vector, while the cDNAs of MORF2/3/5/6 were amplified and cut by XhoI and SalI and cloned into the pSPYCE vector (Walter et al., 2004). For the overexpression of recombinant proteins, the PCR products of MORF2/3/5/6, Δ40aaMORF2, Δ50aaMORF3/5/6, MORF3-T17/S20/T35A, and pF1J6 were digested with NdeI and SalI and cloned into the pET21a vector (Novagen), and the PCR products of STY8 and STY17 were digested with NcoI and SacI and cloned into pET28a vector (Novagen). For GFP analysis, the PCR products of MORF2/3/5/6 containing attB sites were transferred into the pDONR201 vector (Life Technologies) by Gateway BP reaction (Life Technologies) according to the manufacturer’s instructions. The pDONR201 vector containing the fragment of interest was transferred into the C-terminal GFP vector (Life Technologies) by Gateway LR reaction (Life Technologies) according to the manufacturer’s instructions.

Expression of Recombinant Proteins

The coding sequences of STY8 and STY17 were subcloned into the pET28a vector (Novagen), and the coding sequences of pMORF2/3/5/6, mMORF2/3/5/6, and pMORF3-T17/S20/T35A were subcloned into the pET21a vector (Novagen). The overexpression and purification of STY8 and STY17 were performed as described by Lamberti et al. (2011b). The other recombinant proteins were purified as described by Inoue and Akita (2008).

Mitochondria Isolation

Fourteen-day-old seedlings that were grown on MS agar were used for mitochondria isolation as described by Day et al. (1985) and Lister et al. (2007). For immunoblot analysis, wash buffer (300 mM Suc and 10 mM TES) without mitochondria isolation as described by Day et al. (1985) and Lister et al. (2007).

In Vitro Mitochondrial Protein Import

Rabbit reticulocyte TnT in vitro transcription/translation lysate (RRL; Promega) and wheat germ TnT in vitro transcription/translation lysate (WGL; Promega) were used to synthesize [35S]Met-labeled precursor proteins as described previously (Dessi et al., 2003). The in vitro mitochondrial protein import assay was performed as described by Lister et al. (2007).

In Vitro Phosphorylation Assay

This assay was performed as described (Martin et al., 2006). The recombinant substrate proteins and recombinant STY8 and STY17 kinases were expressed in the Esherichia coli BL21 (PlysS) strain and purified. Two micrograms of recombinant substrate proteins was incubated with 2 µg of recombinant STY8/17, 35 µg of WGL, 200 µg of RRL, and 10 µg of LE in the reaction buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 0.5 mM MnCl2, and 2.5 µM ATP) containing 5 µCi of [γ-32P]ATP. The reaction was incubated at 30°C for 15 min and terminated by adding 20 µL of 5X SDS loading buffer. The reaction products were analyzed on a 15% (v/v) SDS-PAGE gel and exposed to Hyperfilm MP (GE Healthcare).

Yeast Two-Hybrid Interaction and BiFC Assays

The Clontech Matchmaker two-hybrid system was used according to the manufacturer’s instructions. Positive interactions were verified on triple dropout medium without Trp, Leu, and His. BiFC assays were performed as described (Walter et al., 2004). The pSPYNE and pSPYCE vectors containing genes of interest were cotransformed into the yeast cells of Nicotiana benthamiana by Agrobacterium tumefaciens infiltration. Biofluorescence was detected with an LSM710 confocal laser scanning microscope (Zeiss).

Immunoblot Assay

Fourteen-day-old wild-type Arabidopsis Col-0, Arabidopsis overexpressing AtPAP2 (OE7), and the pnap T-DNA insertion mutant (Salk_035607) were used for mitochondrial isolation. Mitochondrial proteins were separated on a 10% (w/v) SDS-PAGE gel and transferred to Hybond-C nitrocellulose membranes (GE Healthcare). The membranes containing mitochondrial proteins were immunodetected by anti-AtPAP2 antibodies as described by Sun et al. (2012b).

Phosphorylation of Mitochondrial Precursor Protein

For phylogenetic analysis of PAP2, two steps were conducted to identify PAPs, including AtPAP2/9-like proteins across 64 species ranging from algae to higher plants to human, with genome sequences available. First, BLASTP (e ≤ 1e-5) was used to search homologous protein using 42 seed PAP proteins, including PAPs with a unique C-terminal hydrophobic motif against non-redundant protein sequences of plants (taxid: 3193) in which all PAP candidate sequences with signals of the five conserved motifs (DxG, GDISY, GNHE, QGH, and GHV) within the proteins were identified. Second, an HMM matrix was built for the C-terminal hydrophobic motif of PAP proteins using HMM build, and the candidate PAP sequences in step 1 were used to identify PAP proteins with a C-terminal hydrophobic motif using HMM search, in which the AtPAP2/9-like proteins were identified. For phylogenetic analysis of STYs, a similar strategy was implemented. First, BLASTP (e ≤ 1e-5) was used to search homologous proteins using 20 seed STYs (each with an ACT motif and a kinase activate segment motif) against the 64 species protein database, in which STY candidate sequences were identified. Second, to make sure that each candidate STY contains the ACT and activate segment motifs, an HMM matrix was built for the ACT and kinase activate segment motifs, respectively, and the candidate STYs identified in step 1 were searched using HMM search, in which the STY protein set for each species was determined. For phylogenetic analysis of MORF, a similar strategy to that used for PAP and STY was implemented. First, BLASTP (e ≤ 1e-5) was used to search homologous proteins using nine seed MORF proteins from Arabidopsis against the 64 species protein database in which the MORF candidate sequences were identified. Second, an HMM matrix was used to screen the identified orthologs for a conserved GCT motif.

Sequence data from this work can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AT1G13900 (AtPAP2), AT2G33430 (MORF2), AT3G06790 (MORF3), AT1G32580 (MORF3), AT2G35240 (MORF6), AT2G17700 (STY8), and AT4G39780 (STY17).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Negative controls of subcellular localizations of MORFs in Arabidopsis cell suspension.
Supplemental Figure S2. Mitochondrial import efficiency of RRL-pMORF3 and RRL-A50aaMORF3.

Supplemental Figure S3. The effects of RRL and WGL on the activities of STY8 kinase.

Supplemental Figure S4. The expression level of AtPAP2 in mitochondrial protein of wild-type, OE7, and p2p2 T-DNA line.

Supplemental Figure S5. Distribution of STY, PAP, and MORF genes.

Supplemental Figure S6. Ser is the dominant amino acid at the presequences of MORF family proteins.

Supplemental Figure S7. Growth phenotypes of P2Tom OE lines.

Supplemental Table S1. Summary of STYs, MORFs, and AtPAP2 in different plant sources.

Supplemental Table S2. List of primer sequences used for overproduction of recombinant proteins, site-directed mutagenesis, yeast-two hybrid, GFP, and BiFC studies.

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LITERATURE CITED


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