**F-Type ATP Synthase Assembly Factors Atp11 and Atp12 in Arabidopsis**

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F1p and F2p are members of two chaperone families essential for assembly of the mitochondrial ATP synthase in *Saccharomyces cerevisiae* and *Homo sapiens*. However, the role of their homologs in higher plants is unclear with regard to the assembly of both chloroplast ATP synthase (cpATPase) and mitochondrial ATP synthase (mtATPase). Here, we show that loss of either Atp11 or Atp12 is lethal in Arabidopsis. While Atp12 is only localized in mitochondria, Atp11 is present both in chloroplasts and mitochondria. Yeast two-hybrid analyses showed that, as their homologs in yeast, Atp11 specifically interacts with the β subunit of the mtATPase and cpATPase, and Atp12 interacts with the α subunit of the mtATPase, implying that Atp11 and Atp12 fulfill a conserved task during assembly of ATP synthase. However, the binding sites for Atp11 in the β subunit of mtATPase and cpATPase are slightly different, suggesting that the mechanisms of action may have evolved in different ways. Although Atp11 interacts with cpATPase β subunit as the two assembly factors BFA3 and BFA1, they bind to different sites of the β subunit. These results indicate that Atp11 is involved in the assembly of both cpATPase and mtATPase but Atp12 is specifically required for the assembly of mtATPase in higher plants.

**Keywords:** ATP synthase, assembly, chloroplast, mitochondria, Atp11, Atp12

**INTRODUCTION**

Adenosine triphosphate (ATP) is the major cellular energy storage compound used to drive numerous biochemical reactions in biological systems. Utilizing the proton motive force (pmf) across the membrane, ATP is synthesized by the ubiquitous membrane-embedded F₁Fₒ ATP synthase, also called F-type ATP synthase (Mitchell, 1961; Yoshida et al., 2001; Junge and Nelson, 2015). F-type ATP synthase is a multiprotein complex widely distributed in bacteria, mitochondria (mtATPase) and chloroplasts (cpATPase) (Bertoni, 2018). These enzymes share a similar structure composed of two rotary motors with distinct functionalities. The F₁ subcomplex represents the
hydrophilic motor which protrudes from the thylakoid membrane into the stroma and contains five subunits α, β, γ, δ, and ε, with a stoichiometric ratio of 3:3:1:1:1 (von Ballmoos et al., 2009; Hahn et al., 2018). Three catalytic nucleotide and phosphate binding sites are located at the interfaces between the α and β subunits. The αβ3 hexamer surrounds a central stalk containing the subunits γ and ε (Abraham et al., 1994; Groth and Pohl, 2001). The F₀ motor is a hydrophobic membrane-integrated component with the subunit composition α₂β₂γ₋₁₋₁⁻₁⁻₁⁻₁⁻₁⁻₁ (Nakamoto et al., 2008; von Ballmoos et al., 2009). F₁ is fixed and physically connected to F₀ via the subunits b₂ in F₀ and δ in F₁. The γε stalk is inserted into the αβ3 hexamer and fixed on the c-ring of F₁. During ATP synthesis, the transmembrane electrochemical proton gradient generated by photosynthetic or mitochondrial electron transport drives the rotation of the c-ring and the γε stalk, thereby inducing conformational changes in the catalytic sites of the αβ3 hexamer that lead to synthesis of ATP from ADP and inorganic phosphate (Junge and Nelson, 2015; Kühibrandt, 2019).

Although the structure of the F-type ATP synthase and the functional mechanisms of ATP synthesis have been widely investigated, the assembly of the complex is not well understood. Previous studies revealed that assembly of both mtATPase and cpATPase proceeds in a stepwise manner (Ackerman and Tzagoloff, 2005; Rak et al., 2011; Rühle and Leister, 2015; Zhang et al., 2020). During their assembly, several assembly factors are required at certain steps through specific interactions with individual subunits of the ATP synthase. Atp11p and Atp12p are two assembly factors present in most eukaryotes and they are essential for assembly of mtATPase (Ackerman and Tzagoloff, 2005; Picková et al., 2005; Rak et al., 2011). In *Saccharomyces cerevisiae*, dysfunction of the Atp11p and Atp12p proteins induces aggregation of the F₁β and F₁α subunits, suggesting that Atp11p and Atp12p are required to maintain the β and α subunits in a soluble state at the early stage of ATP synthase assembly (Wang and Ackerman, 2000; Wang et al., 2000). Further investigations revealed that Atp11p binds to the pre-catalytic site (CS) at the surface of unassembled β subunit while Atp12p binds to the pre-non-catalytic site (NCS) interface at the surface of the unassembled α subunit to form Atp11p-β and Atp12p-α heterodimers, respectively (Wang and Ackerman, 2000; Wang et al., 2000; Ackerman and Tzagoloff, 2005). This kind of interaction is believed to prevent the formation of insoluble α-α and β-β homodimers (Wang et al., 2000). Structures of Atp11p from *Candida glabrata* and Atp12p from *Paracoccus denitrificans* have been resolved and it has been proposed that formation of Atp11p-β and Atp12p-α heterodimers may act as decoys preventing the formation of nonphysiological α/β subunit aggregates (Ludlam et al., 2009).

Homologs of Atp11p and Atp12p are also present in higher plants that contain two F-type ATP synthases, cpATPase and mtATPase. We designated them Atp11 and Atp12, respectively. However, their localization and functions in higher plants remain unclear. In this study, we provide evidence that absence of Atp11 or Atp12 is lethal for Arabidopsis. While Atp12 is localized in mitochondria and interacts with the α subunit of mtATPase, Atp11p is localized in both chloroplasts and mitochondria. Both proteins may fulfill similar functions as their orthologs during the assembly of F-type ATP synthase in higher plants.

**RESULTS**

**Isolation and Characterization of the *atp11* and *atp12* Mutants**

To investigate the roles of Atp11 and Atp12 in Arabidopsis, the T-DNA insertion mutants *atp11-1* (SALK_018817) and *atp12-1* (GABI_516A01) were obtained from the Arabidopsis biological resource center (ABRC). To determine the T-DNA insertion sites, genomic DNA was extracted from the mutants and the amplified products were subsequently sequenced (Figures 1A,B). The results showed that the T-DNA was inserted into the third intron and fourth exon of *Atp11* and *Atp12* in the corresponding mutants, respectively (Figure 1A). However, neither *atp11-1* nor *atp12-1* homozygous mutants could be identified (Figure 1B). No visible phenotype was found in the *atp11-1* or *atp12-1* heterozygous mutants. Failure to obtain homozygous mutant plants suggested homozygous lethality for these two genes. We then analyzed the seeds within mature siliques of the heterozygous mutants. Both mutants display a deficiency in endosperm or embryo development (Figure 1C). We then determined the ratio of normal to aborted embryos in the progeny of *atp11* and *atp12* heterozygous plants. The results showed that in both cases, this ratio is about 3:1 (Supplementary Table S1). Moreover, the ratio of wild type to heterozygous plants in the progeny of both *atp11* and *atp12* heterozygous plants. These results indicate that the *atp11* and *atp12* mutants and their corresponding T-DNA insertion are inherited in a Mendelian manner.

Full-length genomic gene sequences including their promoters were used to complement the *atp11-1* and *atp12-1* heterozygous mutants. The complemented transgenic lines in the *atp11-1* and *atp12-1* homozygous mutant background were screened in the second generation by PCR (Figure 1B). As shown in Figure 1C, the complemented plants produced fully developed and viable seeds similar to WT (Figure 1C), confirming that the embryo lethality of the *atp11-1* and *atp12-1* homozygous mutants was indeed due to the disruptions of *Atp11* and *Atp12*, respectively.

**Sequence Alignment of Atp11 and Atp12 Proteins From Eukaryotic Photosynthetic Organisms**

Bioinformatic analysis indicates that there are proteins related to Atp11 and Atp12 in various land plants as well as in the green alga *Chlamydomonas reinhardtii* (Figure 2), but not in cyanobacteria. Multiple sequence alignment analysis showed that the sequences of Atp11 and Atp12 are well conserved (Figure 2). Besides, Atp11 and Atp12 are predicted to have a plastid-
mitochondria of WT plants and this band is shifted to a higher molecular mass in the atp11-HA plants, as expected for the Atp11-HA fusion protein (Figure 3C). Comparable levels of ATP3 were detected in the mitochondria of WT and atp11-HA plants (Figure 3C). These results indicate that the Atp11 protein is localized in the mitochondria and that the addition of the HA-tag fused to the C-terminus of Atp11 does not affect the function of Atp11 during assembly of mtATPase.

The Atp11-HA fusion protein was also detected in the chloroplast stroma fraction of atp11-HA plants (Figure 3C). However, our antibody detected a non-specific band with a molecular mass similar to Atp11 protein in the chloroplast stroma (Figure 3C). To further confirm the presence of Atp11 in the chloroplast stroma of wild-type plants, immunoprecipitation experiments were performed. Chloroplast stroma from WT and atp11-HA plants was incubated with CNBr-activated agarose coupled with preimmune serum and Atp11 antibody overnight. Immunoblot analyses of the immunoprecipitated samples using the Atp11 antibody detected the Atp11 and Atp11-HA bands form WT and atp11-HA stroma, respectively (Figure 3D), indicating that Atp11 is indeed present in the stroma of chloroplasts. The level of Atp11-HA in the atp11-HA plants is higher than the level of Atp11 in the WT plants in both the chloroplast stroma and mitochondria (Figures 3C,3D), which is consistent with the qRT-PCR results of Atp11 transcripts in both genotypes (Figure 3B).

Intact chloroplasts isolated from atp11-HA plants were separated into stroma and thylakoid membrane fractions, which were further used for immunoblot analyses using the HA antibody. The Atp11-HA fusion protein was detected in the stroma fraction of atp11-HA plants, but no signal was detected in the stroma fraction of WT or thylakoid membranes of WT and atp11-HA plants (Figure 3E), indicating that Atp11 is not localized to the chloroplast thylakoids. Localization of Atp11 in chloroplast stroma is consistent with the localization

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1http://www.cbs.dtu.dk/services/ChloroP
FIGURE 2 | Sequence alignment of Atp11 and Atp12 proteins from eukaryotic photosynthetic organisms. (A) Multiple sequence alignment of Atp11 and several orthologs. (B) Multiple sequence alignment of Atp12 and several orthologs. Sequences of Atp11 and Atp12 were obtained from GenBank (http://www.ncbi.nlm.nih.gov/) or Phytozome (http://www.phytozome.net/) and aligned with ClustalW2. The possible cleavage site of Atp11 and Atp12 from Arabidopsis were predicted with the CBS Prediction Servers (http://www.cbs.dtu.dk/services) and are indicated by a black arrow above the sequence. Identical amino acids are shown with white letters on a black background, and similar amino acids are indicated with black letters on a gray background.

of its ortholog in yeast as well as with the observation that no transmembrane domain is predicted in Atp11 (TMHMM program\(^3\)).

Taken together, we conclude that while Atp12 is specifically targeted to the mitochondria, Atp11 is localized both in chloroplasts and mitochondria in Arabidopsis.

Atp11 and Atp12 Interact With the \(\beta\) and \(\alpha\) Subunits of mtATPase, Respectively

Since the amino acid sequences of Atp11 and Atp12 are well conserved and both are localized in mitochondria (Figures 2, 3), it is reasonable to speculate that Atp11 and Atp12 of plants have similar roles as their presumed orthologs in yeast to facilitate the assembly of mtATPase in Arabidopsis. To verify this proposal, we determined interactions between Atp11 and Atp12 with subunits of the Arabidopsis mtATPase using the yeast two-hybrid system (Figure 4 and Supplementary Figure S1A). While mature Atp11 and Atp12 were fused to the bait vector, mtATPase F\(_1\) subunits were fused to the prey construct. As shown in Figure 4, Atp11 and Atp12 were found to specifically interact with the \(\beta\) (ATP2) and \(\alpha\) (ATP1) subunits of the Arabidopsis mtATPase, respectively, indicating that Atp11 and Atp12 have a conserved function as their orthologs in \textit{Saccharomyces cerevisiae}.

Atp11 Also Interacts With the \(\beta\) Subunit of Chloroplast ATP Synthase

Besides mitochondria, Atp11 was also localized in chloroplasts (Figure 3), suggesting that Atp11 may have a dual role in the two organelles in Arabidopsis. To determine whether Atp11 is involved in assembly of the cpATPase, subunits of the cpATPase were fused to the prey vector and were co-transformed with BD-Atp11 into yeast and then cultured on SD/-Leu/-Trp/-His/-Ade/X-a-gal medium to detect their interactions. Our results indicate that Atp11 specifically interacts with CF\(_1\)\(\beta\) among the five CF\(_1\) subunits (CF\(_1\)\(\alpha\) to CF\(_1\)\(\epsilon\)) and two CF\(_{o}\) subunits (CF\(_{o}\)\(\alpha\) and CF\(_{o}\)\(\gamma\)).

\(^3\)http://www.cbs.dtu.dk/severces/TMHMM-2.0/
and CF$_{\beta}$(II) (Figure 5A and Supplementary Figure S1B), similar with its interaction with the $\beta$ subunit of mtATPase (Figure 4A). To further investigate the interaction of Atp11 and CF$_{\beta}$ in vivo, affinity chromatography using Atp11-HA plants was performed (Figure 5B). The results showed that CF$_{\beta}$, but not CF$_{\alpha}$ and CF$_{1}$, co-immunoprecipitated with Atp11-HA, confirming the specific interaction of Atp11 and CF$_{\beta}$ in the chloroplast stoma in vivo (Figure 5B).

Therefore, to further map the binding site of Atp11 in the CF$_{\beta}$ subunit, intact CF$_{\beta}$ subunit was divided into three regions as previously described (Zhang et al., 2016, 2018). The results show that Atp11 strongly interacts with the CF$_{\beta}$II region and only weakly with the CF$_{\beta}$III region (Figure 5C and Supplementary Figure S1C). The CF$_{\beta}$II region was further divided into three parts, CF$_{\beta}$II-1, CF$_{\beta}$II-2, and CF$_{\beta}$II-3 (Zhang et al., 2016, 2018). Y2H assays
detected a weak interaction between Atp11 and CF₁βII-2 and a strong interaction with CF₁βII-3 (Figure 5D and Supplementary Figure S1C).

We previously showed that BFA3 and BFA1 are involved in the assembly of chloroplast ATP synthase and both of them interact with CF₁β (Zhang et al., 2016, 2018). PAB is also an assembly factor required for assembly of chloroplast ATP synthase through its interaction with the CF₁γ subunit (Mao et al., 2015). Our yeast two-hybrid analyses showed that Atp11p does not interact with BFA3, BFA1, and PAB (Figure 5A), although BFA3 and BFA1 also interact with CF₁βII-2 as Atp11p in chloroplasts. Thus, Atp11 may promote the assembly of CF₁β subunit via different mechanisms used by BFA1 and BFA3 in chloroplasts. This hypothesis is consistent with our previous finding that overexpression of Atp11 in bfa3 could not complement the phenotype of bfa3 (Zhang et al., 2016).
**Arabidopsis Atp11 Specifically Interacts With the βII (ATP2II) Sub-Region of the Mitochondrial ATP Synthase**

It has been shown that Atp11p from *Saccharomyces cerevisiae* binds to the nucleotide-binding domain of the β subunit (Wang and Ackerman, 2000). To detect the binding site of Atp11 in the mtATPase β (ATP2) subunit in Arabidopsis, intact mtATPase β subunit was divided into three regions as previously described (Zhang et al., 2016). Using the yeast two-hybrid system, we found that Atp11 specifically interacts with the ATP2II region ([Figure 6A and Supplementary Figure S1D](#)). The ATP2II region was further divided into three subregions and our results identified a weak interaction between Atp11 and ATP2II-2 and a strong interaction between Atp11 and the other two subregions (ATP2II-1 and ATP2II-3) ([Figure 6B and Supplementary Figure S1D](#)). These results differ slightly from those obtained with the cpATPase where Atp11 strongly interacts with the sub-regions of CFβII-3 and slightly with CFβII-2 and CFβIII ([Figures 5C,D](#)). Moreover, Atp11 from *Saccharomyces cerevisiae* seems do not interact with the region of mtATPase β subunit corresponding to ATP2II-3 of the Arabidopsis mtATPase β subunit (Wang and Ackerman, 2000). These results suggest that the binding sites for Atp11 are slightly different in Arabidopsis and yeast mitochondria as well as in Arabidopsis chloroplasts.

**DISCUSSION**

Atp11p and Atp12p are members of two large chaperone families that were first discovered in studies of respiratory-deficient mutants of *Saccharomyces cerevisiae* (Ackerman and Tzagoloff, 1990). Subsequent analyses revealed that Atp11p and Atp12p are essential for assembly of mtATPase by binding to the β and α subunits, respectively (Wang and Ackerman, 2000; Wang et al., 2000; Ackerman and Tzagoloff, 2005), and their function is conserved during mtATPase assembly from yeast to humans (Wang et al., 2001; Ackerman, 2002). A blast search revealed that proteins structurally related to Atp11p and Atp12p are present in all photosynthetic eukaryotes including angiosperms, bryophytes, and the green alga *Chlamydomonas reinhardtii* ([Figure 2](#)) which contain two F-type ATP synthases (cpATP- and mtATP-synthase). However, it is unclear whether Atp11 and Atp12 function in the assembly of both organelar ATP synthases in photosynthetic eukaryotes.

In this work, we show that Atp12 is specifically localized in mitochondria but not in chloroplasts ([Figure 3](#)). Moreover, yeast two hybrid assays showed that Atp12 specifically interacts with mtATPase α subunit ([Figure 4B](#)), which is consistent with the localization and function of the Atp12p ortholog in yeast and *Homo sapiens*. These results imply that the function of Atp12 is conserved in mitochondrial ATPase assembly in higher plants. Disruption of Arabidopsis *ATP12* may lead to the loss of mtATPase, resulting in embryo lethality of *atp12* homozygous mutants ([Figure 1](#)). A comprehensive search of prokaryotic genomes revealed that Atp12p homologs are solely present in α-proteobacteria (Picková et al., 2005). According to the endosymbiotic theory, mitochondria originated from α-proteobacteria and chloroplast from cyanobacteria (Osteryoung and Nunnari, 2003; Timmis et al., 2004). Although cyanobacteria contain F-type ATPases as chloroplasts, they do not have a homolog of Atp12. These facts suggest that Atp12p originated from a mitochondrial ancestor and is consistent with the appearance of mitochondria during the evolution of species.

Previous studies showed that no Atp11p homolog was detected in the α-proteobacterial genome and the origin of Atp11p is thought to be linked to the evolution of aerobic metabolism in eukaryotes (Picková et al., 2005). Our results show that, in contrast to Atp12, Atp11 is localized both in chloroplasts and mitochondria in Arabidopsis and tobacco ([Figure 3](#)). It has been demonstrated that chloroplasts arose from a cyanobacterial ancestor acquired by a eukaryotic host in which mitochondria were already present (McFadden, 2001). Since cyanobacteria do not contain Atp11 gene, chloroplast may have acquired Atp11p during the evolution of photosynthetic eukaryotes. Yeast two-hybrid analysis indicates that Atp11 interacts with both cp and mtATPase β subunits, consistent with the function of its orthologs ([Figures 4–6](#)). In addition, we found that the binding sites for Atp11 in the β subunit of the mt and cp ATP synthases are slightly different ([Figures 5–7](#)). Moreover, these two binding sites are also different from the binding site for Atp11 in the mtATPase β subunit in *Saccharomyces cerevisiae* ([Figure 7](#)). These results imply that Atp11 fulfills a conserved function during ATP synthase assembly but the working mechanisms may have somehow diversely evolved.

Up to now, three cpATPase assembly factors PAB, BFA3, and BFA1 have been reported to transiently bind to subunits of the CF1 subcomplex in chloroplasts. However, we found that Atp11 does not interact with these three proteins ([Figure 5A](#)). These results indicate that Atp11, BFA1, and BFA3 may play different roles during the assembly of ATP synthase (Zhang et al., 2016, 2018, 2020). Moreover, BFA1 and BFA3 bind to CFβIII-2, a region in the catalytic site of CFβ subunit (Zhang et al., 2016, 2018). Atp11, however, was found to strongly interact with CFβII-3 but only weakly with CFβII-2 ([Figure 5](#)). Additionally, a weaker interaction of Atp11 with CFβIII was also detected in yeast two-hybrid assays ([Figure 5](#)). These results indicate that Arabidopsis Atp11 plays a role in assembly of cpATPase but in a different way than BFA3 and BFA1. Further investigations are required to reveal the putative role of Atp11 in regulating cpATPase assembly in higher plants.

In conclusion, our results demonstrate that Arabidopsis Atp12 is specifically localized in mitochondria while Atp11 is present in both mitochondria and chloroplasts. As their orthologous proteins in yeast and humans, Arabidopsis Atp11 and Atp12
interact with the β and α subunits of ATPase, respectively, in their resident organelles, although the binding sites with their partners are slightly different. Thus, Atp11 and Atp12 are likely conserved chaperone family proteins essential for F-type ATPase assembly in all eukaryotes including the plant kingdom.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The heterozygous atp11-1 and atp12-1 mutants were obtained from the NASC (SALK_018817 and GABI_516A01). All the mutants and transgenic Arabidopsis thaliana (Ath) lines used in this study were in the Col-0 background. Plants were grown in soil in a growth chamber under controlled greenhouse conditions with a 16 h-light/8 h-dark photoperiod and at a light intensity of 50 μmol photons m$^{-2}$ s$^{-1}$ at 23°C. Six-week-old plants of atp11-1 and atp12-1 heterozygotes were used to analyze the embryo lethal phenotypes. To produce the transgenic lines, genomic DNA of Atp11 and Atp12 including promoter were cloned into the plant expression vector pCAMBIA1301 (Wang et al., 2020). In addition, the genomic DNA of Atp11 was fused with the sequence encoding the HA tag and then cloned into pCAMBIA1301 to produce the atp11-HA plants. The resulting vectors were introduced into the heterozygous atp11-1 and atp12-1 mutants by the Arabidopsis floral-dip method with Agrobacterium tumefaciens (Clough and Bent, 1998). Transgenic lines were screened on Murashige and Skoog medium with hygromycin B and were further confirmed by PCR.

Multiple Sequence Alignment

Sequences of Atp11 and Atp12 proteins in various species were obtained from GenBank\(^3\) or Phytozome\(^4\). Multiple sequence alignment of these proteins was generated with ClustalW2.

Subcellular Localizations of Atp11 and Atp12

For subcellular localization of Atp11 and Atp12, full size Atp11 or Atp12 was subcloned into the pCambia2300-P35S-GFP vector

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\(^3\)http://www.ncbi.nlm.nih.gov/

\(^4\)http://www.phytozone.net/
with GFP at the C terminus (Xing et al., 2019). For protoplasts transformation, the 35Spro:Atp11-GFP or 35Spro:Atp12-GFP constructs were transformed into protoplasts of Arabidopsis (Zhang et al., 2019). The mitochondrial marker MitoTracker Red CMXRos at a final concentration of 500 nM was incubated with the protoplasts for 15 min in the dark before imaging. The fluorescence of transiently transformed Arabidopsis protoplasts was observed with a Zeiss LSM 780 inverted microscope with a 488-nm laser. For MitoTracker Red, 561 nm excitation and 572–621 nm emission and for chlorophyll auto-fluorescence, 488 and 650–750 nm were used for excitation and emission, respectively. For double track imaging, GFP and chlorophyll auto-fluorescence were activated independently.

### Quantitative RT-PCR

Three-week-old wild-type and atp11-1A plants were used for qRT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse transcribed with M-MLV reverse transcriptase (Promega). qRT-PCR was performed with ChamQ Universal SYBR qPCR Master Mix (Vazyme) and Actin 2 gene was used as control (Qin et al., 2020).

### Isolation of Chloroplast Stromal Proteins and Thylakoid Membranes

Chloroplast stromal proteins and thylakoid membranes were isolated as previously described (Zhang et al., 2016, 2018). Leaves of 3-week-old transgenic lines (atp11-1A) were fully homogenized. Intact chloroplasts were carefully isolated in Medium II buffer (0.33 M sorbitol, 20 mM HEPES/KOH, pH 7.6) and then osmotically ruptured in Medium II buffer without sorbitol. Thylakoid membranes and stromal proteins were separated from each other by centrifugation (12,000 × g for 10 min at 4°C). A Protein Assay Kit (Bio-Rad) was used to determine the concentration of stromal proteins. Chlorophyll content was determined as described by Porra et al. (1989).

### Isolation of Mitochondria

Isolation of mitochondria was carried out as previously described (Sweetlove et al., 2007). Leaves from 3-week-old WT and atp11-1A plants were fully homogenized in isolation buffer containing 0.3 M sucrose, 5 mM tetrasodium pyrophosphate, 10 mM KH2PO4, pH 7.5, 2 mM EDTA, 1% PVP40, 1% BSA, 5 mM cysteine and 20 mM ascorbic acid and centrifuged at 5000 × g for 10 min. Crude mitochondria were collected by centrifugation on a Percoll density gradient consisting of 18%, 25%, and 50% Percoll solution at 40,000 × g for 55 min. The 25–50% Percoll interface containing intact mitochondria was collected and diluted threefold with washing buffer. Mitochondria were collected by centrifugation at 20,000 × g for 10 min. The concentration of mitochondrial proteins was determined by a DC Protein Assay kit (Bio-Rad, 5000116).

### Immunoblot Analysis

For immunoblotting, thylakoid membranes were resuspended in sample buffer (50 mM Tris-HCl, pH 6.8, 5% SDS, 20% glycerol, 8 M urea, 5% 2-mercaptoethanol, 1% bromophenol blue) and incubated for 30 min at room temperature. Stromal and mitochondrial proteins were mixed with an equal volume of the sample buffer (2X). Protein samples were separated by SDS-urea-PAGE and transferred to nitrocellulose membranes. Immunoblot signals were measured by the chemiluminescence method with a LuminoGraph WSE-6100 (ATTO Technology).

### Antibody Production and Antibody Source

To express the mature Atp11 recombinant protein, the pET28a expression vector harboring the cDNA sequence encoding the mature Atp11 protein (amino acids 87–248) was constructed. Recombinant protein was induced with 1mM IPTG in E. coli BL21 (DE3) strain and purified using Ni-NTA agarose under denaturing conditions. The polyclonal Atp11 antibodies were produced in rabbits (PhytoAB, United States) and were used at a 1:1000 dilution. Antibodies against RbcL (PhytoAB, PHY0346), D1 (PhytoAB, PHY0057), HA tag (Abmart), ATP3 (PhytoAB, PHY0959S), RPE (D-RIBULOSE-5-PHOSPHATE-3-EPIMERASE, PhytoAB, PHY0616), CF1α (PhytoAB, PHY0311), CF1β (PhytoAB, PHY0312), CF1γ (PhytoAB, PHY0313) were purchased from a commercial supplier and used at a 1:1000 dilution.

### Immunoprecipitation

For immunoprecipitation, preimmune serum and the antibody against Atp11 were purified with Protein A Sefinose (TM) Resin (Sangon Biotech) and then coupled to CNBr-activated agarose (Sangon Biotech). Chloroplast stroma isolated from WT and atp11-1A plants was denatured with 2% SDS for 10 min at 25°C and then 30 s at 70°C. After centrifugation at 15,000 × g for 10 min, the supernatant was transferred to a new tube and diluted 10-fold with IP buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1% Igepal CA-630). A total of 100 μl antibody-coupled CNBr-activated agarose was added and incubated overnight at 4°C. The beads were washed seven times with IP buffer and one time with washing buffer (50 mM Tris-HCl, pH 7.5). Bound protein was eluted with 50 μl of 1.5X Laemmli buffer (90 mM Tris-HCl, pH 6.8, 3% SDS, 10% glycerol, and 0.01% bromophenol blue) and 10 μl of sample was used for immunoblot analysis with antibodies against Atp11.

### Yeast Two-Hybrid Assays

Yeast two-hybrid assays were conducted following the Matchmaker Gold Yeast Two-hybrid System (Clontech). The sequences encoding mature Atp11 and Atp12 were fused to the bait vector pGBK7T7, respectively. The coding sequences of subunits of the chloroplast and mtATPase were cloned into the prey vector pGADT7. Prey and bait plasmids were transformed...
into the yeast strain Y2H Gold by the small-scale lithium acetate method. Positive colonies were grown on SD-/Trp-Leu medium for 3 days to confirm the transformation with both bait and prey plasmids and cultured on SD-/Leu-/Trp-/His-/ade/X-a-Gal medium to detect protein–protein interactions.

**Other Methods**

Affinity chromatography of Atp11-HA associated protein was performed as previously described (Zhang et al., 2016, 2018). Plastid-targeting signals (cTP) of Atp11 and Atp12 proteins were predicted by ChloroP 1.1 Server (see text footnote 1) and TargetP 2.0 Server².

**Accession Numbers**

Sequence data from this article can be found in the National Center for Biotechnology Information or Phytozome databases database under the following accession numbers: AtATP11, *Arabidopsis thaliana* databases database under the following accession numbers: National Center for Biotechnology Information or Phytozome

**DATA AVAILABILITY STATEMENT**

The datasets generated for this study are available on request to the corresponding author.

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**AUTHOR CONTRIBUTIONS**

ZD, CL, and LP conceived the study and designed experiments. ZD, KL, LZ, LC, and LL performed experiments. All authors analyzed the data. ZD and LZ produced the figures. LZ, J-DR, and LP wrote the manuscript. LP supervised the whole study.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.522753/full#supplementary-material

Supplementary Figure 1 | Growth of indicated yeast strains on Sd/-Leu/Trp plates. As growth and transformation controls, yeast co-transformed with various vectors as in Figures 4–6 were cultured on Sd/-Leu/Trp. 10- to 10,000-fold dilutions are shown.

Supplementary Table 1 | Ratio of normal to aborted embryos in the progony of *Wt*, atp11−/+ and atp12−/+ mutants.

Supplementary Table 2 | Segregation in the progeny of atp11−/+ and atp12−/+ mutants.

Supplementary Table 3 | Primers used in this work.
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