An Arabidopsis Stomatin-Like Protein Affects Mitochondrial Respiratory Supercomplex Organization

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Stomatins belong to the band-7 protein family, a diverse group of conserved eukaryotic and prokaryotic membrane proteins involved in the formation of large protein complexes as protein-lipid scaffolds. The Arabidopsis (Arabidopsis thaliana) genome contains two paralogous genes encoding stomatin-like proteins (SLPs; AtSLP1 and AtSLP2) that are phylogenetically related to human SLP2, a protein involved in mitochondrial fusion and protein complex formation in the mitochondrial inner membrane. We used reverse genetics in combination with biochemical methods to investigate the function of AtSLPs. We demonstrate that both SLPs localize to mitochondrial membranes. SLP1 migrates as a large (approximately 3 MDa) complex in blue-native gel electrophoresis. Remarkably, slp1 knockout mutants have reduced protein and activity levels of complex I and supercomplexes, indicating that SLP affects the assembly and/or stability of these complexes. These findings point to a role for SLP1 in the organization of respiratory supercomplexes in Arabidopsis.

Biological membranes are compartmentalized by the formation of discrete protein complexes and supercomplexes, often aided by specialized scaffolding proteins. Scaffolds facilitate the organization of membranes into specific domains, thereby providing a means for spatial and temporal control. Organellar scaffolding proteins such as mitochondrial prohibitins (PHBs) were discovered due to their wide-ranging roles in various cellular processes in animals (Bos taurus, Homo sapiens), yeast (Saccharomyces cerevisiae), and plants (Arabidopsis [Arabidopsis thaliana]; Coates et al., 1997; Van Aken et al., 2007; Schleicher et al., 2008). PHBs belong to the stomatin, prohibitin, flotillin, HifC/K (SPFH) superfamily of diverse eukaryotic and prokaryotic membrane proteins based on homologies in the central parts of these proteins (Tavernarakis et al., 1999). This conserved domain is named the band-7 domain after the first identified member, human stomatin (or erythrocyte band 7.2b protein). The band-7 family proteins generally form oligomers and regulate the assembly and activity of supramolecular protein complexes in various cellular localizations (Brownman et al., 2007). The precise function of the band-7 domain remains unknown in most organisms, although a role in the organization of membrane proteins by acting as a lipid-protein scaffold is a common theme.

In Arabidopsis, the best characterized band-7 family proteins are PHBs. Loss of the Arabidopsis homolog AtPHB3 affects mitochondrial morphology and function, resulting in meristem activity defects and overall growth reduction (Van Aken et al., 2007). The same gene was identified as the enhanced ethylene response (eer3-1) loss of function point mutation that gives an extreme constitutive response to ethylene (Christians and Larsen, 2007). The PHB mutant allele phb3-3 was identified as a mutant defective in nitric oxide accumulation after hydrogen peroxide treatment (Wang et al., 2010). Several prohibitins were identified together with mAAA-type FtsH (for filamentation thermosensitive) proteases in a 2-MDa complex (Piechota et al., 2010) that is thought to be involved in protein quality control in the mitochondrial matrix. In addition, several prohibitins copurified with complex I subunits (Klodmann et al., 2010; Meyer et al., 2011), although this interaction has not been studied functionally.

Two stomatin-like proteins (SLPs) from Arabidopsis (designated as AtSLP1 and AtSLP2) have been identified in several organelar proteomics studies, mainly of the mitochondrial proteome and plant membrane microdomains (Heazlewood et al., 2004; Borner et al., 2005; Dunkley et al., 2006; Mitra et al., 2007). However, both proteins remain functionally uncharacterized. To investigate the function of AtSLPs, we combined biochemical approaches with reverse genetics. Our results demonstrate mitochondrial membrane localization of AtSLPs and reveal a role in respiratory complex and supercomplex organization that could have implications for the study of band-7 proteins in other species.
RESULTS

Arabidopsis Contains 17 band-7 Family Members

Stomatin-type proteins are characterized by a conserved stomatin signature sequence (InterPro domain IPR001972), as well as the band-7 domain (InterPro domain IPR001107). The Arabidopsis genome encodes three proteins that contain both domains: At4g27585 (AtSLP1), At5g54100 (AtSLP2), and At3g01290 (UniProt ID Q9SRH6). Whereas the two AtSLP proteins are closely homologous to each other based on the amino acid sequence (62% identity, 66% similarity), the third member is less conserved (18% identical, 30% similar to both AtSLPs). This latter protein belongs to the group of hypersensitive induced response-type band-7 proteins (Nadimpalli et al., 2000), of which there are four members in Arabidopsis.

A phylogenetic analysis revealed that the Arabidopsis band-7 family proteins fall into five distinct classes based on homology with animal orthologs (Supplemental Fig. S1A). In addition to two SLPs, Arabidopsis has seven PHBs, three proteins resembling flotillins, one protein that is erlin-like, and four plant-specific hypersensitive induced response-type proteins. The SLPs fall into a clade with the PHB proteins. Further database searches revealed that the two SLPs arose by a gene duplication event (Supplemental Fig. S1B) between chromosomes 4 and 5, and are therefore paralogous genes.

Arabidopsis SLPs Localize to Mitochondria in Vivo

The two Arabidopsis SLPs were previously identified as mitochondrial in a range of proteomics studies (Heazlewood et al., 2004; Borner et al., 2005), as possibly dually localized to chloroplasts and mitochondria (Dunkley et al., 2006), and in detergent-resistant microdomains (Borner et al., 2005). To clarify the subcellular localization of these proteins, the SLP1 open reading frame was fused to a yellow fluorescent protein (YFP) at the C terminus and stably transformed into wild-type Arabidopsis plants, and the transgenic plants were examined by confocal microscopy (Fig. 1, A and B). In at least three independent transgenic lines, SLP1-YFP fluorescence in leaves was localized exclusively to subcellular structures resembling mitochondria (Fig. 1A). To confirm mitochondrial localization, we examined whether SLP1-YFP fluorescence colocalized with the mitochondrial marker dye MitoTracker in root hairs of transgenic plants (Fig. 1B). SLP1-YFP fluorescence correlated with the MitoTracker signal in most instances (Fig. 1, B and C, merge). No evidence for localization to either chloroplasts or the cell periphery was found in leaves and in roots of transgenic lines. We took a similar approach to confirm the subcellular localization of AtSLP2. A fusion construct was generated that contained the complete SLP2 genomic region including a 5' upstream region and the 3' untranslated region with GFP inserted into the last exon. The resulting construct (gSLP2-GFP) was stably transformed into Arabidopsis wild-type plants. Green fluorescence was apparent as mobile organelles resembling mitochondria and was found expressed throughout the plants. Figure 1C shows representative confocal images of SLP2-GFP colocalized with MitoTracker in a root hair. SLP2-GFP also colocalized with MitoTracker in leaves of transgenic plants (Supplemental Fig. S2). No evidence for an extramitochondrial localization was found in independent transgenic lines.

AtSLP1 Is Highly Expressed during Seedling Growth and in Inflorescences

To functionally characterize AtSLPs, two independent homozygous transfer DNA (T-DNA) insertion lines were isolated, each carrying an insertion in the SLP1 gene (designated slp1-1 and slp1-2; Fig. 2A). The
slp1-1 T-DNA was within the last SLP1 exon, whereas slp1-2 had a T-DNA inserted in an intron as identified by DNA sequencing of PCR products spanning the insertion sites. One homozygous T-DNA insertion line was analyzed in the SLP2 gene (Fig. 2A). Line slp1-1 was crossed to slp2-1 and a homozygous double mutant was isolated within the F2 generation.

We tested gene expression in the single and double mutant lines by quantitative reverse transcription PCR (RT-PCR) from plants grown in hydroponic culture. Using this method, we found no expression of SLPs in the corresponding single and double T-DNA insertion lines, confirming homozygosity of our mutants (Fig. 2B). We also did not detect significant changes in SLP1 expression between the wild type and slp2-1, and in SLP2 between the wild type and slp1-1, indicating no compensatory overexpression effects under this growth condition. None of the mutants displayed any obvious growth phenotypes under standard conditions in hydroponic culture or on soil.

We raised a polyclonal antibody recognizing the C-terminal 71 amino acids of SLP1 to assess SLP1 protein characteristics. This epitope was chosen based on its hydrophilic properties within the SLP1 protein, as seen in a hydrophobicity plot (Supplemental Fig. S3A). The SLP1 antibody was tested on crude protein extracts and on mitochondrial fractions from our isolated knockout mutant lines by western blotting (Fig. 2, C and D). A protein band migrating at approximately 45 kD, the predicted size of the SLP1 open reading frame, was detected in crude extracts and in mitochondrial fractions from wild-type control plants but not in any of our mutant lines, confirming specificity of the antiserum.

We used the antibody to test SLP1 protein expression within different Arabidopsis organs (Supplemental Fig. S3B). Western blotting of crude protein extracts revealed that SLP1 is present in all major organs tested (root, leaves, stem, inflorescences, and siliques), but is particularly abundant in buds and fertilized flowers compared with vegetative tissue parts. Attempts to raise an antibody against SLP2 were not successful.

We also investigated tissue-specific expression by fusing the genomic 5' upstream sequences of both AtSLPs to the GUS reporter gene and transformed Arabidopsis wild-type plants. Both promoter SLP-GUS constructs resulted in nearly identical expression patterns. Images taken from promoter AtSLP1-GUS plants are shown (Supplemental Fig. S3C). The AtSLP1 promoter drove

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**Figure 2.** Isolation of slp T-DNA insertion lines and submitochondrial localization. A, T-DNA insertion sites of isolated homozygous plants. B, Quantitative RT-PCR of SLP expression in the wild type and slp mutant lines from hydroponic culture. SLP expression was normalized to actin as an internal standard. Averages of three biological replicates are shown with SDs. No expression of SLPs could be detected in the corresponding knockout lines (shown by lack of bar). C, Western blot (anti-SLP1) of homozygous slp1-1 and slp1-2 plants and the wild-type (qrt1-2 in Col-3 background) control from crude leaf protein extracts. Rubisco bands (Ponceau S) are included as the loading control. Blots were probed with preimmune serum (PI) or crude serum (anti-SLP1). D, Western blot (anti-SLP1) of mitochondrial samples and fractionated mitochondria from the wild-type control (qrt1-2), slp1-1, and slp1-2. Mitochondria were fractionated into peripheral (p.) and integral (i.) membrane proteins. The white arrowhead marks the position of SLP1. Black arrowheads indicate the position of bands with higher molecular weight. Col-3, ecotype Columbia 3 of Arabidopsis; UTR, untranslated region; Wt, wild type.
GLU5 expression highly in very young seedlings (2 d after germination; Supplemental Fig. S3Ca), particularly in hypocotyl and cotyledons. Later in development, GLUS staining was more pronounced in vascular tissue (Supplemental Fig. S3C, b, c, and e) and in stipules (Supplemental Fig. S3Cd). Occasionally, GUS staining occurred in guard cells (Supplemental Fig. S3Cf). The AtSLP1 promoter was also active in the stem of mature plants and at branch points (Supplemental Fig. S3Cg and h), correlating with high expression in flowers (Supplemental Fig. S3B). Overall, it appeared that AtSLPs are highly expressed in developing seedlings and aerial plant parts, particularly in inflorescences. These observed promoter activity profiles are consistent with published microarray data. For example, AtSLP transcripts were found in hypocotyls and in cotyledons, and transcript abundance in rosette leaves was found to decrease after the transition to flowering (Schmid et al., 2005; Winter et al., 2007). Expression in roots is generally lower than in shoots, and is mainly confined to the xylem pole pericycle in 5- to 6-d-old seedlings (Brady et al., 2007). High gene expression is found in young buds (flower stage 9) and sepal (flower stage 15) but not in mature pollen grains, which is in contrast with the promoter-GUS fusion data (Schmid et al., 2005). This discrepancy could be explained by the different methods used or variations in growth conditions. On the other hand, the protein abundance profile revealed by western blotting demonstrated that the protein accumulated to high levels in inflorescences (Supplemental Fig. S3B), which may indicate that the abundance of SLP1 protein is posttranslationally regulated in these tissues.

**AtSLP1 Is an Integral Mitochondrial Membrane Protein**

We searched the SLP1 sequence for motifs that match the published Arabidopsis mitochondrial targeting sequence consensus (Huang et al., 2009), and found a conserved class II (-3 R) cleavage site after Phe-43, corresponding to a mature protein of 40.3 kD (368 amino acids). The SLP2 amino acid sequence harbors a similar class II cleavage site after Phe-41, giving rise to a mature protein of 39.3 kD (360 amino acids; Supplemental Fig. S4). In the pep2pro database (http://fgcz-pep2pro.uzh.ch/), the first detectable tryptic peptide at the N terminus of SLP1 starts at Phe-43 (Baerenfaller et al., 2011). From this finding, we conclude that SLP1 and SLP2 are proteolytically processed before insertion into the inner membrane, and are therefore likely to have access to the matrix as well as the intermembrane space. Thus, it is likely that the protein band seen on the western blots (Fig. 2, C and D) is indeed a mature form of SLP1 that happens to migrate slightly higher than 40 kD.

We used the SLP1 antibody and biochemical fractionation of isolated mitochondria to assess localization within mitochondria. Mitochondria were fractionated after disruption by freeze-thawing into carbonate-soluble (peripheral) and carbonate-insoluble (integral) inner membrane proteins. Figure 2D shows a western blot of whole mitochondria and mitochondrial membrane fractions from control and slp1 single knockout plants. The SLP1 protein fractionates with the membrane-associated proteins, probably aided by its hydrophobic stretch located within the band-7 domain (Supplemental Fig. S3A). Further subfractionation shows that the protein appears mainly in the integral membrane fraction, with a smaller proportion behaving like a peripheral protein. Notably, SLP1 appears as several higher M_r bands within the integral protein fraction not seen in mitochondria from the single knockout lines (Fig. 2D), indicative of SDS-resistant higher protein oligomers or complexes (black arrowheads) within the inner membrane.

**SLP1 Is Present in a High M_r Complex That Comigrates with the Respiratory Supercomplex I1III2**

To investigate the size of native SLP1 protein complexes, we utilized digitonin solubilization of isolated mitochondria fractions and blue-native PAGE (BN-PAGE) followed by western blotting (Fig. 3A). SLP1 was found to reside in at least one high M_r complex migrating near the top of the gel (black arrowhead) that was not present in samples from the slp double mutant. Increasing the ratio of the digitonin to protein amount from 2.5 to 10 did not substantially change this pattern. In all digitonin-treated samples, SLP1 was detected in this high M_r band, as well as in a slightly smaller band visible with higher digitonin/protein ratios (Fig. 3A, white arrowhead).

BN-PAGE-fractionated samples were further analyzed by western blotting and Coomassie brilliant blue staining to compare the position of SLP1 on the blot to the well-established pattern of mitochondrial respiratory complexes seen in the Coomassie stain (Fig. 3B). Complex I (NADH dehydrogenase) and complex III (cytochrome bc1 oxidoreductase) in Arabidopsis are partially present in a supercomplex composed of complex I and a dimer of complex III (I-III2) in BN-PAGE, with a small proportion localized to supercomplex I1III2, a dimer of I-III2 (Eubel et al., 2003). This comparison revealed that the SLP1 complex seems to comigrate with the highest M_r supercomplex, I1III4, which is estimated to be approximately 3 MDa in size (Eubel et al., 2004). Furthermore, we noticed that slp1 knockout mutants have reduced protein levels of supercomplex I1III2 (1.5 MDa) and complex I (1 MDa), as seen on the Coomassie-stained BN gel, implying that loss of SLP1 affects the assembly or stability of complex I and associated supercomplexes. Quantification of the band intensities of complexes I and III2 and supercomplexes (Fig. 3C) revealed a drastic reduction of supercomplex I1III4 compared with the wild type in the slp1/slp2 double mutant (86%). The same supercomplex was on average 67% less abundant than the wild type in the slp1-1 single mutant, and showed a slight but not significant reduction in slp2-1 (20%). Supercomplex I1III2 was also reduced in slp1 mutants only.
(63% in the double mutant, 49% in slp1-1) and complex I was reduced by 54% in the double mutant and 38% in the slp1 single mutant, respectively. By contrast, we found a mild increase of 28% in complex I abundance in the slp2-1 mutant.

We also examined protein levels of complex III2 because it was recently reported that a loss of supercomplexes in mouse (Mus musculus) cells caused a simultaneous increase in free complex III2 (Lapuente-Brun et al., 2013). In both the slp1 single and double mutants, we found a moderate increase of III2 protein to 125% and 153% of wild-type levels, respectively, which we interpret as a consequence of losing supercomplexes. These results indicate that a loss of SLP1 causes a loss of complex I and supercomplexes, with I2III4 being the most severely affected.

We explored the extent to which SLP1 comigrates with respiratory supercomplexes by increasing the resolution from a one-dimensional BN gel to a second dimension run as SDS-PAGE gels. Figure 3D shows two western blots of two-dimensional BN/SDS-PAGE gels probed with anti-SLP1 from wild-type and double mutant mitochondria. The antibody detected the SLP1 monomer at the expected molecular mass of approximately 40 kDa in the second dimension, at the site corresponding to the position of supercomplex I2III4 in the first dimension (black arrowhead). In addition, the signal extended to a second spot of slightly lower Mr (white arrowhead), most likely corresponding to what we observed in the first dimension as a second band (Fig. 3A, white arrowhead). These findings indicate that the native SLP1 complex comigrates with supercomplex I2III4 and suggest that a loss of SLP1 has knock-on effects on the accumulation of lower-order respiratory complexes composed of complex I and dimeric complex III.

**SLP1 Affects the Activity of Complex I and Its Supercomplexes**

Our results thus far suggest that SLP1 comigrates and could be associated with supercomplex I2III4 in a
manner such that it remains attached after digitonin treatment of mitochondrial membranes. This possibility suggests that the loss of SLP1 could affect the catalytic activities of respiratory complexes. Complex I provides the first entry point for electrons into the respiratory chain from NADH oxidation, passing on electrons to complex III via ubiquinol. The assembly of respiratory supercomplexes was recently shown to increase the efficiency of substrate-dependent electron flux for oxidative phosphorylation (Lapuente-Brun et al., 2013). To establish the effect of SLP1 loss on respiratory supercomplex function, we assessed their catalytic activity by in-gel activity staining of BN-PAGE lanes (Fig. 4A). We first compared protein levels of digitonin-solubilized mitochondrial samples in BN gels from the wild type and slp mutant lines (Fig. 4A). We also included a sample from the independent slp1 allele, slp1-2, which showed the same patterns of complex I reduction as slp1-1 and the double mutant (Fig. 4A). In-gel complex I activity was assayed by staining with nitroblue tetrazolium (NBT). When NBT is reduced, it forms a dark blue formazan precipitate by acting as a hydrogen acceptor upon NADH oxidation by complex I. NBT staining intensity revealed that supercomplex I\textsubscript{2}III\textsubscript{4} activity was almost completely abolished in the slp1 mutants (Fig. 4A) compared with the wild type and slp2-1 mutants. This effect was observed with samples from the double slp1/slp2 mutant as well as with a sample from slp1-2, and thus is a property of SLP1. Quantification of band intensities of active complex I demonstrated a decrease of 84% in activity of supercomplex I\textsubscript{2}III\textsubscript{4} in the slp1/2 double mutant, correlating well with the observed decrease in protein abundance (Fig. 4B). By contrast, we found a decrease of 86% in slp1-1 and an increase to 120% of wild-type levels in slp2-1. Supercomplex I\textsubscript{2}III\textsubscript{4} activity was reduced 45% in the slp double mutant and 61% in the slp1-1 single mutant, and remained unchanged in slp2-1. Free complex I activity was decreased less severely in the double and single mutants (57% and 47%, respectively), and slightly increased in slp2-1 (125% activity of wild type; Fig. 4B).

We also observed increased activity of a complex I fragment (probably the 850 kD assembly subcomplex; Meyer et al., 2011) only in the double mutant and in slp1, but not in the slp2 single mutant or in wild-type controls (Fig. 4, A and C, black arrowheads). We also assessed complex II (succinate dehydrogenase) activity in the same way. In-gel staining for complex II activity revealed no substantial differences in staining intensities between wild-type controls and the slp single and double mutants (Supplemental Fig. S5).

Importantly, constitutive overexpression of SLP1 in the slp1/2 double mutant background restored supercomplex protein levels to near the wild type, and also

![Figure 4. Respiratory supercomplex activity in slp mutants. A, Coomassie Brilliant Blue stain (left) and complex I activity stain (right) of wild-type (WT) control and slp mutant samples as indicated. The positions of supercomplexes are indicated on the left. Black arrowheads indicate the appearance of the 850-kD complex I fragment in slp1 mutants. B, Quantification of complex I and supercomplex activities. Band intensities were quantified relative to complex V band (F1 subunit) in the same gel and normalized to wild-type levels. Averages are plotted with the SEM. Significant differences are indicated by an asterisk (two-tailed t test, \(P < 0.05\); \(n = 3\) to \(n = 6\) of independent mitochondria preparations). C, Complementation of slp1/2 supercomplex deficiency by SLP1 overexpression. BN-PAGE analysis of complex I protein abundance (Coomassie Brilliant Blue stain, left), activity (middle), and SLP1 expression by western blotting (right). Relative positions of supercomplexes are indicated on the left. The black and white arrowheads indicates a complex I active fragment and an additional SLP1 band, respectively. [See online article for color version of this figure.]


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rescued the loss in complex I activity (Fig. 4C). Western blot analysis with SLP1 antibody showed increased abundance of the native SLP1 complex at 3 MDa, as well as the appearance of a lower molecular mass band at approximately 1.2 MDa (white arrowhead). We interpret this second band as a possible subcomplex of SLP1 that has not been assembled completely, or this band could represent complex I bound to free SLP1 protein. Together, these results are an indication for specific effects of SLP1 on supercomplex integrity that directly or indirectly determine either the assembly or stability status of complex I and supercomplexes.

The SLP1 Complex Is Distinct from Supercomplex I\textsubscript{2}III\textsubscript{4}

The question remained regarding whether the SLP1 complex is actually part of supercomplex I\textsubscript{2}III\textsubscript{4} or whether it forms an independent structure of a similar size in the inner membrane that affects supercomplex organization by other means. To distinguish these two possibilities, we utilized supercomplex-deficient mutants affected in complex I assembly. We chose 18 kD (18 kDa subunit of complex I), which has been reported to lack complex I activity entirely, and \textit{γ}-\textit{carbonic anhydrase} 2 (\textit{ca2}) a mutant that fails to assemble complex I efficiently (Meyer et al., 2011). We separated mitochondria isolated from soil-grown plants on BN-PAGE and determined complex I activity by activity staining (Fig. 5). As also seen in Figure 4A, we found that supercomplex activity and protein levels were reduced in the \textit{slp1/2} double mutant compared with the wild type and \textit{slp2-1}. Mitochondria from 18 kD lacked supercomplex I activities almost entirely, and \textit{ca2} mitochondria had reduced supercomplex and complex I activities compared with the wild type. To determine the effect of loss of supercomplexes of complex I on the SLP1 complex, we used western blotting and detection with SLP1 antibody (Fig. 5). We found no change in the migration pattern of SLP1 in 18 kD and \textit{ca2} mitochondria, although we detected a smear toward a slightly lower molecular mass and increased SLP1 levels in 18 kD. We conclude from this experiment that the SLP1 complex forms an independent unit of 3 MDa in the inner membrane that does not require supercomplex I\textsubscript{2}III\textsubscript{4} for its assembly. This complex could be purely composed of SLP1-SLP2 homo- or hetero-oligomers, or it could also contain other additional proteins.

Mitochondrial Respiration Is Unaffected in \textit{slp} Mutants

We measured respiration of isolated mitochondria from the \textit{slp} double mutant and a wild-type control with various substrates (Supplemental Table S1). We found no significant differences in oxygen consumption rates between the \textit{slp1/2} mutant and control mitochondria with either malate/pyruvate, succinate, or NADH as substrates in state II and state III and no consistent differences in respiratory control ratios. This suggests that the changes in supercomplex organization occurring in \textit{slp} mutants do not substantially affect respiratory capacities in isolated mitochondria. Respiratory supercomplexes have been implicated in the channeling of electrons and their diversion away from the alternative oxidase (AOX) as the final electron acceptor (Eulber et al., 2003; Ramírez-Aguilar et al., 2011). We sought to test the effect of supercomplex I\textsubscript{2}III\textsubscript{4} loss on components of the alternative respiration pathways: the external NAD(P)H dehydrogenases and AOX. Mitochondrial samples of \textit{slp} mutants and wild-type controls grown as hydroponic seedlings were subjected to SDS-PAGE and western blotting (Supplemental Fig. S6). Total AOX levels were decreased in the \textit{slp1/2} double mutant and in the \textit{slp1-1} single mutant compared with the wild type and \textit{slp2-1} mitochondria. On the other hand, protein levels of external alternative NAD(P)H dehydrogenase B (NDB) isoforms were increased in \textit{slp1} and the double mutant, indicating that a reduction in complex I activity triggers a compensatory response in the alternative pathway that involves NADH oxidation by the external dehydrogenases with a concomitant reduction of AOX levels.

We extended our respiration analysis to intact tissue. Root respiration was measured in control conditions and after a short heat treatment designed to induce oxidative stress (Schwarzländer et al., 2009, 2012; Supplemental Table S2). Respiratory rates were measured both in control conditions and after heat treatment and then again after sequential addition of cyanide to inhibit the cytochrome pathway, and finally salicylhydroxamic acid (SHAM) to inhibit AOX. Heat treatment caused a 30% reduction in the respiration rate of wild-type roots compared with the control temperature. In the \textit{slp1} single and
double mutants, the basal respiration rate was on average slightly decreased (30%) compared with wild-type roots, and heat treatment had only half the inhibitory effect (15% reduction). The addition of potassium cyanide (KCN) reduced basal rates by approximately 60% in the wild type in the control temperature, and there was no difference in slp mutants. After heat treatment, cyanide reduced respiration by 60% in the wild type, and this reduction was significantly less in the double mutant (46%), although the overall remaining respiration rate was similar. The addition of SHAM diminished overall respiration by >95% in all genotypes in both control and heat conditions. These findings indicate that overall basal tissue respiration is slightly impaired in slp mutants, but the responses to heat shock and inhibitors are largely unaffected.

DISCUSSION

SLP Topology in the Inner Membrane

We have demonstrated that SLP1 localizes to mitochondrial membranes (most likely the inner membrane) and probably has domains facing both sides of the inner membrane (Fig. 2; Supplemental Fig. S4). The identification of a processing cleavage site strongly suggests that SLP1 (as well as SLP2) are imported initially into the matrix and processed, and then subsequently inserted into the inner membrane via the hydrophobic stretch (Supplemental Fig. S3A). These properties of Arabidopsis SLPs resemble those of human SLP2. This protein is also proteolytically processed within the first 50 amino acids, but both termini seem to reside in the intermembrane space according to protease K digestion experiments (Hájek et al., 2007). A native complex of mammalian SLP2 was estimated to be 1.8 MDa in rat liver mitochondria (Reifsneider et al., 2006). Although this is smaller than the Arabidopsis SLP complex, SLP2 is also found associated with PHBs in a smaller complex of 250 kD in HeLa cells (Da Cruz et al., 2008). In addition, SLP2 was demonstrated to interact with mitofusin-2, an outer membrane protein involved in mitochondrial fusion (Hájek et al., 2007), and is required for stress-induced hyperfusion by regulating the proteolytic processing of the inner membrane guanosine 5′-triphosphatase optic atrophy1 (Tondera et al., 2009). It is likely that the C termini of the Arabidopsis SLPs are also exposed to the intermembrane space, but no complex of SLPs with PHBs has thus far been identified from Arabidopsis (Van Aken et al., 2007; Piechota et al., 2010), suggesting that different mechanisms or functions may have evolved in plant mitochondria.

SLP1 Protein Complex Formation

Based on the protein migration pattern in BN-PAGE (Fig. 3), we conclude that SLP1 migrates at approximately 3 MDa as an independent complex at the same position as supercomplex I₃II₃, a dimer of the more abundant I·III₂ complex in Arabidopsis (Eubel et al., 2003, 2004). The 3 MDa SLP complex is probably composed of SLP1/SLP2 oligomers and possibly other peripherally associated proteins, whereas the smaller SLP complex seen in two-dimensional PAGE (Fig. 3D) could be formed from SLPs alone. The native migration of these complexes is not altered in the absence of SLP2, suggesting that the hetero-oligomeric complex can be replaced by a homo-oligomeric arrangement of SLP1 alone. We currently do not know what remains of the SLP complex in the absence of SLP1, but evidently a homo-oligomeric SLP2 complex cannot fulfill the same functions with regard to supercomplexes as the hetero-oligomeric SLP complex or SLP1 on its own. Therefore, we conclude that SLP1 is crucial for the formation of the 3-MDa complex, and has the ability to create a suitable environment for the formation/stabilization of complex I and associated supercomplexes. For this reason, we conclude that SLP2 acts merely as a stabilizing accessory subunit, and that SLP1 alone can compensate for the loss of SLP2 but not vice versa. We assume that SLP1 has thus far escaped detection in proteomic studies of native mitochondrial complexes because of its high molecular mass migration, and possibly also because of the choice of detergents used for solubilization.

How Does SLP1 Affect Complex I Abundance?

We interpret the reduction of supercomplex protein and activity levels in slp1 mutants in two ways. First, the native SLP complex could be important in the assembly process of complex I. In line with this hypothesis, slp1 mutants accumulate the NADH-oxidizing smaller complex I fragment of 850 kD (Fig. 4), the same as that reported for the ca2 mutant (Meyer et al., 2011). This intermediate was proposed to lack the distal membrane arm of complex I and could be a stable assembly intermediate or a degradation product. Therefore, it is likely that the SLP complex is crucial in the late assembly stages of complex I. A lower amount of assembled complex I in slp1 (approximately 50%; Fig. 3C) subsequently leads to lower abundance of supercomplexes containing complex I because of equilibrium between them (Li et al., 2013). Alternatively, the near complete loss of supercomplex I₃II₃ could be a consequence of destabilizing effects that occur within the inner membrane in the absence of SLP1. However, supercomplexes do not seem to simply dissociate in slp1 mutants because we do not observe a simultaneous increase in free complexes I and III₂ (Fig. 3C). Therefore, the loss of SLP1 could alter supercomplex dynamics, leading to instability and ultimately degradation of complex I specifically. Interestingly, knockdown of SLP2 in HeLa cells was reported to increase the turnover of PHBs and also of subunits of complexes I and IV by increasing metalloprotease activity (Da Cruz et al., 2008). These findings support a role for mitochondrial SLPs in determining protease activity that could be conserved in Arabidopsis. In addition, based on what is known for human SLP2, it is likely that SLP1 also has a scaffolding role related to...
the binding and organization of lipids within the inner membrane. Cardiolipin availability has been linked to the stability of supercomplexes in yeast, human, and plant mitochondria, providing additional means for controlling supercomplex abundance (for review, see Wittig and Schägger, 2009; Pineau et al., 2013). Lipid binding and microdomain formation are conserved features of several band-7 family proteins. For example, a stomatin from Caenorhabditis elegans was demonstrated to bind cholesterol via its band-7 domain (Huber et al., 2006), and HsSLP2 was demonstrated to bind cardiolipin in an in vitro assay (Christie et al., 2011). Therefore, it is conceivable that in plant mitochondria, SLPs could aid the formation of specialized membrane domains that affect protein-lipid organization on both sides of the inner membrane. In this way, SLP scaffolds could affect the dynamics of supercomplex assembly and disassembly and therefore determine their functioning.

**SLP1 and Supercomplex Function**

The involvement of SLP1 in respiratory supercomplex organization could imply wider roles in the regulation of respiration. The physiological role of supercomplexes was recently elucidated and lies in the creation of spatially separated areas of electron flow, thus allowing rapid adaptation of respiratory pathways to changing metabolic demands (Lapuente-Brun et al., 2013). Other functions include respiratory complex stability and the indirect regulation of the alternative respiratory pathways in plants (Eitbel et al., 2003; Dudkina et al., 2010; Millar et al., 2011; Ramírez-Aguilar et al., 2011; van Dongen et al., 2011). In slp1 mutants, we detected a down-regulation of AOX, an increase in abundance of NDB, the external NAD(P)H dehydrogenases. Although this effect did not translate into detectable differences in overall respiration rates, this type of compensatory behavior was previously reported (e.g. for the complex I mutant NADH dehydrogenase [ubiquinone] fragment S subunit 4; ndufs4); Meyer et al., 2009) and could account for the lack of growth phenotypes in slp1 mutants. Furthermore, we also do not exclude the possibility that PHBs are partially redundant at a functional level, although they are found in a different protein complex in Arabidopsis that associates with mAAA-type proteases (Van Aken et al., 2007; Pichota et al., 2010). By contrast, SLP2 knock-out mice were reported to be embryo lethal, reflecting the greater adaptability of the plant respiratory pathway (Christie et al., 2012). In conclusion, our study has identified a novel SLP complex in Arabidopsis mitochondria, which has an effect on complex I and supercomplexes. An open question is currently whether this effect is a conserved feature of all mitochondrial SLP proteins, or if it is specific to plants.

**MATERIALS AND METHODS**

**Phylogenetic Analysis and Protein Structural Features**

Protein sequences were obtained from the UniProt database (http://www.uniprot.org). Sequences were aligned with the Muscle algorithm and a phylogenetic bootstrap tree was created by the neighborhood-joining method using Mega software (version 5.2.2; Tamura et al., 2011). The Plant Genome Duplication database (Tang et al., 2008) was queried with the AtHg27585 locus. Kyte-Doolittle hydrophobicity profiles were downloaded from ProtScale (http://www.expasy.ch/tools) with a window size of 7 and were reconstructed in Sigma Plot. Putative transmembrane spanning regions were identified with the TMAP algorithm (Persson and Argos, 1994). Predicted coiled-coil regions were identified with features of Protein software (DNA Star) (Parry, 1982).

**Cloning Procedures**

The open reading frame of AtSLP1 was amplified by PCR (SLP1 forward primer, CATGCCATGGGAAAGGACCACCTGCTGTAATAAAGC; reverse primer, CATGCCATGGCCTGCGTCTGGCCG). The amplicon was ligated into pGEM T-easy (Promega) according to the manufacturer's instructions. GFP fusion to the C terminus of AtSLP2 was accomplished by inserting a KpnI site in front of the stop codon using the megaprimer method with NotI and PstI restriction sites (Higuchi et al., 1988). GFP was amplified with KpnI sites (forward primer, CCGGGTACCATGATATGCTGACTAG; reverse primer, CCGGGTACCTCTGGATAGTACATGG). The fusion cassette (gAtSLP2-GFP) was excised from pGEM T-easy with ApI and PstI and ligated into ptkAN. Positive plant transformants were selected on 0.5× Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 0.5% (w/v) Suc and 50 μM/ml kanamycin. Kanamycin-resistant T2 seedlings were used for confocal imaging, SLP promoter regions were amplified from leaf genomic DNA (Edwards et al., 1991), for AtHg27585 2898 bp (forward primer, TAGAGGTAGATGCTGATGCTGTTTACTAC; reverse primer, CTTCTGGGGAGAATCAGAAAAATG) and for At9f54100 1898 bp (forward primer, CGGGTGAGTTTGATGTTTGGT; reverse primer, CTTCTGAAACTGTTACTAATC) upstream of the start codon and were ligated into pDRIVE (Qiagen) Constructs were subcloned into pCAMBIA1301 for stable transformation of Arabidopsis ecotype Columbia 0. Plants were transformed on 50 μg/ml hygromycin and GUS activity was assayed after staining with 1 μM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (cylclohexylammonium) in 50 μM Na-P buffer, pH 7.2, 0.5 mM K ferricyanide, and 0.5 mM K ferrocyanide and destaining in 70% (v/v) ethanol.

The SLP1 overexpression construct was created by amplifying the open reading frame with primers (forward primer, GCAACCTGAACTCCCTGCTGCTGCTGCTGCTGCTG; reverse primer, CTACTCGCAATCTTGTTTGCG). The amplicon was ligated into pENTR-D-TOPO (Invitrogen, Life Technologies) according to the manufacturer's instructions. This entry clone was subsequently used for recombination into the binary vector pTKAN+ (Schaaf et al., 2006) using PspOMI (NEB Biolabs). All insertions were verified by DNA sequencing.

The genomic sequence of AtSLP2 was amplified from genomic Arabidopsis thaliana ecotype Columbia 0 DNA (forward primer, GCGTTTTACTTCTGCGTCTGCCG; reverse primer, AAAGATAACAGGGACCAACTCTTAGAACG) and ligated into pGEM T-easy (Promega) according to the manufacturer's instructions. GFP fusion to the C terminus of AtSLP1 was accomplished by inserting a KpnI site in front of the stop codon using the megaprimer method with NotI and PstI restriction sites (Higuchi et al., 1988). GFP was amplified with KpnI sites (forward primer, CCGGGTACCATGATATGCTGACTAG; reverse primer, CCGGGTACCTCTGGATAGTACATGG). The fusion cassette (gAtSLP1-GFP) was excised from pGEM T-easy with ApI and PstI and ligated into ptkAN. Positive plant transformants were selected on 0.5× Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 0.5% (w/v) Suc and 50 μg/ml kanamycin. Kanamycin-resistant T2 seedlings were used for confocal imaging, SLP promoter regions were amplified from leaf genomic DNA (Edwards et al., 1991), for AtHg27585 2898 bp (forward primer, TAGAGGTAGATGCTGATGCTGTTTACTAC; reverse primer, CTTCTGGGGAGAATCAGAAAAATG) and for At9f54100 1898 bp (forward primer, CGGGTGAGTTTGATGTTTGGT; reverse primer, CTTCTGAAACTGTTACTAATC) upstream of the start codon and were ligated into pDRIVE (Qiagen) Constructs were subcloned into pCAMBIA1301 for stable transformation of Arabidopsis ecotype Columbia 0. Plants were transformed on 50 μg/ml hygromycin and GUS activity was assayed after staining with 1 μM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (cylclohexylammonium) in 50 μM Na-P buffer, pH 7.2, 0.5 mM K ferricyanide, and 0.5 mM K ferrocyanide and destaining in 70% (v/v) ethanol.

The SLP1 overexpression construct was created by amplifying the open reading frame with primers (forward primer, GCAACCTGAACTCCCTGCTGCTGCTGCTGCTGCTG; reverse primer, CTACTCGCAATCTTGTTTGCG). The amplicon was ligated into pENTR-D-TOPO (Invitrogen, Life Technologies) according to the manufacturer's instructions. This entry clone was subsequently used for recombination into the binary vector pENTRTOPO (Invitrogen, Life Technologies) according to the manufacturer's instructions. This entry clone was subsequently used for recombination into the binary vector pH7WG2 (Karimi et al., 2002) with LR clonase II according to the manufacturer's instructions. The resulting construct was verified by DNA sequencing and used for stable transformation as described and selection on 50 μg/ml hygromycin. Homozygous lines were isolated in the T3 generation and used for complementation analysis.

**Confocal Microscopy**

For root imaging of SLP1-eYFP with MitoTracker, seedlings were grown in hydroponic culture in 0.5× MS medium, pH 5.7 (KOH), supplemented with 0.5% (w/v) Suc for 5 d. Seedlings were stained in growth medium with 100 nm MitoTracker Deep Red for 45 to 60 min (Molecular Probes) followed by a brief rinse in fresh MS medium. Confocal images from gAtSLP2-GFP were taken from 30-d-old seedlings grown on MS agar medium. Whole seedlings were incubated for 45 min with 100 nm MitoTracker Orange in MS medium (Molecular Probes). Before imaging, excess dye was briefly washed off in fresh MS medium. Confocal images were collected using a Zeiss CLSM510 Axiovert 200 microscope, a META detector, and ×40 Plan Neofluor or ×63 Planapo objective lenses. Excitation/emission wavelengths and filters were as follows (in nanometers): 514/530 to 590 (YFP), 633/650 longpass (Mito- tracker), 633/659 to 756 (MitoTracker Deep Red), 488/505 to 330 (GFP), and
Plant Growth, Transformation, and Mutant Isolation

Arabidopsis was grown on soil, sterile 0.5% MS agar plates or in 0.5% MS liquid medium, with 0.5% (w/v) Suc under long-day (16-h light:8-h dark) conditions with 150 μmol m⁻² s⁻¹ light 22°C and 60%-70% relative humidity. Plants were transformed by floral dip with Agrobacterium tumefaciens GV3101 (Clough and Bent, 1998). SLPI T-DNA insertion mutants were isolated from the Syngenta Arabidopsis Insertion Library (SAIL) collection (Sessions et al., 2002) and were created in the prt1-2 mutant background (Copenhagen et al., 1998) as follows: slp1-1 (SAIL_210_D11) and slp1-2 (SAIL_65_C05). T-DNA insertions were identified by resistance to BASTA (glufosinate ammonium) and PCR was performed using the following primer sets: slp1-1 (genomic product forward primer, AGCTAGAGCAACAGCACTG; reverse primer, AACCAGACCGGATAA-TAACGG; T-DNA product with reverse primer and SAIL LB3 primer, TAG-CTCTGATATCTCAAACTCTGATAAC; and slp1-2 (genomic product forward primer, GGCAATCCCATATCAACATTG; reverse primer, TTCTC-ACCCATGTCCTCCA; T-DNA product with reverse primer and SAIL LB3 primer). The slp1-2 (Salk_138526) mutant line was confirmed as homozygous with primers (genomic forward, GTTACATTTCCTGAAGAGG; genomic reverse primer, CCAATGGAGCTTCCCTCCT; T-DNA insertion primer Lba1 TGTTCAAGTGGCCCATCG; T-DNA insertion sites were verified by DNA sequencing of PCR products. Double homozygous slp mutant plants were isolated by genotyping PCR from the F2 generation after crossing.

Quantitative RT-PCR

RNA was extracted from hydroponically grown whole seedlings (14-d-old in 1× MS medium, pH 5.7, 1% (w/v) Suc, 0.1% (w/v) agar) using TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. Reverse transcription reaction was performed with 2 μg RNA according to the manufacturer’s instructions (Fermentas RevertAid RT enzyme) after digestion with RNase-free DNase I (NEB). Quantitative RT-PCR was performed using SYBR Select MasterMix (AB Applied Biosystems) in an AB Applied Biosystems 7300 Real-Time PCR System. The 3′ end of SLPI was amplified with primers (forward, GGTACA-GCCAAGCAACGTC; reverse, CTACCTCCGGTCTCCTGTC) and the 5′ end of SLPI2 was also amplified with primers (forward, CAAGAGTGAACTGCTGCG; reverse, CCCTCTAATCCACAAACGGT). Actin28 was amplified as the reference gene with primers (forward, GGTAAGATGTGGACTCTGG; reverse, AACCAGCCTATTCCTGCTG). Amplification was analyzed using a previously described method (Tasi et al., 2007).

Antibody Production and Western Blotting

The SLP1 epitope (residues 341 to 411) was amplified by PCR (forward primer, GCTCAACCTTGGTACCATAAACAGGCTG; reverse primer, CTAG-AAACGTGGTCCTAGGCCTGCC) and ligated into pQ800-L (Quenihy with Spl and PstI sites for expression in Escherichia coli strain M15 (pREP4). After induction with 1 mM isopropyl-β-D-thiogalactoside for 4 h at 37°C, the overexpressed, N-terminally 6× His-tagged fusion protein was purified by nickel-nitrilotriacetic acid agarose chromatography essentially as described by the manufacturer (Qiagen). Purified protein was dialyzed against 20 mM sodium bicarbonate, 0.02% (w/v) SDS, for antibody production in rabbits (Scottish National Blood Transfusion Service). Unpurified serum was used directly on western blots at 1:5000 (crude extracts) or 1:10,000 (mitochondrial samples) dilution.

Crude protein extracts were prepared from leaves or extracts by extraction with 62.5 mM Tris-Cl, pH 6.8, 30% (v/v) glycerol, 5% (w/v) SDS, 1.4 μM β-mercaptoethanol, and quantification using the AmidoBlack protein assay. Proteins (20 μg) were separated by SDS-PAGE in a mini gel format (BioRad protein 3 or Hoefer MiniElV systems) and transferred to nitrocellulose membranes for immunode - tecter includes the Bradford method (BioRad QuickStart Bradford assay kit). Mitochondria were fractionated by freeze-thawing and carbonated washing essentially as described by Millar et al. (2001) and western blots were prepared as described above.

BN-PAGE

One- and two-dimensional BN-PAGE were performed as described by Eubel et al. (2003) using digitonin:protein ratios as indicated. Native gels were blotted onto polyvinylidene difluoride membranes for 3 h at 4°C before standard immunodetection procedures (dilution of anti-SLP1 1:10,000). In-gel activity staining was performed as described by Sabar et al. (2005). Gels were stained with colloidal Coomassie Brilliant Blue overnight as described by Eubel et al. (2003). Gel scans were analyzed for band intensities with the National Institutes of Health ImageJ software Gel Analyzer tool (Schneider et al., 2012).

Respiration Assays

Respiration of isolated mitochondria was recorded using a Hansatec oxygen electrode as described (Schwarzländer et al., 2012) in incubation buffer (0.3 mM mannitol, 10 mM MES KOH, 7.5 mM MgSO4, 10 mM NaCl, 5 mM KH2PO4, 0.1% (w/v) bovine serum albumin) at 25°C. Substrates were added as follows. To measure malate/pyruvate respiration, malate (10 mM), pyruvate (10 mM), thiamine pyrophosphate (0.1 mM), NAD⁺ (0.3 mM), and CoA (0.0125 mM) were added together and the respiration rate was measured after the addition of ADP to give state 3 (0.1 mM). To measure AOX activity, KCN (0.5 mM), dithiothreitol (DTT; 5 mM), and pyruvate (1 mM) were added in state 4 simultaneously, followed by n-propyl-gallate (0.05 mM) to block AOX-mediated oxygen consumption. To measure succinate-linked respiration, succinate (10 mM) was added with ATP (0.25 mM) and oxygen consumption was recorded in state 2, followed by ADP (0.1 mM) to give state 3 as above. AOX activity was measured subsequently by adding, KCN, DTT, and pyruvate as described above. To measure oxygen consumption caused by NADH oxidation, NADH (1 mM) was added and respiration was recorded in state 2 followed by adding ADP as described above to record state 3 respiration. AOX activity was measured after adding KCN, DTT, and pyruvate as described above. Mitochondrial intactness was assayed by measuring the latency of cytochrome c oxidative activity after adding ascorbate (10 mM) followed by cytochrome c (0.05 mM) and Triton X-100 (0.05% (v/v)).

To measure tissue respiration, seedlings were grown for 14 d on vertical agar plates (0.5× MS, pH 5.7, 0.8% (w/v) agar). Heat treatment was performed at 45°C for 10 min; control samples were left untreated. Root tissue was excised and fresh weight was taken immediately afterward before incubation in liquid 0.5× MS medium in the oxygen electrode chamber. KCN was added (1 mM) to measure AOX-linked respiration, followed by SHAM (4 mM) to block AOX activity. Statistical analysis was performed as one-way ANOVA with multiple comparisons by Student-Newman-Keuls post hoc tests.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers A4g27385 (ASLp1) and A5g54100 (ASLp2).

Supplemental Data

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

Supplemental Figure S1. Arabidopsis band-7 family.

Supplemental Figure S2. SLPI2 colocalizes with MitoTracker in leaves.

Supplemental Figure S3. ASLp1 properties and expression.

Supplemental Figure S4. Mitochondrial targeting sequences of SLPs.

Supplemental Figure S5. Complex II activity is unchanged in slp mutants.

Supplemental Figure S6. Changes in the alternative respiratory pathway in slp1 mutants.

Supplemental Table S1. Mitochondrial respiration rates.

Supplemental Table S2. Root respiration rates.
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