Lumen Thiol Oxidoreductase1, a Disulfide Bond-Forming Catalyst, Is Required for the Assembly of Photosystem II in Arabidopsis

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Here, we identify Arabidopsis thaliana Lumen Thiol Oxidoreductase1 (LTO1) as a disulfide bond-forming enzyme in the thylakoid lumen. Using topological reporters in bacteria, we deduced a lumenal location for the redox active domains of the protein. LTO1 can partially substitute for the proteins catalyzing disulfide bond formation in the bacterial periplasm, which is topologically equivalent to the plastid lumen. An insertional mutation within the LTO1 promoter is associated with a severe photoautotrophic growth defect. Measurements of the photosynthetic activity indicate that the lto1 mutant displays a limitation in the electron flow from photosystem II (PSII). In accordance with these measurements, we noted a severe depletion of the structural subunits of PSII but no change in the accumulation of the cytochrome b6f complex or photosystem I. In a yeast two-hybrid assay, the thioredoxin-like domain of LTO1 interacts with PsbO, a lumenal PSII subunit known to be disulfide bonded, and a recombinant form of the molecule can introduce a disulfide bond in PsbO in vitro. The documentation of a sulphydryl-oxidizing activity in the thylakoid lumen further underscores the importance of catalyzed thiol-disulfide chemistry for the biogenesis of the thylakoid compartment.

INTRODUCTION

Thiol-disulfide chemistry is an essential process for the biogenesis of the bacterial periplasm, the mitochondrial intermembrane space (IMS), and the thylakoid lumen. Strikingly, each compartment appears to have unique redox enzymes that oxidize sulphydryls (thio-oxidation) and reduce disulfide bonds (thio-reduction) in target proteins (Hermann et al., 2009; Depuydt et al., 2011; Kadokura and Beckwith, 2010).

In the periplasmic spaces of most proteobacteria, the thio-oxidizing pathway consists of a disulfide bond–catalyzing system defined by soluble DsbA and membrane-bound DsbB (Dsb for disulfide bond) (Heras et al., 2009; Depuydt et al., 2011; Kadokura and Beckwith, 2010). DsbA catalyzes disulfide bridge formation on Cys-containing substrates that are translocated across the membrane into the periplasmic space. DsbB operates by recycling reduced DsbA to its oxidized form with transfer of the electrons to quinones, which are membrane-soluble redox carriers in the respiratory chain. A central component of the thiol-disulfide oxidoreductase is DsbD/CcdA (Ccd for cytochrome c deficiency). This protein conveys reducing power from the cytosol to several periplasmic protein targets whose activity requires reduced thiols. DsbD/CcdA maintains the reduction state of oxidoreductases that shuffle disulfide bonds that are incorrectly formed and protect proteins containing a single Cys from hyperoxidation (Depuydt et al., 2011; Kadokura and Beckwith, 2010). DsbD/CcdA is also needed to reduce the active site of a disulfide reductase involved in the assembly of cytochromes c, a class of metalloproteins with a heme covalently attached to a CXXCH motif. The accepted view is that the CXXCH motif is first oxidized by the Dsb machinery and then reduced by the disulfide reductase to provide free sulphydryls for the heme attachment (Bonnard et al., 2010; Sanders et al., 2010).

While the presence of thiol-metabolizing pathways is well established in bacteria, there was little support for the operation of thiol-based chemistry in the mitochondrial IMS and the thylakoid lumen, which are topologically equivalent to the bacterial periplasm. Recent discoveries in both organelles have now changed this perception. In mitochondria, Mia40p/Erv1p (Mia for mitochondrial intermembrane space import and assembly; Erv for essential for respiration and viability) proteins were found to be key enzymes of a disulfide relay system driving the import of Cys-rich proteins into the IMS (Depuydt et al., 2011; Riemer et al., 2011; Sideris and Tokatlidis, 2010). Although unrelated in sequence, Mia40p/Erv1p are functionally equivalent to bacterial DsbA/DsbB. Mia40p introduces disulfide bonds into protein...
targets and is recycled back to its oxidized form by the flavoprotein Erv1p, which transfers the electrons to cytochrome c, a soluble redox shuttle in the IMS. By analogy to the bacterial pathways, the participation of thio-reducing factors in the IMS is expected. The flavoprotein Cycl2p (Cyc for cytochrome c) and CcmH (Ccm for cytochrome c maturation), an oxidoreductase implicated in cytochrome c maturation, were proposed to act as a disulfide reductase, but this still awaits experimental validation (Bernard et al., 2005; Meyer et al., 2005; Corvest et al., 2010).

In the thylakoid lumen, the involvement of a thio-reducing pathway was established through classical and reverse genetics approaches. Components of this pathway include a thiol/disulfide membrane transporter of the CcdA/DsbD family and CCSS/HCF164 (cytochrome c synthesis/high chlorophyll fluorescence), a membrane-anchored, lumen-facing, thio-reducing-like protein. These proteins define a trans-thylakoid pathway for the delivery of reductants from stroma to lumen (Lennartz et al., 2001; Page et al., 2004; Motohashi and Hisabori, 2006, 2010; Gabilly et al., 2010, 2011). Operation of the trans-thylakoid pathway is needed to reduce disulfides in target proteins, a process essential for photosynthesis (Lennartz et al., 2001; Page et al., 2004; Gabilly et al., 2010, 2011).

The identities of the thio-oxidizing catalysts in the lumen are currently unknown, and no DsbA- or DsbB-like enzymes can be detected in the genomes of cyanobacteria, which are the presumed ancestors of chloroplasts. However, disulfide bonded proteins are present in this compartment and include not only known structural components, such as PsbO, a subunit of the PSII oxygen-evolving complex (OEC) that resides in the lumen.

LTO1 is required for the assembly of PSII through the formation of a disulfide bond in PsbO, a subunit of the PSII oxygen-evolving complex (OEC) that resides in the lumen.

RESULTS

LTO1, a VKOR-Like Protein at the Thylakoid Membrane, Is Conserved in Photosynthetic Eukaryotes

A protein displaying a membrane domain with similarity to VKOR and fused to a thioredoxin-like domain was identified in all sequenced genomes of photosynthetic eukaryotes (Tie and Stafford, 2008). We name this protein LTO1. LTO1-like proteins are predicted to be polytopic membrane polypeptides with five to six transmembrane domains and contain seven strictly conserved cysteine residues (with the exception of the Volvox and Chlorella proteins) (see Supplemental Figure 1 online). Four of the Cys residues are arranged in two motifs: CXXC and WCXXC. The WCXXC motif is part of a thio-reducing domain that is absent from VKOR and some bacterial VKOR-like proteins (Goodstadt and Ponting, 2004).

Topological Analysis of LTO1

To generate a topological model of plastid LTO1, we employed PhoA and LacZ reporters in Escherichia coli (Manol, 1991). We already demonstrated the reliability of this approach in establishing the topological arrangement of three proteins in the thylakoid membrane, which is analogous in a bioenergetic sense to the bacterial plasma membrane (Dreyfuss et al., 2003; Hamel et al., 2003; Page et al., 2004). We opted to engineer PhoA-LacZ sandwich fusions at positions predicted to be at extramembrane locations (see Supplemental Figure 1 online). High PhoA activities indicate a p-side location for the insertion site of the fusion, since PhoA is active only in the periplasm. Reciprocally, fusions with high LacZ activity confirm association of the α and ω fragments of β-galactosidase in the cytoplasm; therefore, an n-side location of the fusion site can be deduced. As shown in Table 1, PhoA activities for fusions 2, 3, 4, and 5 were 2.5- to 67-fold higher than the LacZ activities, and a periplasmic location was assigned for the corresponding positions in Arabidopsis LTO1. Note that it is likely that the stability of each sandwich fusion accounts for the difference in PhoA activities. Fusion
**Table 1. Topological Analysis of Arabidopsis LTO1 from PhoA and LacZ Fusion Analysis**

<table>
<thead>
<tr>
<th>Fusion</th>
<th>Position</th>
<th>LacZ</th>
<th>PhoA</th>
<th>Topology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>74</td>
<td>73 (±22)</td>
<td>4 (±3)</td>
<td>n-side</td>
</tr>
<tr>
<td>2</td>
<td>113</td>
<td>19 (±4)</td>
<td>78 (±7)</td>
<td>p-side</td>
</tr>
<tr>
<td>3</td>
<td>191</td>
<td>16 (±7)</td>
<td>40 (±5)</td>
<td>p-side</td>
</tr>
<tr>
<td>4</td>
<td>195</td>
<td>14 (±8)</td>
<td>80 (±5)</td>
<td>p-side</td>
</tr>
<tr>
<td>5</td>
<td>573</td>
<td>19 (±6)</td>
<td>1269 (±28)</td>
<td>p-side</td>
</tr>
</tbody>
</table>

Measurements of alkaline phosphatase (PhoA) and β-galactosidase (LacZ) activities of sandwich fusions at the indicated positions within LTO1. Measurements were taken on at least two separate bacterial clones and correspond to an average of three representative measurements. The ±SD is indicated for those measurements. Fusion numbers correspond to those indicated in Figure 1. Values are expressed in arbitrary units for LacZ and Miller units for PhoA. The p-side corresponds to the bacterial periplasm and the lumen of the chloroplast, while the n-side corresponds to the bacterial cytoplasm and the stroma of the chloroplast.

1 displayed a high LacZ activity but a low PhoA activity, an indication that the fusion point is located in the cytoplasm. Based on the n- and p-side topological analogy between the compartments of the bacteria (cytoplasm/periplasm) and those of the thylakoid (stroma/lumen), we deduced that the N and C termini of LTO1 face the stroma and the lumen, respectively, while domains containing redox motifs and conserved Cys residues are exposed to the lumen (Figure 1).

**LTO1 Partially Complements for Loss of DsbAB in Bacteria**

To provide further support for the proposed topology and function, we chose a heterologous complementation assay. In this assay, an *E. coli* dsbAB mutant was used to test the ability of LTO1 to restore disulfide bond formation in the periplasmic space. For this experiment, we chose an Ara-inducible promoter to drive the expression of full-length *Arabidopsis LTO1* cDNA and monitored bacterial motility, PhoA activity, and growth on MacConkey agar, which are dependent upon DsbAB. When FlgI, a flagellar motor protein in the periplasmic space, is not disulfide bonded, the bacteria are nonmotile (Dailey and Berg, 1993). This loss of motility can be readily visualized on low concentration agar. As shown in Figures 2A and 2B, LTO1 partially restores the motility phenotype of a dsbAB mutant, demonstrating that the plant protein can promote disulfide bond formation. This restoration is Ara dependent, confirming that LTO1 is indeed responsible for the observed restored motility. The activity of periplasmic PhoA, which requires two intramolecular disulfide bonds (Sone et al., 1997), was also partially restored upon expression of the plant protein (Figure 2C). In addition, while *E. coli* strains lacking DsbA and/or DsbB grow poorly on MacConkey agar, the dsbAB mutant expressing LTO1 grows well on MacConkey agar supplemented with the inducer Ara (Table 2, Figure 2D).

We conclude that LTO1 partially compensates for the loss of bacterial DsbAB and displays sulfhydryl oxidizing activity. Our findings solidify the proposed lumenal location of the redox motifs and conserved Cys residues (Figure 1) and suggest that the relevant targets of action of LTO1 are also lumen localized.

**Knockdown of Arabidopsis LTO1 Impairs Plant Growth**

To address the question of LTO1 function at the thylakoid membrane, we chose a reverse genetics approach and identified a T-DNA insertion in the promoter region of *Arabidopsis LTO1* (Figure 3A). Through PCR analysis and sequencing, we confirmed that the inserted T-DNA is located 45 bp from the predicted initiation codon of the LTO1 open reading frame (ORF). RT-PCR analysis showed that the LTO1 transcript accumulation is strongly reduced in the homozygous insertion line, suggesting that insertion of the T-DNA altered the transcriptional activity of LTO1 (Figure 3B). This promoter region is also shared by SEN1, which was shown to be involved in senescence (Schenk et al., 2005). However, we show that the transcription of *SEN1* is not affected by the T-DNA insertion (Figure 3B). Homozygous lto1 mutants display a severe growth phenotype compared with the wild-type Columbia (Col) ecotype when grown on soil. The phenotype is not as drastic when grown on Suc-supplemented agar, indicating that the slow growth may be due to a defect in photosynthesis.

![Figure 1. Proposed Topological Arrangement of Plastid Arabidopsis LTO1.](image)
Loss of LTO1 Produces a Photosynthetic Defect

To test the hypothesis that the reduced growth seen in the mutant is a result of a photosynthetic defect, we conducted several spectroscopy tests on fresh leaves of lto1 plants. Illumination with far-red light preferentially excites photosystem I (PSI) over PSII, resulting in net oxidation of the plastoquinone (PQ) pool, cytochrome f, plastocyanin (PC), and P700 of PSI (Joliot and Joliot, 2005). The status of P700 was monitored by 10-μs flashes from a 705-nm LED. We found that the leaves of lto1 plants exhibited P700 oxidation by far-red light and rereduction immediately upon termination of illumination (see Supplemental Figure 2 online), indicating that they possessed functioning PSI. The absolute level of the P700 photobleaching signal in lto1 leaves was smaller than in wild-type leaves, but this was likely due to the fact that lto1 leaves tended to be thinner than wild-type leaves. We concluded that the function of PSI is not affected by the mutation. We did, however, notice that the net oxidation of P700 proceeded more quickly in the lto1 leaves. Moreover, they lacked a kinetic inflection point early in the time course (see Supplemental Figure 2A online), which is likely due to an influx of electrons into the electron transfer chain from a light-dependent source. The source is most likely PSII, as the rereduction of P700+ occurred with a higher rate in the lto1 mutant in the dark (see Supplemental Figure 2B and Supplemental Table 1 online). This would seem to rule out any defect in PC or cytochrome b6f levels. The higher rate of P700+ rereduction upon termination of illumination might be taken as an indication that thylakoids of lto1 plants are engaged in cyclic electron transfer to a much higher extent than in wild-type plants.

We also examined directly the changes in the redox state of cytochrome b6f cofactors induced by far-red light. We found that both the oxidation of cytochrome f and the reduction of cytochrome b occurred to a greater extent in the lto1 plants during illumination, indicating a limitation in electron flow to the cytochrome b6f complex in the light (see Supplemental Figure 3 online). The kinetics of decay in the dark were roughly the same. In summary, there appears to be a limitation in light-driven electron flow to cytochrome b6f, which would imply a defect in PSII function.

We tested this hypothesis by fluorescence induction analysis (Figure 4, Table 3). The rise in fluorescence emission from chlorophyll from the initial state in dark-adapted plants (F0) to the

Table 2. Efficiency of Plating on MacConkey Agar of dsbAB Strains Expressing Arabidopsis LTO1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>LB + Amp</th>
<th>Mac</th>
<th>Mac + Ara</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK295 (pBAD24)</td>
<td>Wild type</td>
<td>1.0</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>HK329 (pBAD24)</td>
<td>ΔdsbAB</td>
<td>1.0</td>
<td>10^-5</td>
<td>10^-4</td>
</tr>
<tr>
<td>HK329 (pLTO200)</td>
<td>ΔdsbAB lto1+</td>
<td>0.1</td>
<td>10^-5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Amp, ampicillin; Mac, MacConkey.

*EOP (efficiency of plating) values were calculated with respect to the growth of the wild-type strain HK295 (pBAD24) on LB agar containing ampicillin.*
maximal level ($F_{m}'$) provoked by a 80-ms saturating pulse, otherwise known as the variable fluorescence ($F_v$), is a function of the quantum efficiency of PSII (Rohacek and Bartak, 1999). The $F_v/F_{m}'$ ratio is taken as a measure of the maximal quantum yield of PSII. In wild-type leaves, $F_v/F_{m}'$ was 0.8, which is a typical value for healthy plants, and the variation was $\sim 1$ to 2% of this value. By contrast, the $F_v/F_{m}'$ ratio in lto1 leaves was $\sim 0.5$, with higher variability ($\sim 20$%; Table 3). This indicates a rather severe defect in PSII. Upon continuous illumination, $F_v$ dropped precipitously due to a large decrease in $F_{m}'$ (maximal fluorescence level in the light) in both wild-type and lto1 leaves. However, we found that the level of chlorophyll fluorescence before the saturating pulse ($F_0'$) was actually lower in lto1 plants, which is unusual (see Discussion). Nonphotochemical quenching in the lto1 leaves exhibited enormous variation and was not consistently higher or lower than wild-type leaves at the same light intensity (Table 3). While the lto1 leaves seemed to suffer from higher photoinhibition (loss of PSII activity due to illumination at high light fluxes) than wild-type leaves, the difference was not great (Table 3).

Figure 3. Knockdown of LTO1 Results in a Growth Defect.  
(A) Structure of LTO1 and SEN1. Black boxes represent exons and thin lines correspond to introns in SEN1 and LTO1. The initiation codons (ATG) of SEN1 and LTO1 are indicated. The position of the T-DNA insertion in the SALK_15193C line is indicated.  
(B) Expression of LTO1. Transcripts corresponding to LTO1 and SEN1 were analyzed by RT-PCR in homozygous lto1 and wild-type (WT [Col-0]) plants. TUB2 was used as a control for constitutive expression. To ensure that amplification was in the linear range, the optimal number of cycles was determined for each couple of primers separately. PCR amplification products were separated by electrophoresis in agarose gel and ethidium bromide stained. The gel was imaged using an imaging system. Four independent biological replicates were performed for this experiment, and one representative is shown in the figure.  
(C) The lto1 mutant displays a growth defect. Wild-type (Col-0) and lto1 homozygous plants are shown 6 weeks after seed germination on soil. [See online article for color version of this figure.]

Figure 4. The Photosynthetic Defect in the lto1 Mutant Is Due to a Limitation in the Electron Flow from PSII.  
Fluorescence induction of representative leaves from wild-type (A) and lto1 (B) plants. Fluorescence was measured using a 10-µs pulse of 430-nm light and appropriate filters to transmit red light. Actinic illumination provided by 520-nm LEDs (1.4 mmol photons m$^{-2}$ s$^{-1}$) commenced immediately after taking $F_0$ and $F_m'$ values at time 0. At each time point, fluorescence immediately before ($F_0'$, open circles) and after ($F_m'$, closed circles) a 80-ms saturating pulse was measured during a brief window in which the actinic light was off (see Methods for details). After 2 min, the actinic light was extinguished and the recovery of fluorescence was followed in the same manner. The variable fluorescence at each point ($F_v' = F_m' - F_0'$) is also shown as gray triangles. All fluorescence values were normalized to $F_{m}'$; thus, what is shown is actually $F_m'/F_{m}'$ (closed circles), $F_0'/F_{m}'$ (open circles), and $F_v'/F_{m}'$ (gray triangles). Upward and downward arrows indicate when the actinic light was on or off, respectively.
Actinic Light Intensity pinpoints PSII as the site of the photosynthetic defect caused by irreversible loss of quantum efficiency.

Photoinhibition in Wild-Type and Table 3.

<table>
<thead>
<tr>
<th>Actinic Light Intensity</th>
<th>Wild Type</th>
<th>lto1</th>
</tr>
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<tbody>
<tr>
<td>170 μE m⁻² s⁻¹</td>
<td>0.81 ± 0.01</td>
<td>0.49 ± 0.11</td>
</tr>
<tr>
<td>NPO</td>
<td>1.17 ± 0.08</td>
<td>0.90 ± 0.79</td>
</tr>
<tr>
<td>1400 μE m⁻² s⁻¹</td>
<td>0.80 ± 0.01</td>
<td>0.48 ± 0.11</td>
</tr>
<tr>
<td>NPO</td>
<td>1.37 ± 0.06</td>
<td>1.82 ± 1.15</td>
</tr>
</tbody>
</table>

Photoinhibition (%) = 1 – (FV/FM), where F0 is the initial fluorescence emission after dark adaptation and immediately before a saturating pulse, FM is the maximal fluorescence emission immediately after the saturating pulse, and the variable fluorescence parameter, Fv = Fm – F0. NPQ = (Fm – Fv)/Fm, where Fm is the maximal fluorescence emission after adaptation to the actinic light (after 2 min). Photoinhibition = 1 – (Fv/Fm) (Fv/Fm), where Fv and Fm are the variable and maximal fluorescence, respectively, after 2 min of exposure to 1400 μE m⁻² s⁻¹ actinic light followed by 8 min of dark recovery (i.e., irreversible loss of quantum efficiency).

Fluorescence parameters Fv/Fm (maximum quantum yield of PSII), non-photochemical quenching (NPQ), and photoinhibitions were calculated based on fluorescence measurements like those shown in Figure 4 using formulae described by Rohacek and Bartak (1999). Fv/Fm = (Fm – F0)/Fm, where F0 is the initial fluorescence emission after dark adaptation and immediately before a saturating pulse, FM is the maximal fluorescence emission immediately after the saturating pulse, and the variable fluorescence parameter, Fv = Fm – F0. NPQ = (Fm – Fv)/Fm, where Fv is the maximal fluorescence emission after adaptation to the actinic light (after 2 min). Photoinhibition = 1 – (Fv/Fm) (Fv/Fm), where Fv and Fm are the variable and maximal fluorescence, respectively, after 2 min of exposure to 1400 μE m⁻² s⁻¹ actinic light followed by 8 min of dark recovery (i.e., irreversible loss of quantum efficiency).

Finally, we examined the function of ATP synthase using the decay of the carotenoid bandshift signal at 520 nm as a spectroscopic marker for the transmembrane electric field of the thylakoid membrane (Sacksteder and Kramer, 2000). The rate of decay is a measure of how fast the proton motive force is expended, which is primarily due to ATP synthase activity. Although we saw slight differences between the wild-type and mutant leaves, in terms of the multiphasic decay of the carotenoid bandshift signal, the overall decay was similar (see Supplemental Figure 4 online). Thus, we see no evidence for major defects in the function of ATP synthase. In conclusion, our spectroscopy analysis of lto1 leaves pinpoints PSI as the site of the photosynthetic defect caused by the lto1 mutation. We saw no problems with the function of the other three major thylakoid membrane complexes: PSI, cytochrome b₇₆, or ATP synthase.

Loss of LTO1 Impacts PSII Accumulation

To further detail the impact on photosynthesis due to loss of LTO1, we performed immunoblot analyses using antibodies against the subunits of PSI, PSII, and the cytochrome b₇₆ complex. We found that loss of LTO1 has no impact on the accumulation of the cytochrome b₇₆ subunits, including the cytochromes b₇₆ and f that contain a heme cofactor (Figure 5). The steady state level of PC, the electron carrier between the cytochrome b₇₆ and PSI, was unaffected by the lto1 mutation (Figure 5). We also excluded a defect in PSI based on the fact that the abundance of Psaa, a core subunit whose accumulation is diagnostic of PSI assembly, is unchanged (Figure 5). By contrast, we noted a depletion of the PSII subunits, including D1, D2, PsbB (CP47), and PsbE (cytochrome b₅₅₃), which are the core proteins of the reaction center (Figure 6). Interestingly, there is also a severe reduction in the levels of PsbO, PsbP, and PsbO, three extrinsic proteins bound to the PSII core subunits on the luminal side of the thylakoid membrane (Bricker and Frankel, 2011; Popelkova and Yocum, 2011). Together, these three proteins define a functional module called the OEC, which mediates the light-dependent oxidation of water.

A construct expressing the LTO1 cDNA engineered with a C-terminal C-myc tag complements both the photodetributional growth and PSII defects when introduced in the lto1 mutant (Figures 6 and 7, Table 4). This demonstrates that the molecular lesion in LTO1 is responsible for the PSI-deficient phenotype. Using an anti-C-myc antibody to probe thylakoid membrane extracts, we were able to detect a 40-kD band corresponding to LTO1 in the lines complemented with the cDNA expressing construct (see Supplemental Figure 5 online).

The Thioredoxin-Like Domain of LTO1 Interacts with PsbO1 and PsbO2

The finding that LTO1 exhibits sulfhydryl oxidizing activity and is required for PSII accumulation suggests that the formation of one or more disulfide bonds is needed for the biogenesis of this photosynthetic complex. Based on our heterologous complementation experiments, it is likely this step occurs on the luminal side of the thylakoid membrane. Interestingly, PsbO, a luminal subunit of the OEC, carries a single disulfide bond that is critical for PSII assembly and activity (Burnap et al., 1994; Betts...
et al., 1996; Wyman and Yocum, 2005). To test the hypothesis that PsbO is a relevant target of LTO1, we decided to see if the thioredoxin-like soluble domain of LTO1 (LTO1sol), postulated to carry the sulfhydryl oxidizing activity, could interact with PsbO in a yeast two-hybrid assay. LTO1sol was used as bait and PsbO1 and PsbO2, the two isoforms of Arabidopsis PsbO (Peltier et al., 2002; Schubert et al., 2002), were used as prey in a GAL4-based two-hybrid system. In one study, the second Cys in the WCXXC motif was shown to be critical in detecting the interaction between thioredoxins and their targets using a yeast two-hybrid assay (Vignols et al., 2005). However, we found that both wild-type (WCSHC) and mutant (WCSHS) forms of LTO1sol could interact with either PsbO1 or PsbO2, based on the recovery of GAL4-dependent adenine/His prototrophies and aureobasidin A resistance in the yeast reporter strain (Figure 8). As expected, none of these polypeptides alone elicit such a response (Figure 8). This established both PsbO1 and PsbO2 as relevant targets of LTO1 action in vivo.

The Soluble Domain of LTO1 Can Catalyze Disulfide Bond Formation in PsbO

To test the possibility that the single Cys pair present in PsbO1 and PsbO2 could be oxidized by LTO1, we purified recombinant forms of LTO1sol and PsbOs and performed in vitro redox assays. We attempted to purify PsbO1 as a recombinant protein, but the resulting polypeptide was proteolytically processed when expressed in bacteria. Recombinant PsbO2, on the other hand, was purified in its oxidized form and the disulfide bond present in the molecule could be chemically reduced in a dose-dependent fashion by the action of DTT (Figure 9A). To test the ability of oxidized LTO1sol to introduce a disulfide bond in PsbO2, we incubated air-oxidized LTO1sol with reduced PsbO2. This resulted in the oxidation of PsbO2 (Figure 9B, lane 3). Concomitant with the oxidation of PsbO, oxidized LTO1sol was converted to its reduced form (Figure 9B, lane 3), an expected finding if LTO1sol catalyzes disulfide bond formation. Quantification of the oxidized and reduced species indicates that one molecule of reduced PsbO2 was acted upon by one molecule of oxidized LTO1. It should be noted that, even though DTT treatment fully reduced PsbO2, a small fraction of reduced PsbO2 can become reoxidized, presumably because of traces of oxygen present in solution (Figure 9B, lane 2). Based on our redox assays, we conclude that the thioredoxin-like domain of LTO1 can catalyze disulfide bond formation in the PsbO2 target.

DISCUSSION

The Plastid VKOR-Like Protein Defines a Trans-Thylakoid Thio-Oxidation Pathway

In this article, we explored the function of LTO1, a plastid VKOR-like protein in Arabidopsis. In plastids, VKOR-like proteins are present at the thylakoid membrane and carry a C-terminal, thioredoxin-like domain typical of oxidoreductases belonging to the PDI family (Ellgaard and Ruddock, 2005). By analogy to VKOR in the ER, a plastid VKOR-like protein is presumed to participate in a transmembrane thio-oxidation pathway. One key question in terms of deducing the function of a plastid VKOR-like protein is to define its topological arrangement within the thylakoid membrane, particularly with respect to the domains predicted to catalyze redox chemistry. Using bacterial topological reporters, a luminal localization was assigned for the two Cys pairs

Figure 6. Accumulation of PSII Subunits Is Impacted by Loss of LTO1.
The accumulation of PSII subunits (D1, D2, PsbO, PsbP, PsbQ, PsbB, and PsbE) in the lto1 mutant, the lto1 mutant complemented by the LTO1-C-MYC cDNA (under the control of the 35S promoter), and wild-type (WT) plants was analyzed via immunoblot analyses. PSI subunit PsaA and MFP1 (for MAR binding filament-like protein 1), a thylakoid-associated nucleoid binding protein, were used as control (Jeong et al., 2003). Thylakoid proteins corresponding to 7 μg of chlorophyll were separated on a 12% SDS-acrylamide gel. For an estimation of the protein abundance in the lto1 mutant, dilutions of the wild-type sample were loaded on the gel. Gels were transferred to membranes before immunodetection with antisera against PSII subunits PsaA and MFP1.

Figure 7. Complementation of the lto1 Photosynthetic Defect by LTO1 cDNA.
Phenotypes of the wild type (WT), two independent lto1 lines expressing the LTO1-C-MYC cDNA under the control of the 35S promoter (A and B), and lto1 homozygous plants after 3 weeks of growth on soil. [See online article for color version of this figure.]
Detailed phenotypic analysis of the *Arabidopsis* *lto1* mutant revealed that the function of the plastid VKOR-like protein is required for accumulation of PSII, a photosynthetic complex involved in the light-dependent reactions of photosynthesis (Figures 3 to 7). Moreover, analysis of the decay of variable fluorescence after a 1-ms flash in the presence of the Qb site inhibitor DCMU was consistent with the idea that there was a higher amount of PSII reaction centers with damaged OECs in the *lto1* mutant (see Supplemental Figure 6 online). We reasoned that catalysis of a disulfide bond in a luminal target is a required step for the biogenesis of PSII. Interestingly, PsbO, a luminal subunit of PSII required for stable assembly of the OEC, was shown to carry a single intramolecular disulfide that is strictly conserved in cyanobacteria and photosynthetic eukaryotes. In vitro studies established that this disulfide bond is critical to maintain the tertiary structure of PsbO (Tanaka et al., 1989; Betts et al., 2011). Leaves from five separate plants were removed and immediately assayed.

The Redox Activity of LTO1 Is Required for the Assembly of PSII

Table 4. Quantum Efficiency of Wild-Type, *lto1*, and LTO1-Complemented *lto1* Lines

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>Fv/Fm (±SD), n = 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>MS</td>
<td>0.828 ± 0.007</td>
</tr>
<tr>
<td><em>lto1</em></td>
<td>MS</td>
<td>0.811 ± 0.000</td>
</tr>
<tr>
<td><em>lto1</em> + LTO1</td>
<td>MS + Suc</td>
<td>0.815 ± 0.008</td>
</tr>
<tr>
<td>Wild type</td>
<td>MS + Suc</td>
<td>0.522 ± 0.113</td>
</tr>
<tr>
<td><em>lto1</em></td>
<td>MS + Suc</td>
<td>0.828 ± 0.003</td>
</tr>
<tr>
<td><em>lto1</em> + LTO1</td>
<td>MS + Suc</td>
<td>0.828 ± 0.000</td>
</tr>
</tbody>
</table>

Maximum quantum yield of PSII (Fv/Fm) was measured as described in Table 3 and Methods. Each type of plant was grown from seeds on Murashige and Skoog (MS) or Murashige and Skoog + 2% Suc medium under moderate light (~80 μmol PAR photons m⁻² s⁻¹). Leaves from five separate plants were removed and immediately assayed.

As seen with bacterial VKOR-like proteins, it is expected that the sulphydryl oxidizing activity of LTO1 is linked to the reduction of a quinone in the thylakoid membrane (Li et al., 2010; Kadokura and Beckwith, 2010). A role in disulfide bond formation for LTO1 is inferred from the fact that the plant protein is able to substitute for the function of DsbAB in bacteria (Figure 2). Further support for the involvement of LTO1 in sulphydryl oxidation in the thylakoid lumen comes from our evidence that lumen resident PsbO can be oxidized by the thioredoxin-like domain of the protein in our in vitro assay (Figure 9). Previous studies with mycobacterial and cyanobacterial VKOR-like proteins have demonstrated that the thioredoxin-like domain carries DsbA-like activity, while the VKOR-like central domain is functionally equivalent to DsbB (Singh et al., 2008; Dutton et al., 2010; Wang et al., 2011). In vitro assays show that the *Arabidopsis* LTO1 thioredoxin-like domain can transfer electrons to its VKOR-like central domain (Furt et al., 2010). This supports the view that the plastid protein operates in a manner similar to bacterial VKOR-like proteins in regard to thiol-disulfide chemistry.

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Detailed phenotypic analysis of the *Arabidopsis* *lto1* mutant revealed that the function of the plastid VKOR-like protein is present in the VKOR-like domain and the C-terminal thioredoxin-like domain of LTO1 (Figure 1). This result is in accordance with the topological model deduced for a mycobacterial VKOR-like protein and the crystal structure of a cyanobacterial VKOR-like protein. Both of these methods place the functional domains in the periplasm, which is analogous to the plastid lumen (Dutton et al., 2008; Li et al., 2010; Wang et al., 2011).

In proteobacteria, DsbA introduces disulfide bonds in Cys-containing targets, while DsbB recycles DsbA to its oxidized form with transfer of electrons to a quinone (Depuydt et al., 2011; Kadokura and Beckwith, 2010). A role in disulfide bond formation for LTO1 is inferred from the fact that the plant protein is able to substitute for the function of DsbAB in bacteria (Figure 2). Further support for the involvement of LTO1 in sulphydryl oxidation in the thylakoid lumen comes from our evidence that lumen resident PsbO can be oxidized by the thioredoxin-like domain of the protein in our in vitro assay (Figure 9). Previous studies with mycobacterial and cyanobacterial VKOR-like proteins have demonstrated that the thioredoxin-like domain carries DsbA-like activity, while the VKOR-like central domain is functionally equivalent to DsbB (Singh et al., 2008; Dutton et al., 2010; Wang et al., 2011). In vitro assays show that the *Arabidopsis* LTO1 thioredoxin-like domain can transfer electrons to its VKOR-like central domain (Furt et al., 2010). This supports the view that the plastid protein operates in a manner similar to bacterial VKOR-like proteins in regard to thiol-disulfide chemistry.

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et al., 1996; Wyman and Yocum, 2005). In chloroplast lumenal extracts, reduction of the disulfide in PsbO1 and PsbO2 targets the proteins for degradation. This indicates that the oxidation state of the Cys residues is a key determinant for the subunit stability in vivo (Hall et al., 2010). Further underscoring the importance of this disulfide, a mutation of one of the conserved Cys residues in cyanobacterial PsbO results in complete loss of the subunit and yields a PSII defect (Burnap et al., 1994). In Arabidopsis, loss of PsbO1 and PsbO2 impacts the stability of both extrinsic components of the OEC and core components of the reaction center (Yi et al., 2005). The resulting phenotype is similar to the one displayed by the lto1 mutant lines (Figure 6). Considering that PsbO is a relevant target of LTO1 activity (Figure 9) and that the redox state of the sulfhydryls appears to be determinant for the stability of this subunit (Burnap et al., 1994; Hall et al., 2010), it is conceivable that the PSI assembly deficiency in the lto1 mutant is caused by a sole defect in the oxidation of PsbO sulfhydryls. However, there are proteins required for PSII biogenesis that also have a disulfide bond, such as FKBP20-2, a lumen resident molecule (Lima et al., 2006). Therefore, we cannot rule out the possibility that additional factors might also contribute to the PSII-deficient phenotype in the lto1 mutant.

It is interesting that the fluorescence induction phenotype of the lto1 mutant strongly resembles that of Arabidopsis mutants with low amounts of functional PsbO protein. The quantum yield of PSII fluorescence is decreased significantly in mutants of PSBO1 (Murakami et al., 2002) and in RNA interference mutants largely lacking both PsbO1 and PsbO2 (Yi et al., 2005). However, the PSI maximal quantum yield in the lto1 mutant and in the psbO mutants is not as low as in mutants completely lacking PSII, where it effectively becomes zero (Bennoun et al., 1986). Moreover, in the psbO-1 mutant, Murakami et al. (2002) found that the fluorescence level transiently dropped below the F0 level during illumination, similar to what we saw in the lto1 mutant (Figure 4). This phenomenon is not limited to psbO mutants, as it was also seen recently with mutants in the cyanobacterial b559 subunit of PSII (Bondarava et al., 2010). One possibility is that the PQ pool is partially reduced in the dark in the lto1 mutant and in other mutants with low PSII activity, which would result in an increased F0 level. Upon illumination, PSI activity overwhelms PSII activity (due to the PSII deficiency), and the PQ pool becomes more oxidized, resulting in a fluorescence level lower than the level in the dark. We suggest that in such mutants, the cyclic electron flow pathway has been activated. This would allow the mutants to use PSI and cytochrome b6f to pump protons and thereby synthesize ATP in the absence of significant levels of linear electron flow due to the deficiency in PSI. This would explain both the elevated F0 level and the fact that P700* is reduced much faster in the lto1 mutants compared with the wild type (see Supplemental Figure 2 online), an expected finding if cyclic electron transfer is elevated (Finazzi and Forti, 2004; Iwai et al., 2010; Peeva et al., 2010).

Are There Other Relevant Targets of LTO1 Sulfhydryl Oxidizing Activity?

The lto1 mutant characterized during this study is a knockdown, and additional defects could be revealed in conditions of complete loss of LTO1 function (Figure 3). Conceivably, LTO1 regulates the redox state of additional Cys-containing proteins residing in the thylakoid lumen. An indication that further targets exist comes from the observation that a cyanobacterial VKOR-like null mutant displays a pleiotropic growth defect incompatible with a sole defect in PSII (Singh et al., 2008). However, it is not known if this phenotype can be attributed to impaired disulfide bond formation in targets residing in the thylakoid lumen and/or in the periplasm. Other possible targets of LTO1 are proteins containing lumen-facing Cys residues, which are active...
in the oxidized form. Only a few luminal proteins with known thiol-dependent enzymatic activities have been identified. Examples of such are violaxanthin deepoxidase, an enzyme involved in dissipating excess light (Yamamoto and Kamite, 1972; Sokolove and Marsho, 1976), STT7/STN7, a kinase involved in adaptation to changes in light intensity (Depege et al., 2003; Lemeille et al., 2009), and FKBP13, a prolyl isomerase postulated to act as a foldase for the Rieske protein (Gupta et al., 2002; Gopalan et al., 2004). It has now become apparent that thiol-disulfide chemistry is a catalyzed process not only restricted to bacterial energy-transducing membranes but operating also on the luminal side of the thylakoid membrane. Further genetic and biochemical dissection is needed to elaborate the thiol-metabolizing pathways and understand how they regulate the biogenesis of the thylakoid compartment.

METHODS

Bacterial Strains and Growth Media

Strains HK295 [F Δara-714 galU galK ΔlacZ]74 (pSrl thi) (Kadokura and Beckwith, 2002) and HK329 (HK295 ΔdsbA ΔdsbB) (Eser et al., 2009) were a kind gift from Jonathan Beckwith. Luria-Bertani (LB) broth, LB agar, and MacConkey agar were prepared as described previously (Silhavy et al., 1984). When indicated, ampicillin (50 μg/mL) and L-Ara were used.

Heterologous Complementation of the dsbAB Mutant

The LTO1 ORF was cloned, using the In-Fusion system (Clontech), into the Ara-inducible vector pBAD24 (Guzman et al., 1995) at Ncol and Sphi sites to create plasmid pLTO200. Primers used for In-Fusion cloning and sequencing of the resulting plasmids are listed in a table of primers (see Supplemental Methods 1 and Supplemental References 1 online). The pBAD24 and pLTO200 plasmids were introduced into the ΔdsbA HK329 strain and transformants assessed for complementation of motility and alkaline phosphatase (PhoA)–deficient phenotypes. For motility tests, 10 μL of an overnight culture grown in M9 medium was inoculated into the center of an M9 medium plate (0.25% agar), with or without 0.002% Ara. Plates were incubated overnight at 37°C. Ten plates were measured for swarm in centimeters using a metric ruler. Ten plates were measured for both the control pBAD24 and pLTO200 in both plus and minus Ara conditions. The PhoA activities were measured as described by Manoil (1991) using overnight cultures in M9 medium, with or without Ara.

Efficiency of Plating Assay

Strains HK295 (pBAD24), HK329 (pBAD24), and HK329 (pLTO200) were grown at 37°C overnight in LB broth supplemented with ampicillin. Cultures were serially diluted 1:10 in LB broth, and ~2 μL of these dilutions were spotted onto LB agar and MacConkey agar either lacking or containing L-Ara (0.2%). Plates were incubated overnight at 37°C. Efficiency of plating values were calculated with respect to the growth of the wild-type strain HK295 (pBAD24) on LB agar.

Topological Analysis of LTO1 via PhoA/LacZ Sandwich Fusions

Four PhoA/LacZα sandwich fusion constructs (pLTO212–pLTO215) were generated using In-Fusion recombination with three PCR products as described by Zhu et al. (2007). PCR products were generated using the LTO1 cDNA or the complete PhoA/LacZα fusion cassette in pMA657 as templates (Alexeyev and Winkler, 1999). The PCR products were then cloned into the isopropyl 1-thio-β-d-galactopyranoside ( IPTG)–inducible expression vector pMA657 (Alexeyev and Winkler, 1999) cut by BamHI and XbaI. The pLTO211 plasmid was also generated using In-Fusion recombination at Spel in pLTO210, which was constructed by In-Fusion recombination of the LTO1 ORF into pMA657. PhoA activity measurements were performed as described by Manoil (1991). On the same cultures, LacZ activity measurements were performed as described by Brickman and Beckwith (1975). The leaky nature of the lac promoter allowed measurements to be performed without IPTG induction.

Yeast Two-Hybrid Experiments

The soluble domain of LTO1 (Gln-247 to Gln-376) was used as bait, and the corresponding sequence was cloned via the In-Fusion technique (Clontech) as a PCR fragment at the Ndel-Sall sites of the pGBK7 vector. The sequence corresponding to the soluble domain of wild-type LTO1 was PCR amplified using LTO1-AD-F (5′-CGAGCTTATGCATCGTCGAAGTCGTCAGGCTGCGCGG-3′) and LTO1-AD-R (5′-CGATTCTCTTCGCTGAGGCTGCTGAGCTGCTGCTGCTGCTGAGGCTGCGCGG-3′) as primers and the Arabidopsis thaliana LTO1 cDNA from the ABRC (The Ohio State University; #U25043) as template. Plasmids expressing the mutant form of LTO1 (WCSHS) were constructed via the QuickChange II site-directed mutagenesis kit (Stratagene). Arabidopsis PsbO1 and PsbO2 (Ser-30 to Glu-322 or Ser-29 to Glu-331, respectively) were used as prey, and the corresponding sequences were cloned via In-Fusion as a PCR fragment at the Ndel-XhoI of the pGADT7 vector. The sequence corresponding to PsbO1 was PCR amplified using PsbO1-AD-F (5′-CGAGCTTATGCATCGTCGAAGTCGTCAGGCTGCGCGG-3′) and PsbO1-AD-R (5′-CGATTCTCTTCGCTGAGGCTGCTGAGCTGCTGCTGCTGCTGAGGCTGCGCGG-3′) and the sequence corresponding to PsbO2 was PCR amplified using PsbO2-AD-F (5′-CGAGCTTATGCATCGTCGAAGTCGTCAGGCTGCGCGG-3′) and PsbO2-AD-R (5′-CGATTCTCTTCGCTGAGGCTGCTGAGCTGCTGCTGCTGCTGAGGCTGCGCGG-3′) as primers and Arabidopsis wild-type cDNA as template. The yeast strain Y2HGold (Clontech) was used as a reporter.

In Vitro Redox Assay

The cloned and full-length ATG50820 cDNA was used as template in a PCR to amplify the coding sequence corresponding to the PsbO2 protein with the following oligonucleotides as primers: pET-Psbo2-F (5′-CTTTAAGAAGGATATACATATGTCGAACCAACCAGGGCGAGGTCATCTCT-3′) and pET-Psbo2-R (5′-CGATTCGTCGAAGTCGTCAGGCTGCGCGG-3′) and pET24b-AD-F (5′-CGAGCTTATGCATCGTCGAAGTCGTCAGGCTGCGCGG-3′) as primers and the Arabidopsis thaliana LTO1 cDNA from the ABRC (The Ohio State University; #U25043) as template. Plasmids expressing the soluble domain of Chlamydomonas reinhardtii LTO1 was codon optimized for expression in Escherichia coli using the DNA2:0 Gene Designer software (Villalobos et al., 2006). The optimized sequence was synthesized (Mr Gene). The resulting plasmid (pMA-LTO1opt) was used as template in PCR with Ndel- and Xhol-engineered oligonucleotides pET24b-LTO1opt-F (5′-CTTTAAGAAGGATATACATATGTCGAACCAACCAGGGCGAGGTCATCTCT-3′) and pET24b-LTO1opt-R (5′-CGATTCGTCGAAGTCGTCAGGCTGCGCGG-3′) as primers. The PCR products of both reactions were cloned at the Ndel-Xhol sites of the hexahistidinyl-tag vector pET24b (Novagen) using In-Fusion (Clontech), resulting in the pET24b-PsbO2 and pET24b-LTO1opt plasmids. For expression of the recombinant HisΔ-tagged proteins, 1 liter of LB broth (with 30 μg/mL kanamycin) of E. coli BL21(DE3) strain (Novagen) carrying pET24b-PsbO2 or pET24b-LTO1opt was grown from a 20-mL LB broth overnight starter culture. To induce the recombinant proteins, IPTG was added to a final concentration of 0.5 mM at A600 = 0.4, and the cultures were further grown for 3 h at 37°C. Cells were then harvested by centrifugation at 4000g for 20 min at 4°C, and the pellet was stored at −20°C. Batch purification of the HisΔ-tagged proteins was performed under denaturing conditions (6 M

Disulfide Bond Formation in the Lumen
urea) using nickel-nitrilotriacetic acid resin (Qiagen). Recombinant PsbO2 and LTO1 were dialyzed in a refolding buffer (25 mM Tris, pH 7.5) and stored at −80°C.

PsbO2 in 25 mM Tris-HCl, pH 7.5, was reduced by 200 μM DTT during 1 h on ice. DTT was eliminated by buffer exchange using the Amicon Centricon system (Ultragel-10 membrane; Millipore). Reduced PsbO2 (10 μM) in 25 mM Tris-HCl, pH 7.5, was incubated for 60 min at 25°C in the absence or presence of oxidized soluble LTO1 (16 μM) in 25 mM Tris-HCl, pH 7.5. After incubation, proteins were precipitated with trichloroacetic acid (final 5%), washed with ice-cold acetone and then dissolved in buffer containing 50 mM Tris-HCl, pH 6.8, 2% SDS, 10 mM 4-acetamido-4′-maleimido-7-stibene-2,2′-disulfonic acid (AMS). After a 90-min incubation, reduced (AMS derivative) and oxidized forms of PsbO2 and LTO1 were separated by 15% nonreducing SDS-PAGE and visualized by Coomassie Brilliant Blue staining.

**Growth of Arabidopsis**

The Col-0 ecotype of Arabidopsis was used as the wild type. The T-DNA line SALK_151963C (ito1) was provided as a confirmed homozygote by ABRC. Seeds were surface sterilized and sown on Murashige and Skoog plates (with or without 2% Suc) or on soil and stratified at 4°C for 48 h in the dark before germination. Plants were grown in controlled-environment chambers at a room humidity of 50% and provided daily with 16 h of light (80 to 120 μE m−2 s−1) and 8 h of dark at 22°C.

**Molecular Characterization of the ito1 Mutant Lines**

The SALK_151963C (ito1) line was analyzed for the presence and orientation of the T-DNA via PCR using genomic DNA as a template and the following primer pairs: S-Exon1D (5′-AGGAAACCACCTGTCTTAAAC-3′) and Exon2R (5′-TCAGATGAAGACATTATAC-3′); RB1 (5′-AGTGTGGATGGATGATTGG-3′) and Exon2R (5′-TCAGATGAAGACATTATAC-3′); RB1 (5′-AGTGTGGATGGATGATTGG-3′) and Stop (5′-TTACTGAAGTTGATTGGTCT-3′); and LB1 (5′-CGTGAGGGCCTTGCTGCAACT-3′) and S-Exon1D (5′-ATGGAAACCACCTGTCTTAAAC-3′).

**RNA Extraction and RT-PCR**

The leaves of 21-d-old ito1 and wild-type plants propagated on soil were used for total RNA isolation (Iratni et al., 1997). One microgram of DNase I–treated RNA was reverse transcribed using 200 units of Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. The reaction was performed in the presence of 1 μL of 100 μM oligo(dT)18 in a total volume of 15 μL at 42°C for 50 min. Aliquots (1 μL) were used as template for the PCR reaction with LTO1-specific primers Exon1D (5′-ATGATGGGCGGATTGGTTTTCGTC-3′) and Stop (5′-TTACTGAAGTTGATTGGTCT-3′); SEN1-specific primers S-Exon1D (5′-ATGGAAACCACCTGTCTTAAAC-3′) and S-Stop R (5′-TCACCTCTTCTACCCGCGACTC-3′); and TUB2-specific primers TUB2F (5′-CTCAAGAGGATTTTCAGCAGTA-3′) and TUB2R (5′-TCACCTCTTCTACCCGCGATT-3′).

**Plasmids for Plant Transformation**

For the complementation experiments, the LTO1 full-length cDNA (#U25043) was obtained from the ABRC. The LTO1 coding sequence (excluding the stop codon) was amplified by PCR with platinum Pfx DNA polymerase (Invitrogen) using the primers pENT/LTO1-F (5′-CACCATGATGGGCGGATTGGTTTTCGTC-3′) and pENTR/LTO1-R (5′-CTGAAAGTGGATTGGTTTTCGTC-3′). The amplified product was cloned using Gateway Technology into the pENTR/D-TOPO vector (Invitrogen) and then transferred into the pGW2B20 destination vector (T. Nakagawa, Shimane University, Matsue, Japan) previously linearized by XhoI. The resulting plasmid (p35S-LTO1-C-MYC) was transferred to Agrobacterium tumefaciens GV3101 by electroporation. The ito1 plants were transformed using the floral dip method (Clough and Bent, 1998), and selection of the T1 generation of transgensics was performed on Murashige and Skoog plates with kanamycin (30 μg·mL−1) and hygromycin B (30 μg·mL−1). Seedlings were transplanted onto soil 2 weeks after germination and grown in controlled-environment chambers.

**Protein Preparation and Analysis**

Total plant protein extracts were prepared following the method described by Hurkan and Tanaka (1988), and protein concentrations were determined using the Bio-Rad DC protein kit (with BSA as a standard). Total protein was extracted with an SDS-containing solubilization buffer, and chlorophyll concentration was then determined. When necessary, Coomasie Brilliant Blue–stained gels were used to assess equal loading. For immunoblot analyses, the proteins were separated by SDS-PAGE, blotted onto Immobilon-P membranes (Millipore), and immunodecorated with antibodies. Commercially available antibodies against PC, Psaa, D1, D2, PsbD, PsbO, PsbP, PsbQ (Agrisera), and C-myc (Sigma-Aldrich) were used. The anti-PetC and anti-PetD antibodies were provided by Barkan (University of Oregon). Antisera against cytochrome f, cytochrome b6, cytochrome b559, and MFP1 were gifts from Saebesta Merchant (University of California, Los Angeles), Catherine deVitry (Centre National de la Recherche Scientifique), Karen Meierhoff (Heinrich-Heine-Universität), and Iris Meier (The Ohio State University), respectively.

**Fluorescence Induction**

A freshly cut leaf was placed in the leaf cuvette of a JTS-10 LED spectrometer (Bio-Logic). Fluorescence emission was measured using a 10-μs pulse of light from a 520-nm LED as the excitation source, and a 670-nm high-pass filter was placed in front of the sample detector. A B639 filter was placed in front of the reference detector to measure the excitation pulse, allowing proper normalization of fluorescence emission. Actinic light and saturating pulses were provided by a 520-nm LED array. Before each run, leaves were allowed to dark adapt for 30 to 60 s followed by a 2-s treatment with far-red light (743-nm peak with full width at half maximum of 30 nm; photon flux = 25 mmol m−2 s−1) to drive complete oxidation of the PQ pool. At each time point, fluorescence was measured (F0 or F0′), followed immediately by an 80-ms saturating pulse to fully oxidize the PQ pool, and then fluorescence was measured 100 μs after the pulse was over (Fm or Fm′). The first pair of points should thus provide F0 and Fm. The actinic light was turned on 100 ms later, fluorescence measurements were taken 2 s after that, and then 24 more pairs of measurements were taken in an exponentially spaced fashion for 2 min total. The actinic light was turned on 100 ms later, fluorescence measurements were taken 2 s after that, and then 24 more pairs of measurements were taken in an exponentially spaced fashion for 2 min total. The actinic light was then extinguished, and 24 more time points were taken every 20 s. Note that the actinic light was briefly turned off while fluorescence measurements were made: At each time point, the actinic light was turned off, the F0′ measurement was taken 100 μs later, the 80-ms saturating pulse was given 20 μs later, and then 100 μs later the Fm′ measurement was taken, 20 μs after which the actinic light was turned back on. Thus, all fluorescence measurements were taken in the absence of any light besides the excitation source, avoiding complications or the necessity of subtraction of additional signals induced by the actinic or saturating lights. We found that Fm′ after 2 s of actinic light was slightly higher (~4%) than Fm in the wild-type case, indicating that the 80-ms pulse is not completely saturating. The difference was greater in the case of the ito1 mutant, consistent with the idea that it has a smaller antenna size. Thus, for the purposes of normalization of the data shown in Figure 4, we used the Fm′ value measured at 2 s, which was the highest fluorescence value measured in all cases.
Accession Numbers
Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers ACN43307 and ACN43308 for C. reinhardtii LTO1 cDNAs and accession number AAM65737 for Arabidopsis LTO1 cDNA.

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

Supplemental Figure 1. Alignment of LTO1-Like Proteins from Photosynthetic Eukaryotes.
Supplemental Figure 2. P700 Oxidation and Rereduction Kinetics.
Supplemental Figure 3. Oxidation and Reduction of Cytochrome b6 and Cytochrome f.
Supplemental Figure 4. Decay of Signal at 520 nm upon Termination of Actinic Light.
Supplemental Figure 5. Accumulation of LTO1-c-myc in the LTO1 Complemented lto1 Line.
Supplemental Figure 6. Decay of Flash-Induced Fluorescence in the Presence of DCMU.
Supplemental Table 1. Parameters of P700 Oxidation and Rereduction.
Supplemental Table 2. Parameters of Decay of Flash-Induced Fluorescence Rise in the Presence of DCMU.
Supplemental Methods 1. Spectroscopy Measurements and Table of Primers.

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AUTHOR CONTRIBUTIONS
P.P.H., K.R., and N.R. designed the experiments, analyzed the data, and wrote the article. M.K. and S.C. performed the bulk of the experiments with the exception of the in vivo spectroscopy measurements, which were performed by K.R.

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