Evidence for a Role of Chloroplastic m-Type Thioredoxins in the Biogenesis of Photosystem II in Arabidopsis

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Chloroplastic m-type thioredoxins (TRX m) are essential redox regulators in the light regulation of photosynthetic metabolism. However, recent genetic studies have revealed novel functions for TRX m in meristem development, chloroplast morphology, cyclic electron flow, and tetrapyrole synthesis. The focus of this study is on the putative role of TRX m1, TRX m2, and TRX m4 in the biogenesis of the photosynthetic apparatus in Arabidopsis (Arabidopsis thaliana). To that end, we investigated the impact of single, double, and triple TRX m deficiency on chloroplast development and the accumulation of thylakoid protein complexes. Intriguingly, only inactivation of three TRX m genes led to pale-green leaves and specifically reduced stability of the photosystem II (PSII) complex, implying functional redundancy between three TRX m isoforms. In addition, plants silenced for three TRX m genes displayed elevated levels of reactive oxygen species, which in turn interrupted the transcription of photosynthesis-related nuclear genes but not the expression of chloroplast-encoded PSII core proteins. To dissect the function of TRX m in PSII biogenesis, we showed that TRX m1, TRX m2, and TRX m4 interact physically with minor PSII assembly intermediates as well as with PSII core subunits D1, D2, and CP47. Furthermore, silencing three TRX m genes disrupted the redox status of intermolecular disulfide bonds in PSII core proteins, most notably resulting in elevated accumulation of oxidized CP47 oligomers. Taken together, our results suggest an important role for TRX m1, TRX m2, and TRX m4 proteins in the biogenesis of PSII, and they appear to assist the assembly of CP47 into PSII.

Thioredoxins (TRXs), the ubiquitous small (approximately 12 kD) thiolo:disulfide oxidoreductases, are essential redox regulatory elements in plant metabolism (Schürmann and Buchanan, 2008; Dietz and Pfannschmidt, 2011). All TRXs have a redox-active site that contains two conserved Cys residues in the peptide motif WC (G/P)C (Holmgren, 1989). In the reduced state, TRXs are able to reduce disulfide bridges in the target proteins, thereby modulating their functions and stability (Dietz and Pfannschmidt, 2011).

In contrast with other organisms, plants have a large number of TRX isoforms. At least 20 TRX isoforms have been identified in Arabidopsis (Arabidopsis thaliana), and these are targeted to various cellular compartments, including the chloroplast, mitochondria, and cytosol (Meyer et al., 2005; Lemaire et al., 2007). Depending on their subcellular localization, TRXs are reduced by different electron donor systems. Both cytosolic and mitochondrial TRXs are reduced by compartment-specific NADPH:thioredoxin reductases (NTRs), whereas chloroplastic TRXs are reduced by the ferredoxin:thioredoxin reductase with electrons provided by photosynthetic electron transport (Schürmann and Jacquot, 2000). In addition, chloroplasts have a modified type of NTR, called NTRC, which contains a C-terminal TRX domain and acts as a bifunctional enzyme with NTR/TRX activity for the reduction of target proteins (Serrato et al., 2004).

The chloroplastic TRX system is quite complicated and includes five major groups (2f, 4m, 1x, 2y, and 1z) in Arabidopsis (Collin et al., 2003, 2004; Schürmann and Buchanan, 2008; Arsova et al., 2010). The diversity of TRXs implies the existence of an intricate TRX-modulated redox regulatory network in the chloroplast. Previously, in vitro studies using purified TRX proteins have shown that the f- and m-type TRXs are required for carbon metabolism mainly through the regulation of some Calvin cycle enzymes, while the x- and y-type TRXs seem to serve as hydrogen donors for antioxidant enzymes (Collin et al., 2003, 2004; Schürmann and Buchanan, 2008; Chibani et al., 2011). However, the physiological significance and functional specificity of these different TRX isoforms remain to be determined. Recently, the genetic characterization of TRXs in mutant plants has revealed their novel physiological functions in vivo. The inactivation of TRX m1 leads to decreased...
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light activation of ADP-Glc pyrophosphorylase and altered diurnal starch turnover (Thormählen et al., 2013). The *trx m3* mutation hampers meristem development, leading to a seedling-lethal phenotype (Benitez-Alfonso et al., 2009), and TRX y2 functions as an electron donor to Met sulfoxide reductases for protein repair (Laugier et al., 2013). Collectively, these data reveal the potential for functional diversity in chloroplastic TRXs.

The primary reactions of photosynthesis are mediated by three pigment-protein complexes, PSII, the cytochrome *b*/*f* (Cyt *b*/*f*) complex, and PSI, which are embedded in the thylakoid membranes of chloroplasts and connected in series by small, mobile electron carriers like plastoquinone and plastocyanin (Rascher and Nedbal, 2006; Eberhard et al., 2008). A characteristic feature of these photosynthetic apparatuses is that all consist of multiple nucleus- and chloroplast-encoded subunits as well as numerous pigments, such as chlorophylls and xanthophylls. Hence, the biogenesis of the photosynthetic complexes depends upon a tight coordination between protein and pigment synthesis as well as the spatially and temporally coordinated assembly of the different subunits and the proper incorporation of various cofactors (Rochaix, 2011). Notably, mounting evidence suggests that TRXs play an essential role in the biogenesis of the photosynthetic apparatus. Global proteomic analyses have revealed that some photosynthetic apparatus subunits, such as D1 and PsbO in PSII, cytochrome *f* and Rieske FeS protein in the Cyt *b*/*f* complex, and PsA, PsAF, and PsaN in PSI, may be TRX partners (Motohashi and Hisabori, 2006, 2010, 2013). Although *trx m4* mutants exhibited elevated cyclic electron flow capacity, all *trx m1* and *trx m4* mutant plants showed no obvious growth defects when compared with wild-type plants under normal growth conditions (Courteille et al., 2013; Laugier et al., 2013), implying that functional redundancy may exist between TRX m1, TRX m2, and TRX m4. To test this hypothesis and to avoid the lethality of multiple TRX m deficiency, the tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) approach was applied to efficiently silence one or more TRX m genes (Liu et al., 2002; Burch-Smith et al., 2006; Arsova et al., 2010, 2012).

To verify the phenotypes in *trx m* single mutants, the coding regions of *TRX m1*, *TRX m2*, and *TRX m4* were individually cloned into the pTRV2 vector (pTRV2-TRX *m1*, pTRV2-TRX *m2*, and pTRV2-TRX *m4*) for efficient silencing of single *TRX m* genes (Burch-Smith et al., 2006). Three weeks after infection, the time interval used for all biological experiments, the mRNA levels of *TRX m* genes in VIGS plants were analyzed by quantitative real-time reverse transcription (RT)-PCR. In comparison with VIGS-GFP plants (the negative control), the expression of *TRX m4* was specifically reduced by more than 90% in the plants infiltrated with the pTRV2-TRX *m4* vector (called VIGS-TRX *m4* plants). Unexpectedly, we found that *TRX m2* mRNA levels in plants infiltrated with the pTRV2-TRX *m1* vector decreased by approximately 74%, and vice versa, whereas the expression of *TRX m4* was not influenced in these plants (Fig. 1B; Supplemental Table S1). This off-target silencing effect on *TRX m1* and *TRX m2* could be attributed to the significantly higher coding sequence homology between *TRX m1* and *TRX m2* (76.1% identity) than between *TRX m1* and *TRX m4* (57.9% identity; Supplemental Fig. S1A; Jackson et al., 2003; Chibani et al., 2009). Therefore, plants infected with the pTRV2-TRX *m1* and pTRV2-TRX *m2* vectors exhibited double *TRX m1* and *TRX m4* silencing.

RESULTS

Triple Inactivation of Three M-Type TRX Genes Causes a Pale-Green Leaf Phenotype in Arabidopsis

To examine the physiological functions of the TRX m1, TRX m2, and TRX m4 proteins in vivo, transfer DNA (T-DNA) insertional mutant libraries were screened, and homozygous *trx m1* and *trx m4* mutants were identified in previous studies (Courteille et al., 2013; Laugier et al., 2013). Although *trx m4* mutants exhibited elevated cyclic electron flow capacity, all *trx m1* and *trx m4* mutant plants showed no obvious growth defects when compared with wild-type plants under normal growth conditions (Courteille et al., 2013; Laugier et al., 2013), implying that functional redundancy may exist between TRX m1, TRX m2, and TRX m4. To test this hypothesis and to avoid the lethality of multiple TRX m deficiency, the tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) approach was applied to efficiently silence one or more TRX m genes (Liu et al., 2002; Burch-Smith et al., 2006; Arsova et al., 2010, 2012).

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In this study, we aimed to further investigate the role of chloroplastic TRXs in the biogenesis of the photosynthetic complexes. Among the numerous chloroplastic TRXs, the m-type TRX proteins have been suggested to be involved in leaf development, chloroplast morphology, cyclic electron flow, and tetrapyrrole synthesis (Ikegami et al., 2007; Chi et al., 2008; Benitez-Alfonso et al., 2009; Luo et al., 2012; Courteille et al., 2013). Besides these, the TRX m1, TRX m2, and TRX m4 proteins have been demonstrated to peripherally associate with the stroma-exposed thylakoid membranes (Peltier et al., 2002; Friso et al., 2004). All of these findings encouraged us to comprehensively investigate the impact of TRX m1, TRX m2, and TRX m4 deficiency on chloroplast development and the accumulation of the thylakoid protein complexes. Based on the pale-green leaf phenotype and the specifically impaired PSII complex in plants triply silenced for *TRX m1*, *TRX m2*, and *TRX m4*, we have established the redundant role of the TRX m1, TRX m2, and TRX m4 proteins in PSII accumulation. Furthermore, biochemical studies in vivo and in vitro indicate that the three TRX m proteins directly interact with PSII and modulate the reduction of intermolecular disulfide bonds in CP47.
m2 silencing and were designated VIGS-TRX m1/m2 and VIGS-TRX m2/m1 plants, respectively. Similar to the single TRX m mutant plants, VIGS-TRX m4, VIGS-TRX m1/m2, and VIGS-TRX m2/m1 plants showed no obvious differences from VIGS-GFP plants (Fig. 1; Supplemental Table S1). Furthermore, we introduced the coding regions of TRX m1 and TRX m2 into the pTRV2 vector (pTRV2-TRX m1m2 vector) for simultaneous inactivation of these two TRX genes. Plants infected with the pTRV2-TRX m1m2 vector showed more than 90% silencing efficiency for both TRX m1 and TRX m2 (called VIGS-TRX m1m2 plants) and also exhibited no visible phenotype (Fig. 1A; Supplemental Table S1). Therefore, we propose that silencing of individual m-type TRX genes and concurrent silencing of TRX m1 and TRX m2 have no obvious impact on leaf development.

To further investigate the influence of triple TRX m gene silencing on leaf development, we cloned the coding regions of TRX m4 with TRX m1 or TRX m2 into the pTRV2 vector (pTRV2-TRX m1m4 and pTRV2-TRX m2m4 vectors). Plants infected with either pTRV2-TRX m1m4 or pTRV2-TRX m2m4 showed the triple TRX m-silenced phenotype and were designated VIGS-TRX m1m4/m2 or VIGS-TRX m2m4/m1 plants, respectively (Fig. 1B; Supplemental Table S1). Interestingly, these plants had pale-green leaves with strongly reduced (approximately 45%) chlorophyll contents compared with the VIGS-GFP plants; the chlorophyll a/b ratio was 1.5 in VIGS-TRX m1m4/m2 and VIGS-TRX m2m4/m1 plants versus 2.1 in VIGS-GFP plants (Fig. 1, C and D). Taken together, the phenotypic comparison between single, double, and triple TRX m-silenced plants indicates
that the TRX m1, TRX m2, and TRX m4 proteins possess a functional redundancy in leaf greening, and only the inactivation of the three m-type TRX genes can result in pale-green leaves.

**PSII Activity Is Dramatically Reduced in Plants Triply Silenced for TRX m1, TRX m2, and TRX m4**

To gain insight into the primary target of the triple TRX m1, TRX m2, and TRX m4 gene silencing, noninvasive chlorophyll fluorometric analyses were performed to investigate photosynthetic electron transport in the VIGS plants (Baker, 2008). The ratio of variable fluorescence to maximal fluorescence ($F_v/F_m$), which is an indicator of the maximum efficiency of PSII photochemistry, was drastically decreased in the VIGS-TRX m1m4/m2 and VIGS-TRX m2m4/m1 plants (0.46 ± 0.02 versus 0.83 ± 0.01 in the VIGS-GFP controls; Fig. 2A; Table I). The decreased $F_v/F_m$ below 0.5 indicates that the triple TRX m-silenced plants have primary defects in electron transfer within PSII or a partial loss of PSII capacity (Meurer et al., 1996). Consistently, the actual PSII photochemical efficiency ($\Phi_{PSII}$) and photochemical quenching (qP) could not be measured in VIGS-TRX m1m4/m2 and VIGS-TRX m2m4/m1 plants (Table I). In contrast, nonphotochemical quenching (NPQ) in the VIGS-TRX m1m4/m2 and VIGS-TRX m2m4/m1 plants was higher than in the VIGS-GFP plants (Table I); NPQ is viewed as a compensatory mechanism in response to overexcited chlorophyll to prevent irreversible damage to PSII (Takahashi and Badger, 2011). Furthermore, inactive PSII always affects the light-induced redox kinetics of PSI (Meurer et al., 1996). The redox kinetics of $P_{700}$ showed that $P_{700}$ can be oxidized in VIGS-TRX m1m4/m2 and VIGS-TRX m2m4/m1 plants, but the amplitude of changes in $A_{820}$ induced by far-red light were lower than in the VIGS-GFP plants, and the $\Delta A/\Delta A_{max}$ value was greater than 1 (Fig. 2B), implying that PSI in the triple TRX m-silenced plants is functional but with low activity. These spectroscopic analyses suggest that the photosynthetic changes in the triple TRX m-silenced plants primarily reflect a reduction in PSII activity.

**Accumulation of the PSII Complex Is Specifically Impaired in Plants Triply Silenced for TRX m1, TRX m2, and TRX m4**

The defect in PSII photosynthetic electron transport could be associated with altered protein levels in the PSII complex. To examine the steady-state levels of thylakoid proteins in the VIGS plants, we performed immunoblot analyses using antibodies raised against diagnostic subunits from four thylakoidal membrane photosynthetic protein complexes (i.e. PSII, Cyt b6f complex, PSI, and ATP synthase). Our results showed that levels of the chloroplast-encoded PSII core subunits D1, D2, CP43, and CP47 in thylakoids isolated...
from VIGS-TRX m1m4/m2 and VIGS-TRX m2m4/m1 plants were reduced to approximately 58%, 58%, 18%, and 28% of VIGS-GFP control levels, respectively (Fig. 3). The nucleus-encoded 33-kD subunit of the oxygen-evolving complex (Psbo) accumulated to approximately 68% and 65% of control levels, 64% of VIGS-GFP levels, and the light-harvesting complex II (LHCII) and PSI antenna protein (Lhca1) were reduced to approximately 68% and 65% of control levels, respectively (Fig. 3). In contrast, the diagnostic subunits of PSI (PsaA), the Cyt b6f complex (cytochrome f), and ATP synthase (CFI β) in the VIGS-TRX m1m4/m2 and VIGS-TRX m2m4/m1 plants accumulated to levels similar to those in the control VIGS-GFP plants (Fig. 5).

To further investigate the putative structural alterations of thylakoid membrane protein complexes in the triple TRX m gene-silenced plants, thylakoid membranes from VIGS plants were solubilized with n-dodecyl β-d-maltoside (DM), and the chlorophyll-protein complexes (with equal amounts of chlorophyll) were separated by blue native (BN)-PAGE (Schägger et al., 1994). Seven protein complexes were resolved in the first dimension in the presence of Coomassie blue dye; these represent PSII supercomplexes (band I), PSI monomers and PSII dimers (band II), PSI monomers (band III), CP43-minus PSII (band IV), trimeric (band V) and monomeric (band VI) LHCII, and unassembled proteins (band VII; Fig. 4A). The BN-PAGE analysis clearly showed that the amount of PSII complexes (bands I, II, III, and IV) per unit of chlorophyll was significantly lower in thylakoids isolated from the VIGS-TRX m1m4/m2 and VIGS-TRX m2m4/m1 plants than that from the VIGS-GFP control plants (Fig. 4A). Analyses of the second-dimension SDS-urea-PAGE gels after Coomassie blue staining confirmed that the relative amounts of the PSII core subunits D1, D2, CP43, and CP47 were considerably reduced in the VIGS-TRX m1m4/m2 and VIGS-TRX m2m4/m1 plants, while no significant changes were observed in the amounts of Cyt b f and ATP synthase (Fig. 4, D and E). Collectively, our results indicate that the dramatically decreased PSII activity seen in plants silenced for the three TRX m genes could be attributed to the impaired accumulation of the PSII complex.

To assess whether the triple silencing of TRX m genes causes ultrastructural changes in chloroplasts, transmission electron micrographs of ultrathin sections of leaves from various VIGS plants were analyzed. In agreement with the phenotypic comparisons between VIGS-TRX m and VIGS-GFP plants, chloroplasts from VIGS-TRX m4, VIGS-TRX m1/m2, VIGS-TRX m2/m1, and VIGS-TRX m1m2 plants were similar in size to those from VIGS-GFP plants, and they contained well-developed membrane systems composed of grana connected by stroma lamellae (Fig. 5). In contrast, the thylakoid membranes from VIGS-TRX m1m4/m2 and VIGS-TRX m2m4/m1 chloroplasts were disrupted, such that the grana stacks in VIGS-TRX m1m4/m2 and VIGS-TRX m2m4/m1 chloroplasts appeared to be less connected by stroma lamellae than those in VIGS-GFP control chloroplasts (Fig. 5).

Expression of Chloroplast-Encoded PSII Subunits Is Not Altered in Plants Triply Silenced for TRX m1, TRX m2, and TRX m4

The chloroplast transcriptional regulation system in Arabidopsis depends upon both the plastid-encoded plastid RNA polymerase (PEP) and the nucleus-encoded plastid RNA polymerase (NEP), which have different target genes (Shiina et al., 2005). Chloroplast genes, therefore, can be classified into three groups according to their promoter structures: most of the photosynthetic genes depend on PEP (class I), whereas RNA Polymerase Subunit Alpha (RpoA), RpoB, Acetyl-CoA Carboxylase Carboxyl Transferase Subunit Beta (AccD), and Uncharacterised Protein Family Ycf2 (Ycf2) genes are exclusively transcribed by NEP (class III), and the nonphotosynthetic housekeeping genes are generally transcribed by both PEP and NEP (class II; Hajdukiewicz et al., 1997).

The recently characterized TRX z participates in the PEP-dependent expression of chloroplast genes through the redox regulation of two fructokinase-like proteins (Arsova et al., 2010). To test whether the significant reduction in PSII core subunits observed in the triple TRX m-silenced plants resulted from impaired transcription of chloroplast genes, we used quantitative real-time RT-PCR to analyze mRNA levels of genes that encode the representative chloroplast proteins transcribed by NEP and/or PEP in the VIGS plants (Table II). The PsbA, PsbB, PsbC, and PsbD genes that encode photosystem subunits were selected as representatives of the PEP-dependent genes (class I), RpoA, AccD, and Ycf2 were selected as NEP-dependent genes (class III), and Caseinolytic Protease P (ClpP) and NADH Dehydrogenase ND2 were chosen as class II genes. Quantitative

Table I. Chlorophyll fluorescence parameters in VIGS-TRX m and VIGS-GFP plants

<table>
<thead>
<tr>
<th>Parameter</th>
<th>VIGS-GFP</th>
<th>VIGS-TRX m4</th>
<th>VIGS-TRX m1m2</th>
<th>VIGS-TRX m2m1</th>
<th>VIGS-TRX m2m1</th>
<th>VIGS-TRX m1m4/m2</th>
<th>VIGS-TRX m2m4/m1</th>
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<tbody>
<tr>
<td>$F_{v}/F_{m}$</td>
<td>0.830 ± 0.001</td>
<td>0.825 ± 0.003</td>
<td>0.824 ± 0.004</td>
<td>0.824 ± 0.006</td>
<td>0.835 ± 0.001</td>
<td>0.463 ± 0.021**</td>
<td>0.461 ± 0.030**</td>
</tr>
<tr>
<td>$\Phi_{psii}$</td>
<td>0.352 ± 0.014</td>
<td>0.553 ± 0.018</td>
<td>0.554 ± 0.015</td>
<td>0.589 ± 0.011</td>
<td>0.536 ± 0.005</td>
<td>0**</td>
<td>0**</td>
</tr>
<tr>
<td>NPQ</td>
<td>0.721 ± 0.071</td>
<td>0.751 ± 0.067</td>
<td>0.684 ± 0.035</td>
<td>0.601 ± 0.037</td>
<td>0.633 ± 0.076</td>
<td>1.877 ± 0.256**</td>
<td>1.913 ± 0.226**</td>
</tr>
<tr>
<td>$1 - qP$</td>
<td>0.281 ± 0.118</td>
<td>0.242 ± 0.023</td>
<td>0.249 ± 0.021</td>
<td>0.209 ± 0.015</td>
<td>0.277 ± 0.015</td>
<td>1**</td>
<td>1**</td>
</tr>
</tbody>
</table>

Note: *, **, and *** denote significance relative to VIGS-GFP plants at 0.05, 0.01, and 0.001 levels, respectively.
real-time RT-PCR analysis revealed that the expression of class I genes in VIGS-TRX m plants was no different from that in VIGS-GFP control plants (Table II), suggesting that TRX m proteins are not involved in the regulation of PEP-dependent gene expression. In spite of this, triple silencing of TRX m1, TRX m2, and TRX m4 altered the mRNA levels of some chloroplast-encoded genes. The ClpP and Ycf2 transcript levels were significantly different in comparison with levels in the VIGS-GFP plants (Table II). In addition, expression of the nucleus-encoded genes PsaE, PsbO, and PSII Light Harvesting Complex Gene2.4 (Lhcb2.4), which encode proteins that are targeted to the chloroplast, was substantially reduced in VIGS-TRX m1m4/m2 and VIGS-TRX m2m4/m1 plants (Table II). These data indicate that silencing the three TRX m genes does not result in a general inhibitory effect on the transcription of chloroplast protein genes.

VIGS-TRX m Plants Accumulate More Reactive Oxygen Species Than VIGS-GFP Plants

TRX m proteins function as electron donors for Cys-containing proteins and regulate the functions of target proteins by reducing their disulfide bonds. Among the target proteins of TRX m, 2-Cys peroxiredoxin is an important antioxidant for hydrogen peroxide (H₂O₂) detoxification in chloroplast (König et al., 2002; Dietz and Pfannschmidt, 2011; Tovar-Méndez et al., 2011). Previous studies suggest that TRX m proteins are able to reduce the oxidative state of 2-Cys peroxiredoxin to maintain the metabolic balance of reactive oxygen species (ROS; Collin et al., 2003; Chi et al., 2008). Therefore, we hypothesized that simultaneous inactivation of the Arabidopsis TRX m1, TRX m2, and TRX m4 genes could influence the ROS level in vivo. The results of histochemical staining demonstrated that elevated levels of H₂O₂ and superoxide anion radical (O₂⁻) were detected in all VIGS-TRX m plants, especially in the triply silenced VIGS-TRX m1m4/m2 and VIGS-TRX m2m4/m1 plants (Fig. 6). ROS accumulation at similar levels has been detected in other TRX m gene-silenced plants, such as the Arabidopsis TRX m3 knockout mutant (Benitez-Alfonso et al., 2009) and in rice (Oryza sativa) OsTRX m RNA interference plants (Chi et al., 2008). Furthermore, it is widely accepted that ROS serve as an essential redox signal for the transcriptional regulation of photosynthesis-related nuclear genes (Nott et al., 2006; Ruckle et al., 2007). In line with this, our expression analysis showed that mRNA levels for the nucleus-encoded chloroplast...
protein genes PsAE, PsbO, and Lhcb2 were down-regulated (Table II). Taken together, our data suggest that triple silencing of TRX m1, TRX m2, and TRX m4 not only results in a defect in the PSII complex but also affects ROS metabolism.

**Confirmation of Impaired PSII Accumulation in Triple TRX m-Silenced Plants**

The above results strongly indicated that the triple inactivation of TRX m1, TRX m2, and TRX m4 leads to pale-green leaves and impaired PSII accumulation. To further confirm these observations in TRX m mutant plants, the homozygous trx m1trx m4 double mutant was isolated by crossing and was found to have a similar phenotype to wild-type plants (Supplemental Fig. S2; Supplemental Tables S1 and S2). In combination with the phenotype observed in plants silenced for both TRX m1 and TRX m2, we propose that the simultaneous inactivation of TRX m1 and either TRX m2 or TRX m4 has no conspicuous effect on leaf development.

Furthermore, we introduced the TRV-TRX m2 vector into trx m1trx m4 double mutant plants to silence the TRX m2 gene. Three weeks after infection, plants infiltrated with the pTRV2-TRX m2 vector displayed evidently reduced expression of TRX m1 and TRX m2 genes in comparison with VIGS-GFP (wild-type) plants and were designated VIGS-TRX m2/m1 (WT) and VIGS-TRX m2/m1 (trx m1trx m4) plants (Fig. 7B; Supplemental Table S1). The VIGS-TRX m2/m1 (trx m1trx m4) plants also had pale-green leaves and sharply reduced chlorophyll contents (Fig. 7, A–D). The two-dimensional BN/SDS-PAGE analysis indicated that the accumulation of PSII complexes (bands I, II, III, and IV) and PSII core proteins was specifically and markedly reduced in thylakoid membranes isolated from VIGS-TRX m2/m1 (trx m1trx m4) plants (Fig. 7, E–I).

**M-Type TRX Proteins Associate with Minor PSII Assembly Intermediates and Interact with PSII Core Subunits D1, D2, and CP47**

The observed reduction in the abundance of PSII complexes in the triple TRX m-silenced plants implies a direct regulatory function for m-type TRX proteins in the biogenesis of PSII. To test this hypothesis, we asked whether the TRX m1, TRX m2, and TRX m4 proteins are associated with the PSII complex. Because of the high degrees of similarity between the amino acid sequences of these three TRX proteins (Supplemental Fig. S1B), we were unable to prepare antibodies raised specifically against each of the three individual TRX m proteins. Alternatively, we combined 2 × FLAG-tagged proteins at the C termini of TRX m amino acid sequences for immunoblot analysis. Moreover, to strengthen the interaction between TRX m and their target proteins, the C-terminal
VIGS-GFP plants.

given, where 3.32 corresponds to a 10-fold up-regulation and represents means

Student’s

Table II. Changes in transcript abundance of chloroplast-encoded and nucleus-encoded genes in VIGS-TRX m plants and VIGS-GFP plants

<table>
<thead>
<tr>
<th>Class I genes</th>
<th>Accession No.</th>
<th>VIGS-TRX m4</th>
<th>VIGS-TRX m1/m2</th>
<th>VIGS-TRX m2/m1</th>
<th>VIGS-TRX m1/m2</th>
<th>VIGS-TRX m1/m2</th>
<th>VIGS-TRX m1/m2</th>
<th>VIGS-TRX m1/m2</th>
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<tbody>
<tr>
<td>PsaA</td>
<td>Atcg00350</td>
<td>0.215 ± 0.127</td>
<td>−0.040 ± 0.134</td>
<td>−0.145 ± 0.021</td>
<td>−0.085 ± 0.021</td>
<td>−0.228 ± 0.244</td>
<td>−0.412 ± 0.087</td>
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<tr>
<td>PsbA</td>
<td>Atcg00020</td>
<td>0.350 ± 0.113</td>
<td>0.325 ± 0.085</td>
<td>0.235 ± 0.226</td>
<td>0.270 ± 0.014</td>
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<td>PsbB</td>
<td>Atcg00680</td>
<td>0.068 ± 0.214</td>
<td>−0.202 ± 0.427</td>
<td>−0.188 ± 0.238</td>
<td>−0.202 ± 0.427</td>
<td>−0.105 ± 0.200</td>
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<td>PsbC</td>
<td>Atcg00280</td>
<td>−0.308 ± 0.351</td>
<td>0.070 ± 0.627</td>
<td>−0.152 ± 0.837</td>
<td>−0.310 ± 0.113</td>
<td>−0.478 ± 0.511</td>
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<td>Atcg00270</td>
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<td>0.130 ± 0.177</td>
<td>−0.153 ± 0.032</td>
<td>0.210 ± 0.156</td>
<td>−0.367 ± 0.618</td>
<td>−0.183 ± 0.154</td>
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<td>ClpP</td>
<td>Atcg00670</td>
<td>−0.615 ± 0.163</td>
<td>−0.245 ± 0.219</td>
<td>−0.485 ± 0.120</td>
<td>−0.225 ± 0.057</td>
<td>−1.595 ± 0.054**</td>
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<td>0.475 ± 0.001</td>
<td>N.D.</td>
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<td>0.145 ± 0.042</td>
<td>0.190 ± 0.078</td>
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<td>0.090 ± 0.057</td>
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<td>−1.212 ± 0.174**</td>
<td>−1.248 ± 0.215**</td>
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<td>−0.050 ± 0.064</td>
<td>−0.075 ± 0.014</td>
<td>0.140 ± 0.007</td>
<td>−1.050 ± 0.228**</td>
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<td>−3.563 ± 0.191**</td>
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Cys residue in the TRX active-site motif (WCGPC) that catalyzes the dissociation of target proteins from TRXs was substituted with a Ser residue (Motohashi et al., 2001; Balmer et al., 2003). The TRX m1C105S-FLAG, TRX m2C113S-FLAG, and TRX m4C119S-FLAG fusion proteins were transiently expressed separately in Arabidopsis mesophyll protoplasts, and TRX m3C105S-FLAG was also used as a reference protein (Supplemental Fig. S3A). Thylakoid membranes were then isolated from protoplasts and solubilized using 1% (w/v) DM for two-dimensional BN/SDS-PAGE analysis (Fig. 8A). After electrophoresis, immunoblot analyses were performed using antibodies

Figure 5. Electron micrographs of chloroplasts from VIGS plants. Images of intact chloroplasts and closeup views were obtained from VIGS-TRX m and VIGS-GFP plants. Leaves were collected 3 weeks after infection. Two biological replicates were performed, and similar results were obtained. Scale bars are as indicated.
raised against FLAG-tagged TRX m proteins and PSII subunits (D1, D2, CP43, and CP47). The results showed that the vast majority of the TRX m1, TRX m2, and TRX m4 proteins, but not TRX m3, could comigrate with minor PSII assembly intermediates such as the PSII reaction center (RC), the CP43 precomplex, and the CP47 precomplex, and the immunoblot signals for the TRX m1, TRX m2, and TRX m4 proteins were also detected in the CP43-minus PSII monomers (band IV; Fig. 8B). To confirm the association of TRX m with the PSII complex, we examined the interactions of TRX m with PSII subunits by means of pull-down assays. For this purpose, the immobilized His-TRX m1C107S, His-TRX m2C113S, His-TRX m4C119S, and His-TRX m3C100S recombinant proteins were used as baits to trap potential targets in the thylakoid membranes (Supplemental Fig. S3B). The trapped TRX m target proteins were eluted and subjected to immunoblot analysis. The results showed that TRX m1, TRX m2, and TRX m4, but not TRX m3, could directly interact with PSII core subunits D1, D2, and CP47, but not CP43. In addition, the four TRX m proteins could interact with the ATP synthase subunit (CF1 β), which is a well-known TRX target protein (Motohashi and Hisabori, 2006; Ströher and Dietz, 2008; Montrichard et al., 2009; Lindahl et al., 2011), but not with representative subunits of PSI (PsaA) and the Cyt b₆f complex (cytochrome f; Fig. 8C).

The Deficiency of TRX m1, TRX m2, and TRX m4 Interrupts the Redox Status of PSII Core Subunits

Based on the interactions between TRX m and PSII, we wondered whether the triple inactivation of TRX m1, TRX m2, and TRX m4 could have an effect on the redox status of the PSII complex in vivo. Amino acid sequence analyses showed that many PSII subunits, including D1, D2, CP43, CP47, and PsbO, have at least two conserved Cys residues that can form the intramolecular or intermolecular disulfide bonds (Shimada et al., 2007). Therefore, we investigated the redox states of intramolecular and intermolecular disulfide bonds in PSI proteins isolated from VIGS plants.

To analyze the redox status of intramolecular disulfide bridges in PSII subunits, 4-acetoamido-4-formimidylstilbene-2,2-disulfonate (AMS; Invitrogen) was used to bind the reduced Cys residues (thiol group/sulfhydryl group) in reduced PSII proteins. In contrast, oxidized proteins with disulfide bridges or sulfenic acids cannot bind AMS; thus, the AMS-labeled reduced proteins migrate slower on nonreducing SDS-PAGE gels than do the oxidized proteins (Ikegami et al., 2007; Karamoko et al., 2011; Richter et al., 2013). To comprehensively analyze the exchanges of intramolecular disulfide bonds in PSII subunits, protein extracts from VIGS-GFP plants were treated with either the oxidant CuCl₂, to induce disulfide bond formation, or the reducing agent dithiothreitol (DTT), for reduction of Cys residues, prior to AMS treatment. The mobility of AMS-labeled PSII subunits on nonreducing SDS-PAGE gels was then analyzed by immunoblotting. For D1, D2, CP43, and CP47, only one band was detected in the VIGS-GFP protein extracts, and the protein mobility was unaltered following CuCl₂ or DTT treatment, whereas the mobility of AMS-labeled PsbO proteins showed a response to different redox conditions in that two oxidized PsbO bands were detected in the CuCl₂-treated and nontreated VIGS-GFP protein extracts, and the protein mobility was unaltered following CuCl₂ or DTT treatment, whereas the mobility of AMS-labeled PsbO proteins showed a response to different redox conditions in that two oxidized PsbO bands were detected in the CuCl₂-treated and nontreated VIGS-GFP protein extracts, and DTT could reduce partially oxidized PsbO to reduced PsbO (Supplemental Fig. S4). This suggested that only PsbO proteins are capable of forming intramolecular disulfide bridges and is supported by the evidence that LUMEN THIOL OXIDOREDUCTASE1 (LTO1) mediates the formation of intramolecular disulfide bonds in PsbO, which is essential for the assembly of PSII (Karamoko et al., 2011). However, the mobility of AMS-labeled PsbO in VIGS-TRX m1m4/m2 and VIGS-TRX m2m4/m1 plants was not different from that in VIGS-GFP and VIGS-TRX m1m2 plants (Supplemental Fig. S4). These results suggest that the triple inactivation of TRX m genes does not influence the reduction of intramolecular disulfide bonds in PSII subunits.
Figure 7. Characterization of VIGS-TRX m2 (trx m1trx m4) plants. A, Representative photographs of the wild-type VIGS-GFP (WT), VIGS-GFP (trx m1trx m4), VIGS-TRX m1/m2 (WT), and VIGS-TRX m2/m1 (trx m1trx m4) plants, which were infected with the pTRV2-GFP and pTRV2-TRX m2 vectors via agroinfiltration in wild-type and trx m1trx m4 double mutant backgrounds, respectively. Representative leaves are shown in the insets. At least three biological replicates were performed, and similar results were obtained. Bars = 1 cm. B, The suppression rate of TRX m genes in VIGS-TRX m plants was analyzed by quantitative real-time RT-PCR. The relative mRNA levels of TRX m1, TRX m2, and TRX m4 in the VIGS-TRX m2/m1 (WT), VIGS-GFP (trx m1trx m4), and VIGS-TRX m2/m1 (trx m1trx m4) plants were normalized to the levels in VIGS-GFP (WT) plants (100%). The data represent means ± SD of three biological replicates. C and D, Total chlorophyll (Chl) contents (C) and chlorophyll a/b ratios (D) of the VIGS plants in wild-type and trx m1trx m4 double mutant backgrounds. FW, Fresh weight. The data represent means ± SD of three independent experiments. Statistical significance compared with the VIGS-GFP (WT) plants is indicated by asterisks (**P ≤ 0.01, Student’s t test). E, BN-PAGE analyses of thylakoid chlorophyll-protein complexes. Equal amounts of thylakoid membranes (8 μg of chlorophyll) from the VIGS plants in wild-type and trx m1trx m4 double mutant backgrounds...
Furthermore, we also examined the role of TRX m proteins in reducing the intermolecular disulfide bridges in PSII core subunits. With the formation of intermolecular disulfide bonds, two or more proteins can be aggregated to form homooligomeric/heterooligomeric complexes (Buchanan and Balmer, 2005; Dietz and Pfannschmidt, 2011). To improve the mobility and visualization of intermolecular disulfide bond-mediated macromolecular complexes, protein extracts from VIGS plants were separated by nonreducing SDS-PAGE without AMS labeling (Kirchsteiger et al., 2009; Arsova et al., 2010; Richter et al., 2013). On nonreducing SDS-PAGE gels, the monomers and oligomers of PSII core proteins D1, D2, CP43, and CP47 were observed in VIGS-GFP protein extracts, and the vast majority of PSII core subunits were present as monomeric proteins (Fig. 9). Moreover, we found that the oligomerization of D1 and CP47 was redox sensitive. CuCl2 treatment could significantly increase the aggregation of D1 and CP47 proteins, while DTT could reduce the oligomers to monomers (Fig. 9, A and C), suggesting that D1 and CP47 can form redox-regulated intermolecular disulfide bridges. One possible explanation for the observed oligomeric D2 and CP43 proteins could be the physical interactions between the four PSII core subunits (Wollman et al., 1999; Iwata and Barber, 2004; Nelson and Yocum, 2006). Interestingly, more oxidized CP47 oligomers were detected in VIGS-TRX m1m4/m2 and VIGS-TRX m2m4/m1 plants in comparison with VIGS-GFP and VIGS-TRX m1m2 plants. In contrast, the levels of oligomers and monomers of D1, D2, and CP43 detected in VIGS-TRX m1m4/m2 and VIGS-TRX m2m4/m1 plants were significantly lower than in VIGS-GFP and VIGS-TRX m1m2 plants (Fig. 9C). These data suggest that TRX m1, TRX m2, and TRX m4 are required for modulating the redox status of intermolecular disulfide bridges in PSII core subunits, and the simultaneous inactivation of the three TRX m genes leads to elevated levels of oxidized CP47 oligomers.

DISCUSSION

PSII is an integral membrane, multisubunit protein complex that catalyzes the light-driven water-splitting reaction and reduction of plastoquinone to initiate photosynthetic electron flow. In addition to structural studies of PSII, an emphasis has been placed on the identification of auxiliary proteins involved in the biogenesis and maintenance of the PSII complex (Mulo et al., 2008; Nixon et al., 2010; Komenda et al., 2012). Understandably, the identification of PSII accessory factors and the characterization of their functions will greatly improve our understanding of regulatory mechanisms by which the PSII complex accumulates and is maintained. Here, we report previously unknown functions for the classical chloroplastic TRX m1, TRX m2, and TRX m4 in the regulation of PSII biogenesis in Arabidopsis.

TRX m1, TRX m2, and TRX m4 Are Required for the Accumulation of PSII in Arabidopsis

To investigate the functions of TRX m1, TRX m2, and TRX m4 in the accumulation of the photosynthetic apparatus, we comprehensively characterized the phenotypes of plants silenced for one, two, or three of these TRX m genes. Interestingly, single deficiencies of either TRX m1 or TRX m4, and double inactivation of TRX m1 along with either TRX m2 or TRX m4, resulted in no macroscopic alteration in phenotype, whereas plants silenced for all three genes had pale-green leaves (Figs. 1 and 7; Supplemental Fig. S2; Supplemental Table S1), suggesting the existence of mutual functional compensation between TRX m1, TRX m2, and TRX m4. This could be associated with their similar protein structures and analogous thylakoid membrane localization (Supplemental Fig. S1; Peltier et al., 2002; Friso et al., 2004). In addition, our hypothesis is supported by the fact that the expression of TRX m1, TRX m2, or TRX m4 in yeast can rescue the oxidant-hypersensitive phenotype of the trx null strain (Issakidis-Bourguet et al., 2001). Functional compensation between TRX isoforms has also been observed in tetrapyrrole biosynthesis (Ikemoto et al., 2007; Luo et al., 2012).

Furthermore, triple inactivation of the three TRX m genes has pleiotropic effects on photosynthetic electron transport and chloroplast development. Chlorophyll fluorescence measurements showed that Fv/Fm was dramatically reduced below 0.5 in the triple-silenced plants (Fig. 2; Table I). The decrease in Fv/Fm is due to specific impairment of the PSII complexes (Figs. 4 and 7). In line with this, no PSI efficiency and elevated NPQ were detected, indicating that the electron flow within PSI is severely inhibited in the triple-silenced plants (Table I). In addition, determination of absorbance changes of P700 at 820 nm demonstrates that PSI in the triple-silenced plants is functional but has low activity (Fig. 2B). These spectroscopic properties also have been observed in a number of mutants with defects in PSI, such as low PSII accumulation1 (lpa1; Feng et al., 2006), lpa2 (Ma et al., 2007), lpa3 (Cai et al., 2010), photosynthesis affected mutant68 (pam68; Armbruster et al., 2010), and hec243 (Zhang et al., 2011). Moreover, immunoblot analysis confirmed that essential subunits of the PSII complex

Figure 7. (Continued.)
are severely reduced in plants silenced for TRX m1, TRX m2, and TRX m4 (Fig. 3). Finally, the specifically impaired PSII complex in the triple-silenced plants had a strong effect on the organization of the thylakoid membranes. The amount of stroma lamellae and the stacking of grana thylakoids in the triple-silenced plants are obviously impaired in comparison with that in VIGS-GFP plants (Fig. 5). Taken together, our data on spectroscopy measurements, biochemical analyses, and chloroplast ultrastructure reveal a specific defect in PSII accumulation in the triple TRX m-silenced plants, thus providing a straightforward explanation for the observed pale-green leaf phenotype (Figs. 1 and 7).

The analyses described above indicate that TRX m1, TRX m2, and TRX m4 together participate in the accumulation of the PSII complex. However, the mechanism involved remains to be elucidated. Considering the hydrophilic nature of these three TRX m proteins, the possibility that they may be integral constituents of PSII can be excluded. Thus, these three TRX m proteins probably interact transiently with the PSII complex and some PSII subunits, in a manner similar to several PSII auxiliary factors that have been shown to interact with specific PSII subunits and assist their assembly, but are absent in the functional complexes (Peng et al., 2006; Ma et al., 2007; Armbruster et al., 2010; Cai et al., 2010).

Indeed, TRX m1, TRX m2, and TRX m4, but not TRX m3, comigrate with minor PSII assembly intermediates and interact with PSII core subunits D1, D2, and CP47 (Fig. 8). All of these data suggest that the three TRX m proteins participate directly in the biogenesis of the PSII core complex.

Assembly of the PSII Core Complex Is Redox Dependent and Can Be Modulated by m-Type TRXs

Thiol-disulfide chemistry plays an essential role in the biogenesis of the photosynthetic apparatus and in the maintenance of photosynthetic efficiency (Dietz and Pfannschmidt, 2011; Rochaix, 2011; Kieselbach, 2013). While the formation of disulfide bonds in the oxygen-evolving complex has been shown to be essential for the assembly of the PSII complex (Burnap et al., 1994; Karamoko et al., 2011), relatively few studies have examined the mechanisms of disulfide bridge exchanges in PSII core proteins. This is hampered by multiple limitations such as the hydrophobic nature of PSII subunits and the lack of PSII subunits in mutant plants. Our biochemical analyses in vivo show that two PSII core subunits, D1 and CP47, can form redox-sensitive intermolecular, but not intramolecular,
disulfide bridges (Fig. 9; Supplemental Fig. S4), implying that the assembly of the PSII core complex also requires proper redox status of the PSII core subunits and the involvement of redox regulators. This hypothesis is supported by several lines of evidence. The assembly of D1 proteins into the PSII RC and PSII core complex depends on proper redox conditions. The thiol reactants DTT, N-ethylmaleimide, and iodosobenzoic acid could reduce the stability of early PSII assembly intermediates, inhibit C-terminal processing of the D1 precursor, and prevent the reassembly of CP43 into PSII (Zhang et al., 2000). Moreover, SNOWY COTYLEDON2 and LOW QUANTUM YIELD OF PHOTOSYSTEM II1 (LQY1), with protein disulfide isomerase activity, interact with PSII core subunits CP43 and CP47 and are suggested to function in the biogenesis of thylakoid membranes in cotyledons and in PSII disassembly and/or reassembly during the PSII repair cycle, respectively (Shimada et al., 2007; Lu et al., 2011; Tanz et al., 2012). Intriguingly, the triple silencing of TRX m1, TRX m2, and TRX m4 disrupted the redox state of PSII subunits and led to elevated levels of oxidized CP47 oligomers (Fig. 9C), indicating the important role of TRX m proteins in modulating the redox status of PSII core subunits.

Biogenesis of the PSII complex is a highly ordered process in which a series of assembly steps occur sequentially and are well coordinated in different compartments of the thylakoid membranes (Komenda et al., 2012; Nickelsen and Rengstl, 2013). Thus, it is interesting to consider which step(s) of PSII assembly might involve TRX m1, TRX m2, and TRX m4. In the early stages of PSII assembly, PSII core subunits always combine with distinct low-molecular-mass proteins to form minor PSII assembly intermediate subcomplexes, such as the PSII RC, the CP47 precomplex, and the CP43 precomplex. In two-dimensional BN/SDS-PAGE, TRX m1, TRX m2, and TRX m4 can associate with these PSII assembly intermediates (Fig. 8B). Similar combinations with small PSII complexes have been observed for some PSII accessory factors that are involved in the early steps of PSII biogenesis; HCF136 and YCF48 localize to the thylakoid lumen and participate in the stabilization of newly synthesized D1 precursor and mediate the formation of the PSII RC (Plücken et al., 2002; Komenda et al., 2008; Rengstl et al., 2011). The intrinsic thylakoid proteins PAM68 and SII0933 play an essential role in the conversion of the PSII RC into larger PSII complexes (Armbruster et al., 2010; Rengstl et al., 2011). Furthermore,
the chloroplast thylakoid membrane system is particularly complex in plants, because it is organized into nonappressed stroma lamellae and appressed grana stacks. The various PSII complexes are characterized in distinct thylakoid membrane domains. The functionally dimeric PSII, including PSII supercomplexes and PSII core dimers, dominate in the thylakoid grana, whereas the PSII assembly intermediate subcomplexes, such as PSII monomers, the CP47-containing PSII RC, and the PSII RC, prevail in the stroma lamellae (Danielsson et al., 2006). Thus, it is reasonable that the triple silencing of the three TRX m genes resulted in the conspicuous impaired stroma thylakoid membranes and grana (Fig. 5). Taken together, these results suggest

Table III. Summary of conserved Cys residues in described PSII assembly factors in Arabidopsis

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<th>Assembly Factor</th>
<th>Accession No.</th>
<th>Sizeb (kDa)</th>
<th>Localizationc</th>
<th>No. of Conserved Cys Residuesd</th>
<th>Site of Conserved Cys Residuesd</th>
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<td>44</td>
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<td>174</td>
<td>Fu et al. (2007); Sirpiö et al. (2008); Vasudevan et al. (2012) Karamoko et al. (2011)</td>
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<td></td>
<td>THYLAKOID LUMEN PROTEIN18.3 (TLP18.3)Psb32</td>
<td>At1g54780</td>
<td>23</td>
<td>Intrinsic TM</td>
<td>0</td>
<td>–</td>
<td>Sirpiö et al. (2007)</td>
</tr>
</tbody>
</table>

aPSII assembly factors are classified according to their proposed functions in the biogenesis and repair of the PSII complex. bPredicted molecular mass of mature proteins. cL, Lumen; S, stroma; TM, thylakoid membrane. dThe number of conserved Cys residues in the mature proteins. eThe site of conserved Cys residues in amino acid sequences of mature proteins. fAlb3 is involved in the integration of LHCII into PSII supercomplexes. gTHYLAKOID LUMEN PROTEIN18.3/Psb32 functions in the assembly of PSII supercomplex. hLPA19 and Pb29/Thylakoid Formation1 play roles in PSII repair.
that TRX m1, TRX m2, and TRX m4 may play an essential role in the early steps of PSII biogenesis for proper assembly of the PSII core complex.

Previous studies of PSII mutants, mainly performed in Chlamydomonas reinhardtii, indicated that D2 and CP47 serve a central function in the stable assembly of the PSII complex (Erickson et al., 1986; Vermaas et al., 1986; Lang and Haselkorn, 1989). However, the regulatory mechanism underlying the integration of CP47 into the PSII complex is an open question. At present, only two auxiliary proteins, PAM68 in Arabidopsis and Psb28 in cyanobacterium (Synechocystis PCC 6803), have been identified as being involved in the assembly of CP47 into PSII (Dobakova et al., 2009; Armbruster et al., 2010). Considering that TRX m1, TRX m2, and TRX m4 interact with PSII assembly intermediates and CP47 proteins, and that their deficiency leads to drastically reduced CP47 protein levels and the accumulation of redox-sensitive CP47 oligomers (Figs. 3, 8, and 9), we tentatively propose that these three m-type TRX proteins play a role in assisting the proper assembly of CP47 into the PSII core complex.

**Are There Other Relevant Targets of m-Type TRX Disulfide-Reducing Activity in PSII Biogenesis?**

Despite evidence supporting the disulfide-reducing activity of TRX m1, TRX m2, and TRX m4 in modulating the redox state of PSII subunits, which may in turn correlate with the reduced accumulation of PSII core subunits and PSII complexes (Figs. 3, 4, 7, and 9), the physiological significance of redox-sensitive CP47 oligomerization seems rather unclear. In principle, three scenarios for CP47 oligomerization can be envisioned: (1) several CP47 proteins can form CP47-CF47 homo-oligomers through intermolecular disulfide bridges; (2) CP47 combines with other PSII subunits such as D1 to form CP47-D1 heterooligomers; and (3) CP47 transiently associates with PSII assembly factors by means of intermolecular disulfide bridges. Based on crystalline structure analyses of the PSII complex and the low amounts of PSII protein oligomers in comparison with their monomers (Fig. 9; Iwata and Barber, 2004; Nelson and Ben-Shem, 2004; Dekker and Boekema, 2005), the first two possibilities seem unlikely. Interestingly, as shown in Table III, many PSII auxiliary proteins that are involved in the early steps of PSII biogenesis, such as Albino3 (Alb3), C-Terminal Processing Protease for D1 Protein of PSII, HCF243, LPA1, PAM68, and LPA3, all have conserved Cys residues. Strikingly, Degradation of Periplasmic Proteins1 and FK-506 BINDING PROTEIN20-2 have been suggested to be target proteins of chloroplastic TRXs (Lima et al., 2006; Ströher and Dietz, 2008). Therefore, in order to clarify the m-type TRX-mediated redox regulation of PSII biogenesis, we will further identify the potential reducing acceptors that are essential for PSII biogenesis and assembly of the PSII complex in a future study.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

Arabidopsis (Arabidopsis thaliana ecotype Columbia) was grown in soil in a growth chamber (110 μmol photons m⁻² s⁻¹, 16-h photoperiod, 21°C, and 60% relative humidity). The T-DNA insertion lines for two m-type TRX genes (trx m1 [Salk_087118C] and trx m4 [Salk_025385]) were obtained from the Arabidopsis Biological Resource Center as described by Courtelle et al. (2013). The homozygous T-DNA insertion lines were confirmed by PCR using the gene-specific and T-DNA-specific primers described in Supplemental Table S3. The trx m1dx and trx m4dx double mutant was obtained by crossing single mutants of trx m1 and trx m4 and screening the F2 population by PCR using the primers described above.

**VIGS Assay**

Plasmids pTRV1 and pTRV2 are VIGS vectors based on Tobacco rattle virus as described by Liu et al. (2002). To construct the pTRV2-TRX m vectors, TRX m1, TRX m2, and TRX m4 complementary DNA chat (cDNAs) were generated by RT-PCR with the primers described in Supplemental Table S3 and inserted into the pTRV2 vector. The pTRV2-GFP vector was used as a negative control. pTRV1 and pTRV2 derivatives were introduced into Arabidopsis plants by Agrobacterium tumefaciens infiltration as described by Burch-Smith et al. (2006).

**Transmission Electron Microscopy**

Transmission electron microscopy was performed as described by Yao and Greenberg (2006). Fully expanded leaves from the VIGS-TRX m and VIGS-GFP plants were collected 3 weeks after infection. Micrographs were taken using a transmission electron microscope (JEM1400; JEOL).

**Pigment Analysis and Chlorophyll Fluorescence Measurements**

Chlorophyll was extracted from whole leaves with 80% acetone in 25 mM HEPES-KOH (pH 7.5), and the chlorophyll content was determined as described by Wellburn (1994).

Chlorophyll fluorescence was measured using Maxi-Imaging PAM and Dual-PAM 100 (Walz; http://www.walz.com). Arabidopsis leaves were dark adapted for 15 min prior to fluorescence measurements. The minimum fluorescence yield was measured under a weak measuring light. The maximum fluorescence yield was determined by applying a saturating light pulse (0.5 × 10² μmol photons m⁻² s⁻¹). Leaves were illuminated for 6 min under actinic light illumination (110 μmol photons m⁻² s⁻¹). Thereafter, the maximal fluorescence of light-adapted leaves was measured after applying a second saturating pulse. The ΦPSⅠ excitation pressure (1 - qP), and NPQ were recorded. Measurement of light-induced Fₘₐₓ absorbance changes at 820 nm was performed as described by Meurer et al. (1996). Absorbance changes induced by saturating far-red light represent the relative amounts of phototoxicizable Fₕₐₚ.  

**Thylakoid Membrane and Total Protein Preparations**

Thylakoid membranes were prepared according to Robinson et al. (1980). Isolated thylakoid membranes were quantified based on total chlorophyll as described by Porra et al. (1989). Total proteins extracted from leaves of VIGS plants or from thylakoid membrane preparations were prepared according to Martinez-Garcia et al. (1999). Protein concentrations were determined using the Bio-Rad detergent-compatible colorimetric protein assay according to the manufacturer’s protocol.

**BN/SDS-PAGE and Immunoblot Analyses**

BN-PAGE was performed essentially according to Schägger et al. (1994) with the modifications described by Peng et al. (2006). For two-dimensional analysis, the excised BN-PAGE lanes were soaked in SDS sample buffer (100 mM Tris-HCl [pH 6.8], 2% [w/v] SDS, 1% [v/v] glycerol, and 2.5% [v/v] β-mercaptoethanol) for 15 min at room temperature and then layered onto 1.5-mm-thick 12% SDS-PAGE gels containing 6 μl urea. The gels were stained with Coomassie Brilliant Blue G250 and scanned using a GS-800 densitometer (Bio-Rad).

For immunoblot analysis, total proteins extracted from VIGS leaves or thylakoid membrane preparations were separated on 12% SDS-PAGE gels

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containing 6 M urea. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Millipore) and probed using antibodies specifically directed against individual PSII subunits (D1, D2, CP43, CP47, and PsbO), PSI subunits (PsaA and PsaO), the light-harvesting antenna proteins (Lhcb1 and Lhca1), the Cyt b6/f complex (cytochrome PsbO), PSI subunits (PsaA and PsaO), the light-harvesting antenna proteins— 

Determination of ROS
In situ detection of H2O2 and O2− was performed mainly according to Lu et al. (2011) with minor modifications. For H2O2 visualization, leaves from VIGS plants were vacuum infiltrated with a solution containing 1 mg mL−1 3,3′-diaminobenzidine (pH 3.8) at room temperature for 1 h and incubated in the same solution under growth light illumination for 1 h. The stained leaves were then boiled in a solution of ethanol:acetic acid:glycerol (3:1:1, v/v/v) for 10 min. For O2− detection, leaves were vacuum infiltrated for 30 min with 50 mM phosphate buffer (pH 7.5) containing 1 mg mL−1 nitroblue tetrazolium and subsequently boiled in 90% (v/v) ethanol for 10 min.

Transient Expression of Mutant TRX m Proteins in Arabidopsis Protoplasts

The pull-down assay was performed essentially according to the method of Tada et al. (2008) with some modifications. The full-length cDNAs for TRX m1, TRX m2, TRX m3, and TRX m4 were amplified from Arabidopsis cDNA by RT-PCR. Site-directed mutagenesis of the C-terminal Cys residues in the TRX m active-site motif (WCFCP) were introduced by overlap-extension PCR. Full-length cDNA fragments of TRX m1C107S, TRX m2C113S, TRX m3C100S, and TRX m4C119S were then individually cloned into the pUC19-FLAG transient expression vector. Primer sequences are listed in Supplemental Table S3. TRX m3 and TRX m4 mutant protoplasts were isolated from 14-d-old Arabidopsis seedlings according to Zhai et al. (2009) and transformed with the pUC19-TRX m1C107S, pUC19-TRX m2C113S, pUC19-TRX m3C100S, and pUC19-TRX m4C119S-FLAG plasmids according to Yoo et al. (2007). Transformed protoplasts were collected by centrifugation for 2 min at 100 g, resuspended in WS solution, and kept in the dark for 16 h prior to further analysis.

Expression and Purification of Recombinant His-Tagged Mutant TRX m Proteins

The cDNA fragments encoding the mature TRX m mutant proteins were amplified from pUC19-TRX m1C107S, pUC19-TRX m2C113S, pUC19-TRX m3C100S, and pUC19-TRX m4C119S-FLAG plasmids by RT-PCR and individually cloned into the pET28a expression vector (Novagen). Primer sequences are listed in Supplemental Table S3. The pET28a-TRX m1C107S, pET28a-TRX m2C113S, pET28a-TRX m3C100S, and pET28a-TRX m4C119S plasmids were transformed into Escherichia coli BL21 (DE3; Stratagene) and induced to express with 0.6 mM isopropyl β-D-thiogalactoside. Puriﬁcation of His-TRX m proteins was carried out with Ni2+ afﬁnity chromatography according to the manufacturer’s protocol (GE Healthcare) and incubated with thylakoid membrane proteins solubilized in 1% (w/v) DM overnight at 4°C. The empty resin without TRX m protein was used as the negative control. The resins were then extensively washed with washing buffer containing 0.5 M NaCl until the A280 reached almost zero. The TRX m target proteins linked by the newly formed heterodisulﬁde bonds were eluted with buffer containing 10 mM DTT and 0.5 M NaCl and analyzed by immunoblotting.

Determination of the in vivo Redox State of PSII Proteins

The in vivo redox state of PSII proteins was determined as in previous studies (Ikegami et al., 2007; Tada et al., 2008) with some modiﬁcations. Total leaf proteins were extracted from VIGS plants 3 weeks after inﬁltration without reducing agents. The extracts were divided into three equal parts and incubated with the oxidizing agent (1 mM CuCl2), the reducing agent (10 mM DTT), or the corresponding amount of distilled, deionized water at 23°C for 30 min, and the proteins were then precipitated with 5% (w/v) TCA. The denatured protein pellets were washed twice with 100% acetone and resuspended in 1 volume of 1% (w/v) SDS buffer and 1 volume of SDS-PAGE gel loading buffer without DTT. One part of each sample was incubated with AMS (Invitrogen) for 20 min at room temperature. Both AMS-treated and untreated samples were separated by nonreducing SDS-PAGE and immunoblotted with antibodies against PSII subunits. For a loading control, the third part of the leaf protein extracts was boiled in reducing SDS-PAGE gel loading buffer containing DTT and probed with antibodies against PSII subunits and the large subunit of Rubisco.

Supplemental Data

The following materials are available in the online version of this article. Supplemental Figure S1. Phylogenetic tree and amino acid sequence alignment of TRX m proteins in Arabidopsis and rice. Supplemental Figure S2. Phenotype and identiﬁcation of trx m1trx m4 double mutant plants. Supplemental Figure S3. Expression of TRX m-FLAG proteins in Arabidopsis protoplasts and puriﬁcation of His-TRX m proteins. Supplemental Figure S4. Visualization of the redox status of intramolecular disulﬁde bonds in PSII subunits from VIGS plants. Supplemental Table S1. Summary of TRX m silencing plants and TRX m mutant plants in this study. Supplemental Table S2. Chlorophyll content and chlorophyll ﬂuorescence parameters in TRX m mutant plants. Supplemental Table S3. List of primers used for vector construction in this study.

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