Thioredoxins are ubiquitously distributed proteins that regulate numerous cellular processes. All thioredoxins have a highly conserved active-site sequence that contains two redox-active Cys residues in a conserved motif, Trp-Cys-Gly-Pro-Cys (Holmgren, 1989). In the reduced form, thioredoxins are excellent catalysts for the reduction of intramolecular disulfide bonds of target proteins (Jacquot et al., 2002). In contrast to other eukaryotic cells, plants contain several thioredoxin isoforms that are characterized by subcellular localization and substrate specificity. Here, we describe the functional characterization of a rice (Oryza sativa) thioredoxin m isoform (Ostrxm) using a reverse genetics technique. Ostrxm showed green tissue-specific and light-responsive mRNA expression. Ostrxm was localized in chloroplasts of rice mesophyll cells, and the recombinant protein showed dithiothreitol-dependent insulin β-chain reduction activity in vitro. RNA interference (RNAi) of Ostrxm resulted in rice plants with developmental defects, including semi dwarfism, pale-green leaves, abnormal chloroplast structure, and reduced carotenoid and chlorophyll content. Ostrxm RNAi plants showed remarkably decreased $F_v/F_m$ values under high irradiance conditions ($1,000 \mu$mol m$^{-2}$ s$^{-1}$) with delayed recovery. Two-dimensional electrophoresis and matrix-assisted laser-desorption/ionization time-of-flight analysis showed that the levels of several chloroplast proteins critical for photosynthesis and biogenesis were significantly decreased in Ostrxm RNAi plants. Furthermore, 2-Cys peroxiredoxin, a known target of thioredoxin, was present in oxidized forms, and hydrogen peroxide levels were increased in Ostrxm RNAi plants. The pleiotropic effects of Ostrxm RNAi suggest that Ostrxm plays an important role in the redox regulation of chloroplast target proteins involved in diverse physiological functions.
activate several chloroplast enzymes, including those involved in the Calvin cycle (FBPase, sedoheptulose-1,7-bisphosphatase, phosphoribulokinase, glyceraldehyde-3-P dehydrogenase, Rubisco [via Rubisco activase], ATP synthesis [CF1ATP synthase], and fatty acid synthesis [acetyl-CoA carboxylase]; Nishizawa and Buchanan, 1981; Wolosiuk et al., 1993; Sasaki et al., 1997; Schwarz et al., 1997; Zhang and Portis, 1999). Thioredoxin has been shown to be involved in the inactivation of Glc-6-P dehydrogenase, the first enzyme of the oxidative pentose phosphate cycle (Wenderoth et al., 1997). Both thioredoxins \( f \) and \( m \) can activate FBPase and NADP-MDH in vitro, suggesting that there is some overlap in the substrate specificity of the isoforms (Hodges et al., 1994; Geck et al., 1996). However, disruption of the thioredoxin \( m \) gene in cyanobacteria (\( \textit{Aspergillus nidulans} \)) is lethal, suggesting that the protein may have a distinctive in vivo function. This result also highlights the importance of using genetic tools to define the role of thioredoxins in intact organisms (Muller and Buchanan, 1989).

Although the molecular characteristics and biochemical properties of chloroplast thioredoxins have been well studied in vitro (Scheibe and Anderson, 1981; Geck et al., 1996; Mestres-Ortega and Meyer, 1999; Duek and Wolosiuk, 2001; Issakidis-Bourguet et al., 2001; Collin et al., 2003, 2004), less is known about their physiological role in intact plants. In this study, we have cloned a rice (\( \textit{Oryza sativa} \)) gene encoding a thioredoxin \( m \) and used RNA interference (RNAi) to investigate the function of Ostrxm in rice. The results indicate that Ostrxm has an essential role in chloroplasts for plant growth and development.

**RESULTS**

**Isolation and Expression Pattern of Ostrxm**

A search of The Institute for Genomic Research rice genome database (http://www.tigr.org/tigr-scripts/e2k1/osa1/putative_function_search.pl) with the search term “thioredoxin \( m \)-type” revealed matches with three genes (Os02g42700, Os04g44830, and Os12g08730).

![Figure 1](http://www.plantphysiol.org)  
**Figure 1.** Genomic complexity and mRNA expression of Ostrxm. A, Southern-blot analysis of the Ostrxm gene. Genomic DNA was digested with \( \text{Bam} \text{HI} \) (B), \( \text{Eco} \text{RI} \) (E), or \( \text{Hin} \text{dIII} \) (H). B, Northern-blot analysis of Ostrxm gene expression. Total RNA was prepared from 21-d-old root, shoot apical meristem (SAM), leaf sheath and leaf, late boot to heading stage flower and reproductive stage node. C, Induction of the Ostrxm mRNA transcript by de-etiolation. Twelve-day-old rice seedlings were etiolated for 24 h and exposed to light for the indicated times under a photosynthetic flux of 200 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) at 30°C. RNA was prepared from leaf tissues.

![Figure 2](http://www.plantphysiol.org)  
**Figure 2.** Subcellular localization of Ostrxm and insulin reduction activity of the recombinant protein. A, Subcellular localization of Ostrxm. Ostrxm::smGFP is indicated by green fluorescence (GFP) and the auto fluorescence of chloroplasts is indicated by red fluorescence (TRITC). The bright-field image (Bright) was obtained using the bright-field filter. Colocalized GFP and chloroplasts (Merged) appear as yellow. B, DTT-dependent insulin reduction activity of Ostrxm. Reaction mixtures contained 10 \( \mu \text{g} \) of bovine serum albumin as a control (●), 0.5 \( \mu \text{g} \) of \( \textit{E. coli} \) thioredoxin (○), 1 \( \mu \text{g} \) of Ostrxm (▲), 5 \( \mu \text{g} \) of Ostrxm (▲), or 10 \( \mu \text{g} \) of Ostrxm (●). [See online article for color version of this figure.]
Alignment and phylogenetic analysis of the corresponding cDNA sequences of the three genes revealed that AK099517 (Os02g42700) and AK069195 (Os04g44830) share a sequence identity of 52.8%, but that they significantly diverge from AJ005841 (Os12g08730), sharing only 24.2% and 23.9% sequence identity with this gene, respectively (Supplemental Fig. S1). A cDNA corresponding to Os12g08730 designated as Ostrxm was isolated from a rice leaf cDNA library with the goal of investigating the in vivo function of the protein. A rice functional genomics database (http://signal.salk.edu/cgi-bin/RiceGE) search using the Ostrxm cDNA sequence as a query revealed that the gene comprises two exons interrupted by an intron and that it spans about 1 kb. The high degree of cDNA sequence divergences of Os02g42700 and Os04g44830 from Ostrxm are consistent with a Southern-blot analysis of the T₁ progeny of Ostrxm RNAi transgenic and wild-type plants.

Figure 3. The Ostrxm RNAi construct and analysis of Ostrxm expression in transgenic rice. A, Schematic representation of the Ostrxm RNAi construct. B, Southern-blot analysis of the T₁ progeny of Ostrxm RNAi transgenic and wild-type plants using an [α-32P]ATP-labeled HPT (hygromycin phosphotransferase) cDNA as a probe. C, Northern-blot analysis of the T₁ progeny of Ostrxm RNAi transgenic and wild-type plants.

Subcellular Localization and Disulfide Oxidoreductase Activity of Recombinant Ostrxm

To determine the subcellular localization of Ostrxm in rice, we generated transgenic rice plants expressing Ostrxm-smGFP or ΔN-Ostrxm-smGFP (Ostrxm without the N-terminal transit peptide) and examined their localization in protoplasts by confocal laser-scanning microscopy. Ostrxm-smGFP appeared to colocalize with the red autofluorescence of chloroplasts, whereas ΔN-Ostrxm-smGFP localized to the cytosol (data not shown), indicating that Ostrxm synthesized in the cytosol is translocated into chloroplasts by virtue of its N-terminal transit peptide (Fig. 2A).

In an experiment to investigate the disulfide reductase activity of the protein, recombinant Ostrxm was tested for its ability to reduce the insulin β-chain, an artificial substrate of thioredoxins. Recombinant Ostrxm reduced the insulin β-chain only in the presence of dithiothreitol (DTT; Fig. 2B). In the absence of DTT or when DTT was replaced with ascorbate, the protein was completely unable to reduce insulin (data not shown), indicating that Ostrxm possesses thiol-dependent disulfide reductase activity. However, the disulfide reductase activity of Ostrxm was lower than that of Escherichia coli thioredoxin. These results indicate that Ostrxm is a bioactive disulfide oxidoreductase in chloroplasts.

Phenotypic Analysis of Ostrxm RNAi Transgenic Rice

To study the physiological role of Ostrxm in rice, we generated Ostrxm RNAi transgenic plants that showed a significantly reduced expression pattern of Ostrxm (Fig. 3). Fifteen hygromycin-resistant T₀ transformants were transferred into soil and grown in a greenhouse and self-pollinated to obtain segregating T₁ progeny for genetic analysis. Integration and copy number of the T-DNA were confirmed by Southern-blot analysis (Fig. 3B). Among the 15 hygromycin-resistant T₀

<table>
<thead>
<tr>
<th>Line</th>
<th>Hygromycin−/−</th>
<th>Ratio</th>
<th>Pale-Green Seedlings</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₁-3</td>
<td>22+/8</td>
<td>2.93:1.07</td>
<td>22</td>
</tr>
<tr>
<td>T₁-4</td>
<td>24+/6</td>
<td>3.20:0.80</td>
<td>24</td>
</tr>
<tr>
<td>T₁-14</td>
<td>23+/7</td>
<td>3.07:0.93</td>
<td>23</td>
</tr>
<tr>
<td>T₁-15</td>
<td>22+/8</td>
<td>2.93:1.07</td>
<td>22</td>
</tr>
</tbody>
</table>

Table 1. Segregation analysis of the T₁ generation of Ostrxm RNAi plants.

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transformants, only four lines survived and produced T₁ seeds. The four lines showed a segregation pattern of 3:1 for hygromycin resistance (Table I). Northern-blot analysis of the four surviving transgenic lines showed that Ostrxm expression was efficiently suppressed in all the RNAi transgenic lines (Fig. 3C). Two lines, 4 and 14, were selected for further analysis. T₂ and T₃ Ostrxm RNAi transgenic lines used for experiments were easily distinguished from segregating wild-type plants by their pale-green and semidwarf phenotypes. When gene-specific RT-PCR was performed to check the specificity and efficiency of the targeted knock-down of the Ostrxm gene, about 90% of Ostrxm transcripts was found to be specifically decreased, whereas transcripts of Os0242700 and Os04g44830 were not affected by Ostrxm RNAi (Supplemental Fig. S1C).

Germination was scored following seed imbibition and the subsequent development of roots and shoots was monitored. Both wild-type and Ostrxm RNAi seeds had germinated, with slight differences in germination time (Fig. 4A). When roots and shoots were examined, the RNAi transgenic rice seedlings exhibited marked dwarf phenotype compared to the wild-type seedlings (Fig. 4B). The Ostrxm RNAi transgenic lines also had pale-green leaves with light-yellow stripes, and exhibited reduced plant mass, tiller number (about 70%), and semidwarfism (Fig. 4, C and D). However, there was no significant difference in the leaf number. Ostrxm RNAi transgenic rice produced immature panicles and empty spikelets and had significantly reduced grain yields compared to wild-type plants (Fig. 4E). The average spikelet number per panicle in Ostrxm RNAi transgenic rice was 46.7 ± 3.6 compared with 67.0 ± 4.1 in the wild type.

**Structural Defect of the Photosynthetic Apparatus in Ostrxm RNAi Plants**

The pale-green and semidwarf phenotypes urged us to investigate chloroplast structure and the photosynthetic apparatus of Ostrxm RNAi plants. When leaves were examined under a light microscope, we observed decreased numbers of chloroplasts per mesophyll cell in Ostrxm RNAi plants compared to wild-type plants, and the shape and size of chloroplasts were irregular in Ostrxm RNAi plants compared to those of wild-type plants (Fig. 5, A and B). When chloroplasts of wild-type leaves were investigated by transmission electron microscopy, well-defined grana and stroma-thylakoid...
boundaries and regularly shaped starch grains were observed (Fig. 5, C and E). In contrast, chloroplasts from Ostrxm RNAi plants were of abnormal shape, their thylakoid boundaries were vague, and they lacked regularly shaped starch grains (Fig. 5, D and F). Overall, the ultrastructural defects observed in the leaves and chloroplasts of Ostrxm RNAi transgenic plants suggested that Ostrxm is required for chloroplast biogenesis and differentiation.

Pigment Content and Photosynthetic Efficiency of Ostrxm RNAi Plants

In Ostrxm RNAi plants, chlorophyll (Chl) content per fresh weight was significantly decreased to 76% in the case of RNAi 4 or 53% in the case of RNAi 14. The amount of carotenoids and xanthophyll cycle pigments was also decreased; however, their relative levels with respect to total Chl were different. Thus, the relative amounts of neoxanthin and lutein were decreased in Ostrxm RNAi plants compared to wild-type and vector plants, but those of β-carotene and the pool size of the xanthophyll cycle pigments were increased in Ostrxm RNAi plants (Table II). In addition, the Chl a/b ratio was slightly higher in the leaves of Ostrxm RNAi plants compared to wild-type plants.

Because Ostrxm RNAi plants had reduced pigment content and structurally defective chloroplasts, we measured the potential quantum yield of PSII (Fv/Fm). Under high light irradiance of 1,000 μmol m⁻² s⁻¹ for 4 h, Ostrxm RNAi plants showed significant decreases in Fv/Fm by about approximately 30%, whereas wild-type plants showed about approximately 15% decrease (Fig. 6). After 4 h, the Fv/Fm of wild-type plants recovered to about 95%, whereas that of Ostrxm RNAi plants recovered to about 75%.

Figure 5. Ultrastructural examination of Ostrxm RNAi transgenic rice. A and B, Light microscope images of leaves from wild-type and Ostrxm RNAi rice plants, respectively (magnification = 1,000×). C to F, Transmission electron micrographs of chloroplasts from wild-type (C and E) and Ostrxm RNAi rice plants (D and F). Scale bar = 1 μm (C–F). Cp, Chloroplast; VB, vascular bundle cell; Ep, epidermis; SG, starch grain; CW, cell wall; Thy, thylakoid. Plant tissues were harvested from 10-d-old seedlings grown in a growth chamber with supplemental lighting (8 h dark/16 h light at 200 μmol m⁻² s⁻¹ and a day/night temperature regime of 30°C/25°C).
and it recovered thereafter almost fully, but relatively slowly.

**Redox State of Ostrxm RNAi Plants**

Thioredoxin *m*, acting as an electron donor for Cys-containing proteins, may regulate the activities of target proteins by reducing their disulfide bonds. To explore this hypothesis, the redox state of endogenous 2-Cys peroxiredoxin (2-Cys Prx; Motohashi et al., 2001; König et al., 2002) was monitored by nonreducing electrophoresis and western blotting using an antibody against 2-Cys Prx. In wild-type plants, both the 24-kD reduced monomeric form and the 48-kD oxidized dimeric form of 2-Cys Prx were detected, whereas only the oxidized dimeric form was detected in *Ostrxm* RNAi plants (Fig. 7A). This result suggests that Ostrxm regulates the catalytic activity of 2-Cys Prx by reducing redox-active Cys residues. Because the amount of reduced 2-Cys Prx is related to hydrogen peroxide (H$_2$O$_2$) catabolism, we wondered whether the H$_2$O$_2$ level in *Ostrxm* RNAi plants was increased. The level of H$_2$O$_2$ in leaves of *Ostrxm* RNAi plants in the dark or sunlight conditions was about 2-fold higher than that of wild-type plants (Fig. 7B). The increased H$_2$O$_2$ level in *Ostrxm* RNAi plants suggests that Ostrxm acting as a disulfide reductase plays a crucial role in the 2-Cys Prx-coupled detoxification of plastid-derived peroxides. Therefore, the defective flow of electrons to Ostrxm substrates in *Ostrxm* RNAi plants might be considerably implicated in their severe phenotypes.

**DISCUSSION**

Despite extensive in vitro characterization of thioredoxin *m* in plants, its in vivo function remains to be determined. Thus, we generated *Ostrxm* RNAi transgenic rice plants to investigate the in vivo function of Ostrxm in planta. *Ostrxm* RNAi plants showed phenotypes such as pale-green leaves, growth inhibition, reduced Chl and pigment content, decreased photosynthetic efficiency, defective chloroplast development, and inactivation of 2-Cys Prx. These results raise the possibility that some of the important biosynthetic pathways in chloroplasts are linked to the light-dependent thioredoxin electron transfer chain.

In higher plants, carotenoids mainly accumulate in the thylakoid membrane of chloroplasts, where they function to harvest light and protect the photosynthetic apparatus from oxidative damage by quenching the triplet excited state of Chl (3Chl) and reactive singlet oxygen (1O$_2$) and dissipating excess energy (Demmig-Adams et al., 1996; Tracewell et al., 2001). Thus, the reduced photosynthetic pigments of *Ostrxm*

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**Table II. Pigment content in the leaves of wild-type, Vec, and Ostrxm RNAi transgenic rice plants (mmol/mol Chl)**

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Wild Type</th>
<th>Vec</th>
<th>RNAi 4</th>
<th>RNAi 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neoxanthin</td>
<td>53.4 ± 0.8</td>
<td>55.6 ± 1.3</td>
<td>42.2 ± 0.4</td>
<td>41.7 ± 0.3</td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>134.2 ± 1.6</td>
<td>165.6 ± 1.0</td>
<td>165.1 ± 3.9</td>
<td>167.5 ± 0.5</td>
</tr>
<tr>
<td>Antheraxanthin</td>
<td>14.6 ± 1.7</td>
<td>20.1 ± 1.8</td>
<td>50.3 ± 0.4</td>
<td>40.6 ± 1.2</td>
</tr>
<tr>
<td>Lutein</td>
<td>213.0 ± 2.5</td>
<td>213.5 ± 0.8</td>
<td>178.9 ± 2.7</td>
<td>160.5 ± 2.4</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>9.4 ± 0.1</td>
<td>12.2 ± 0.4</td>
<td>29.6 ± 1.2</td>
<td>18.8 ± 0.4</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>135.7 ± 1.8</td>
<td>151.6 ± 2.0</td>
<td>195.7 ± 4.7</td>
<td>179.7 ± 3.5</td>
</tr>
<tr>
<td>V + A + Z $^b$</td>
<td>158.1 ± 3.4</td>
<td>198.0 ± 3.2</td>
<td>245.0 ± 5.5</td>
<td>226.8 ± 2.1</td>
</tr>
<tr>
<td>Chl $^{ab}$</td>
<td>3.6 ± 0.1</td>
<td>3.6 ± 0.1</td>
<td>3.9 ± 0.2</td>
<td>3.9 ± 0.1</td>
</tr>
</tbody>
</table>

$^a$Pigments were separated and quantified by HPLC as described in “Materials and Methods”. All values are the mean ± SD of at least three independent experiments. Secondary leaves from primary tillers of 56-d-old plants are used for analysis. $^b$Total xanthophyll cycle pigments including violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z).
RNAi plants might cause structural defects of chloroplasts and reduced photosynthetic efficiency under high irradiance conditions, finally producing the observed pale-green leaf and growth defect phenotypes. It has been experimentally demonstrated that the xanthophyll cycle is closely associated with nonphotochemical quenching (NPQ), which is an essential protective mechanism against high light stress (Gilmore and Yamamoto, 1993; Müller et al., 2001; Kühlheim et al., 2002). Formation and relaxation of NPQ is controlled not by the concentration of zeaxanthin, but by the extent of de-epoxidation of the xanthophyll pool (Johnson et al., 2008). The relative xanthophyll pool size (V + An + Z)/Chl, where V is violaxanthin, An is antheraxanthin, and Z is zeaxanthin, and the de-epoxidation state of xanthophyll was significantly higher in Ostrxm RNAi transgenic plants than in wild-type plants and empty-vector plants (Table II), indicating a higher capacity for xanthophyll cycle-dependent thermal energy dissipation by NPQ in Ostrxm RNAi plants. Xanthophyll pool size and the de-epoxidation state seem to be regulated to protect against the damage caused by suppression of Ostrxm in RNAi transgenic rice plants. Thus, a limitation in the acceptor side of PSI in Ostrxm RNAi plants results in a reduced antenna size of PSII, finally making Ostrxm RNAi plants more susceptible to high irradiance.

Recent approaches for identifying targets of chloroplast thioredoxins have provided new insights into the functions of plant thioredoxins (Motohashi et al., 2001; Balmer et al., 2003). An approach using immobilized thioredoxins m and f as baits, 26 identified targets were related to established (Calvin cycle, nitrogen and sulfur metabolism, translation, and pentose phosphate cycle) or unrecognized (isoprenoid, tetrapyrrole and vitamin biosynthesis, protein assembly/folding, protein and starch degradation, glycolysis, HCO₃⁻/CO₂ equilibration, plastid division, and DNA replication/transcription) cellular reactions (Balmer et al., 2003). Interestingly, two-thirds of the identified targets contained conserved Cys residues, increasing the probability that chloroplast thioredoxin regulates the catalytic activities of targets through the reduction of specific disulfide groups. To determine whether Ostrxm affects the expression of proteins that are involved in photosynthesis and chloroplast biogenesis, we examined the expression levels of both nuclear-encoded and chloroplast-encoded chloroplast genes by northern-blot analysis. However, we could not detect any significant differences in the transcript expression levels of these genes in Ostrxm RNAi transgenic plants as compared to wild-type plants (Supplemental Fig. S2). Thus, we performed two-dimensional electrophoresis (2-DE) and matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) analyses to identify differentially expressed proteins in Ostrxm RNAi plants. Ten protein spots resolved on...
2-DE gels, which showed decreased levels in Ostrxm RNAi plants were subjected to MALDI-TOF analysis (Fig. 8), which led to the identification of eight proteins (Table III). Interestingly, the localization of seven identified proteins coincided with that of Ostrxm, and Rubisco small subunit and peptidyl-prolyl cis-trans isomerase, which is designated TLP40, were previously identified as target proteins of thioredoxin (Motohashi et al., 2001). Their possible implications in photosynthetic metabolisms help explain the observed phenotypic changes of Ostrxm RNAi plants.

Photoreduction of oxygen in PSI of chloroplasts generates superoxide anion (O$_2^-$) as the primary product of reduced oxygen species. O$_2$ is converted to H$_2$O$_2$ and O$_2$ by superoxide dismutase. To avoid photoinhibition and protect target molecules in chloroplasts, the immediate removal of H$_2$O$_2$ at the site of production is essential. H$_2$O$_2$ in chloroplasts is rapidly reduced to water by ascorbate catalyzed with ascorbate peroxidase, which is activated by thioredoxin, may also participate in the scavenging of H$_2$O$_2$ in chloroplasts and that it is important for photosynthetic electron transport. Ostrxm is an important regulator of redox homeostasis in chloroplasts and that it is important for photosynthetic CO$_2$ assimilation. Our study using Ostrxm RNAi plants indicates that Ostrxm, an active disulfide oxidoreductase in chloroplasts, is important for photosynthesis and chloroplast development. The observed severe phenotypes and physiological changes in Ostrxm RNAi plants indicate that Ostrxm may regulate target proteins through the reduction of Cys disulfides of targets involved in various chloroplast processes such as isoprenoid biosynthesis, Calvin cycle, and pentose phosphate cycle, etc. However, further genetic investigations are required to expand our understanding of the pleiotropic effects observed in Ostrxm RNAi plants.

**MATERIALS AND METHODS**

**Plant Materials**

Rice (Oryza sativa ‘Dong-jin’) was used for all experiments. Ostrxm RNAi transgenic rice was obtained by Agrobacterium-mediated transformation of embryonic callus using the RNAi technique. Hygromycin selection and plant regeneration were carried out as described (Dai et al., 2001). Surface-sterilized wild-type, vector-transformed (Vec), and transgenic seeds were germinated in a growth chamber conditioned with supplemental lighting (8 h dark/16 h light; 200 µmol m$^{-2}$ s$^{-1}$) and a day/night temperature regime of 30°C/25°C, or in a greenhouse (sunlight).

**RT-PCR Analysis**

RT-PCR was performed to measure the mRNA expression of the Os02g42700, Os04g44830, and Os12g08730 genes and to clone the Ostrxm cDNA (AJ005841). PCRs were performed on a PTC-0202 PCR machine (MJ Research) using 100 ng cDNA, 5 pmol each oligonucleotide primer, 200 µM each dNTP, 1 unit Ex Taq polymerase (TaKaRa Bio) and 1× Ex Taq polymerase buffer in a 20-µL volume. The PCR program initially started with a 95°C denaturation for 5 min, followed by 25 to 35 cycles of 95°C/1 min, 60°C/30 s, 72°C/1 min. The less expressed transcripts of Os02g42700 and Os04g44830 required more than 30 cycles of PCR for detection, whereas 25 cycles were enough for Os12g08730. Specific PCR primers for genes encoding Ostrxm isoforms were used (Os02g42700F: 5’-TGGCCAAGCAGGCCCGCTC-3’; Os02g42700R: 5’-CAGGCTTCCTGGAGTTATCCT-3’; Os04g44830F: 5’-CCGTCTGCTCTGCTCTGGA-3’; Os04g44830R: 5’-GGAGGTCTGGCAGGCTTG-3’; Os12g08730F: 5’-CGCCACTTAATGAGGTCTACTGAGGTA-3’).

**Southern and Northern-Blot Analysis**

For Southern-blot analysis, 10 µg of rice genomic DNA were digested with BamHI, EcoRI, and HindIII, separated on a 0.8% agarose gel, and transferred to a nylon membrane (Amersham). The membrane was hybridized and washed as described previously (Cheong et al., 1999). Total RNA (15 µg) was separated on a 1.2% formaldehyde agarose gel and transferred to a nylon membrane. The RNA bands were used as loading controls. The blot was hybridized with the same probe used for Southern-blot analysis.

**Expression of Recombinant Ostrxm and Insulin Reduction Assay**

cDNA encoding mature Ostrxm was subcloned into pGEX and expressed in Escherichia coli strain BL21 (DE3), and recombinant protein was purified as described previously (Jang et al., 2004). The disulfide oxidoreductase activity of the recombinant Ostrxm protein was assayed at 25°C in the presence of

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### Table III. Identification of differentially expressed proteins in Ostrxm RNAi plants

<table>
<thead>
<tr>
<th>Spot</th>
<th>Identified Protein</th>
<th>Accession No.</th>
<th>Est. Z</th>
<th>p/E/M</th>
<th>MM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ribulose bisphosphate carboxylase large chain</td>
<td>CAG31474</td>
<td>1.15</td>
<td>6.2/53.35</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Peptidyl-prolyl cis-trans isomerase</td>
<td>BAD05657</td>
<td>2.31</td>
<td>4.8/46.81</td>
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</tr>
<tr>
<td>4</td>
<td>33-kD oxygen evolving protein of PSII</td>
<td>BAB64069</td>
<td>2.39</td>
<td>5.2/30.10</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Ribulose-5-P 3-epimerase</td>
<td>AAL84303</td>
<td>0.99</td>
<td>5.7/25.02</td>
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<tr>
<td>6</td>
<td>Carbonic anhydrase 3</td>
<td>AAD56038</td>
<td>2.24</td>
<td>6.3/22.30</td>
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<tr>
<td>7</td>
<td>23-kD polypeptide of PSII</td>
<td>AAC98778</td>
<td>2.13</td>
<td>6.0/23.33</td>
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<td>8</td>
<td>Putative benzotheiazole-induced somatic embryogenesis receptor kinase 1</td>
<td>BAD53863</td>
<td>1.15</td>
<td>5.6/44.21</td>
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<tr>
<td>9</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>10</td>
<td>Rubisco small subunit</td>
<td>AAR19268</td>
<td>1.91</td>
<td>6.8/15.05</td>
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</table>
0.5 mM DTT (Holmgren, 1989). Reactions were initiated by the addition of DTT to a 1 mL reaction mixture, which contained 100 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, and 0.5 mg/mL insulin and various amounts of Ostrxm. Oxidoreductase activity was expressed as an increase in \( \Delta A_{660} \) using a spectrophotometer (Beckman DU 800).

**Subcellular Localization of the Recombinant smGFP-Ostrxm**

PCR was used to generate cDNA fragments encoding the full-length Ostrxm protein (amino acids 1–172) and the mature form of Ostrxm (amino acids 65–172). The cDNA fragments were ligated in frame with smGFP to create Ostrxm::smGFP and ΔN-Ostrxm::smGFP fusion constructs. The Ostrxm::smGFP and ΔN-Ostrxm::smGFP cassettes were then ligated into the expression vector pCAMBIA1300. Transgenic rice plants expressing smGFP fusion constructs were generated by *Agrobacterium*-mediated transformation as described (Dai et al., 2001). Protoplasts were isolated from the leaves of 10-d-old transgenic rice plants grown on Murashige and Skoog plates in a growth chamber as described previously (Gupta and Pattanayak, 1993).

**Generation of Ostrxm RNAi Transgenic Rice**

A 1.4-kb PCR fragment derived from the first intron of OsCDPK (accession no. AF 194414) was ligated into the Pst I/EcoRI restriction sites of pBluescript (Stratagene). An open reading frame (519 bp) of the Ostrxm cDNA with EcoRI/ClaI restriction sites was inserted downstream of the intron, in the sense orientation. An open reading frame of the Ostrxm cDNA with BamHI/PstI restriction sites was inserted upstream of the intron in the antisense orientation. The complete Ostrxm RNAi construct contained an antisense fragment of the Ostrxm cDNA, followed by the OsCDPK intron, and then by the sense fragment of the Ostrxm cDNA, which included a stop codon. The Ostrxm RNAi construct was inserted into the BamHI/ClaI sites of pCAMBIA1300 between the cauliflower mosaic virus 35S promoter and the Ocs terminator to generate pCAMBIA1300-Ostrxm. The Ostrxm RNAi plasmid was then transformed into rice using *Agrobacterium* strain LBA4404, as described previously (Dai et al., 2001).

**Pigment Analysis**

Photosynthetic pigments were analyzed according to Gilmore and Yamamoto (1991a). Briefly, leaf segments from 56-d-old plants grown in a greenhouse were collected after a 3-h dark adaptation period. Three individual plants were used for an experiment and the segments were immediately frozen in liquid nitrogen and ground with a Mixer-Mill (Qiagen). Pigments were extracted from the leaf powder by gentle agitation in ice-cold acetone for 1 h. To minimize pigment degradation, extraction was performed in darkness at 4°C. Cell debris was removed by centrifugation two times at 19,000g for 10 min at 4°C. Extracts were filtered through a 0.2-μm syringe filter and pigments were separated using an HPLC system (HP 1100 series; Hewlett-Packard) equipped with Spherisorb ODS-1 columns (Altech). The pigment concentration was estimated by using the conversion factors for peak areas (in nmol) that were previously calculated for this solvent mixture (Gilmore and Yamamoto, 1991b).

**Microscopy**

Plant tissues were fixed in a solution containing 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 4 h. They were then rinsed in 0.1 M phosphate buffer (pH 7.4) and treated with 1% (w/v) osmium tetroxide for 4 h at 4°C. After rinsing with the phosphate buffer, the samples were dehydrated and embedded in LR White resin (London Resin Company) Thin sections (40- to 50-μm thickness) were prepared with an ultramicrotome (RMC MTXL) and collected on nickel grids (1-GN, 150 mesh). The sections were stained with uranyl acetate and lead citrate and examined using a JEM 100CXII transmission electron microscope (Gothandam et al., 2005).

**Photoinhibitory Treatment and Chl Fluorescence Measurement**

Leaf segments from the same plants described in the pigment analysis section were floated on tap water to avoid water loss, and photoinhibitory treatments were performed using a metal-halide lamp at 28°C. The photon flux density reaching the leaf surfaces was about 1,000 μmol m⁻² s⁻¹, and a 15-cm-deep water bath was placed under the lamp to avoid overheating. \( F_{v}/F_{m} \) is a Chl fluorescence parameter used to evaluate the maximum or potential quantum efficiency of PSI and its decrease in plant leaves is reliable evidence that the leaves are subjected to stress. Therefore, photoinhibition was assessed by measuring \( F_{v}/F_{m} \) before and after high light treatments using a portable fluorometer. A saturating light pulse (3,000 μmol m⁻² s⁻¹; 800-ms pulse duration) was applied to measure \( F_{m} \). \( F_{v}/F_{m} \) was measured using leaf blade after dark adaptation for 15 min. After photoinhibition, leaves were allowed to recover to 20 μmol m⁻² s⁻¹ for the indicated time periods. At least three leaf segments were used and three independent experiments were carried out, giving nine replications for each time point.

**2-DE, In-Gel Digestion, and Protein Identification**

Two grams of rice plant leaves grown in liquid nitrogen were homogenized in 10 mL ice-cold Mg₂⁺/Nonidet P-40 (NP-40) extraction buffer containing 0.5 mM Tris-HCl (pH 8.3), 2% (v/v) NP-40, 20 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 1% polyvinyl polypyrrolidone, and 0.7 M Suc. 2-DE, in-gel digestion, and protein identification were performed according to previously reported methods (Kim et al., 2004).

**H₂O₂ Measurement**

To measure H₂O₂ levels, plants grown in sunlight conditions were used. Leaves of 60-d-old rice harvested at the midnight (dark) or midday (sunlight, 1,750 μmol m⁻² s⁻¹) were analyzed. Leaves (0.2 g) were ground with mortar and pestle in 2 mL of 0.2 M HClO₄. The slurry was centrifuged at 20,000g for 5 min. The supernatant was neutralized with 4% KOH to pH 7.5 and centrifuged at 20,000g for 1 min. An aliquot (100 μL) of supernatant was applied to a 2-mL column of AG-1-X2 resin (Bio-Rad) and the column was eluted with 2.5 mL of distilled water. The eluate was used for the assay of H₂O₂. Assays were performed with an Amplex red H₂O₂/peroxidase assay kit (Molecular Probes) according to the manufacturer’s instructions.

**Supplemental Data**

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

**Supplemental Figure S1. Phylogenetic analysis of rice thioredoxin m isoforms and their expression in Ostrxm RNAi transgenic rice.**

**Supplemental Figure S2. Northern-blot analysis of nuclear- and chloroplast-encoded chloroplast genes.**

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**LITERATURE CITED**


