One of the mechanisms plants have developed for chloroplast protection against oxidative damage involves a 2-Cys peroxiredoxin, which has been proposed to be reduced by ferredoxin and plastid thioredoxins, Trx x and CDSP32, the FTR/Trx pathway. We show that rice (Oryza sativa) chloroplast NADPH THIOREDOXIN REDUCTASE (NTRC), with a thioredoxin domain, uses NADPH to reduce the chloroplast 2-Cys peroxiredoxin BAS1, which then reduces hydrogen peroxide. The presence of both NTR and Trx-like domains in a single polypeptide is absolutely required for the high catalytic efficiency of NTRC. An Arabidopsis thaliana knockout mutant for NTRC shows irregular mesophyll cell shape, abnormal chloroplast structure, and unbalanced BAS1 redox state, resulting in impaired photosynthesis rate under low light. Constitutive expression of wild-type NTRC in mutant transgenic lines rescued this phenotype. Moreover, prolonged darkness followed by light/dark incubation produced an increase in hydrogen peroxide and lipid peroxidation in leaves and accelerated senescence of NTRC-deficient plants. We propose that NTRC constitutes an alternative system for chloroplast protection against oxidative damage, using NADPH as the source of reducing power. Since no light-driven reduced ferredoxin is produced at night, the NTRC-BAS1 pathway may be a key detoxification system during darkness, with NADPH produced by the oxidative pentose phosphate pathway as the source of reducing power.

INTRODUCTION

Oxygenic photosynthesis is the source of atmospheric oxygen and provides organic material for virtually all life forms (Nelson and Ben-Shem, 2004). However, photosynthesis involves transport of electrons in the presence of oxygen; therefore, it is a process that inevitably produces reactive oxygen species (ROS), which are harmful to the cell (Mittler et al., 2004). Consequently, the evolution of photosynthesis has occurred along with the evolution of nonenzymatic and enzymatic ROS-scavenging mechanisms (Ledford and Niyogi, 2005). Nonenzymatic antioxidants include glutathione, ascorbate, carotenoids, or tocopherols, whereas enzymatic scavenging mechanisms include ascorbate peroxidase, superoxide dismutase, glutathione peroxidase (GPX), and catalase (Apel and Hirt, 2004). Plants contain gene families for superoxide dismutase, ascorbate peroxidase, and GPX. The isoforms encoded by the members of these gene families are targeted to different subcellular locations, cytosol, mitochondria, and chloroplasts, as has been shown for GPX genes in Arabidopsis thaliana (Rodriguez Milla et al., 2003). By contrast, catalase is mainly localized in peroxisomes (Apel and Hirt, 2004).

The ROS produced by the chloroplast include singlet oxygen, superoxide and hydroxyl radicals, and hydrogen peroxide (Mittler, 2002). Of these species, singlet oxygen is highly reactive and probably generates oxidative damage near the site of its production (Ledford and Niyogi, 2005). By contrast, superoxide radicals are rapidly converted to hydrogen peroxide in a reaction catalyzed by superoxide dismutase (Asada, 1999). Hydrogen peroxide is a relatively stable and permeable molecule with a double effect for the cell. When it accumulates at a high level, it becomes toxic because it can be converted to highly reactive hydroxyl radicals (Apel and Hirt, 2004); in addition, hydrogen peroxide is an important signaling molecule (Wood et al., 2003a). Indeed, global expression analysis revealed that hydrogen peroxide is involved in the regulation of a large number of genes in tobacco (Nicotiana tabacum) (Vandenabeele et al., 2003) and Arabidopsis (Vanderwaera et al., 2005). As a signaling molecule, hydrogen peroxide, most of which is produced by the chloroplast in plant cells, is involved in the regulation of several processes, including photosynthesis (Pfannschmidt, 2003) and the response of the plant to different environmental stimuli (Laloi et al., 2004).

Therefore, the mechanism to control the production of hydrogen peroxide by chloroplasts is important to balance its signaling role and deleterious effects. In this regard, peroxiredoxins, a family of antioxidant enzymes able to reduce hydrogen or organic peroxides, play an important role (Dietz, 2003; Wood et al., 2003b). Of this family of enzymes, 2-Cys peroxiredoxins (2-Cys Prxs) have been proposed to play a key role as antioxidants and for regulating the level of hydrogen peroxide for signal transduction (Wood et al., 2003a). The catalytic mechanism of 2-Cys Prxs
involves the formation of a disulfide, which needs to be reduced for a new catalytic cycle. In prokaryotes, this reduction is catalyzed by an NADH-dependent peroxiredoxin oxidoreductase termed AhpF (Poole et al., 2000a). In eukaryotes, however, no homologue of AhpF has been described so far, and it is considered that 2-Cys Prxs are reduced by a two-component system formed by thioredoxins (Trxs) and NADPH thioredoxin reductase (NTR).

Previous analysis of the chloroplast detoxification system identified a 2-Cys Prx, termed BAS1, involved in chloroplast protection against oxidative damage (Baier and Dietz, 1997). The redox state of BAS1 depends on the NAD(P)H system, and oxidized BAS1 can be reduced by plastid thioredoxins (Konig et al., 2002), among which, x-type Trx is the most efficient electron donor (Collin et al., 2003). In addition, in vitro and in vivo interaction of the plastid Trx CDSP32 with BAS1 has been shown (Broin et al., 2002; Rey et al., 2005). Furthermore, BAS1 displayed CDSP32-dependent peroxidase activity, though the rate of peroxide reduction catalyzed by BAS1 in the presence of CDSP32 (Broin et al., 2002) was much lower than in the presence of Trx x (Collin et al., 2003). Based on these results, it was proposed that CDSP32 and Trx x are physiological electron donors to BAS1, the expected source of reducing power being ferredoxin reduced by the photosynthetic electron chain. In this regard, Arabidopsis mutants lacking the variable subunit of the ferredoxin thioredoxin reductase (FTR) are more sensitive to oxidative damage than the wild type (Keryer et al., 2004), suggesting that the FTR-Trx (type-x and CDSP32) system is involved in chloroplast protection against oxidative damage.

Recently, we identified a gene in Arabidopsis and rice (Oryza sativa) encoding a novel type of NADPH thioredoxin reductase, termed NTRC (Serrato et al., 2004). The gene was cloned from rice, and the encoded protein, NTRC, was shown to be a bimodular enzyme formed by an NTR-like module at the N terminus and a Trx-like module at the C terminus. Since NTRC is localized to the chloroplast stroma and shows a high affinity for NADPH (Serrato et al., 2004), we hypothesized that this enzyme might function as an alternative electron donor for the chloroplast detoxification system, allowing the use of NADPH for such a protective role. To test this possibility, homologs of CDSP32, Trx x, and the 2-Cys Prx BAS1 were cloned from rice and expressed in Escherichia coli. Here, we show that rice NTRC is a high-efficiency system to transfer electrons from NADPH to the plastid 2-Cys Prx (BAS1) from rice. An Arabidopsis knockout mutant for NTRC shows abnormal chloroplast structure, reduced content of photosynthetic pigments, and a lower rate of photosynthesis. The mutant also is hypersensitive to prolonged darkness. Based on these results, we propose that NTRC is an alternative system, which allows the use of NADPH for chloroplast detoxification.

RESULTS

Cloning and Expression in E. coli of the 2-Cys Prx, BAS1, and the Thioredoxins CDSP32 and Trx x from Rice

We have previously described NTRC as a bifunctional enzyme showing NTR and Trx activity. However, NTRC was unable to catalyze the NADPH-dependent reduction of insulin; that is, NTRC did not function as an NTR/Trx system under these assay conditions (Serrato et al., 2004). Given that NTRC is localized to the chloroplast stroma and shows a high affinity for NADPH, we tested its possible role as an alternative electron donor to the chloroplast detoxification system and its interaction with the FTR-Trx (Trx x and CDSP32) system. To that end, the plastid 2-Cys Prx, BAS1, and thioredoxins CDSP32 and Trx x were cloned from rice, expressed in E. coli with a His-tag at the N terminus, and purified by nitritetriacetic acid (NTA) chromatography (Figure 1A).

The thioredoxin activity of the purified His-tagged Trx x and CDSP32 was determined with the insulin reduction assay in the presence of DTT. Trx x showed a high rate of insulin reduction, even higher than wheat (Triticum aestivum) Trx hA (Serrato et al., 2001), which was used as a positive control (Figure 1B). However, CDSP32 displayed a rather low insulin reduction activity compared with Trx x and Trx hA (Figure 1B). It should be noted that though rice CDSP32 showed low activity, it was detectable,
in contrast with CDSP32 from Arabidopsis, which did not show any insulin reduction activity (Broin et al., 2002). In addition, the C-terminal domain of CDSP32 was produced in E. coli as an N-terminal His-tagged protein (Figure 1A). This truncated polypeptide, containing a Trx active site, showed higher insulin reduction activity than the full-length protein CDSP32 (Figure 1B), in agreement with previous results showing that the C-terminal Trx fold of CDSP32 from Arabidopsis, but not the full-length enzyme, displayed insulin reduction activity (Broin et al., 2002).

NTRC Diverts Reducing Power to the Chloroplast Detoxification System

To test the possible function of NTRC as electron donor to the chloroplast detoxification system, purified recombinant NTRC and BAS1 were incubated in the presence of NADPH and hydrogen peroxide. Figure 2A shows that the system formed by NTRC and BAS1 efficiently catalyzed the oxidation of NADPH when hydrogen peroxide was present. No activity was observed in the absence of NTRC, and a residual rate of NADPH oxidation occurred in the absence of BAS1. This residual activity was also detected in the absence of hydrogen peroxide and therefore is most probably due to diaphorase activity, characteristic of flavoproteins, of NTRC. The initial rate of NADPH oxidation by the NTRC-BAS1 system (40.3 μmol NADPH min⁻¹ μmol⁻¹ BAS1) was remarkably higher than the rate obtained with standard two-component assay conditions in the presence of wheat NTR and Trx HA (2.1 μmol NADPH min⁻¹ μmol⁻¹ BAS1). NTRC activity was confirmed by direct determination of hydrogen peroxide in the in vitro assay (Figure 2B). The residual activity of NTRC in the absence of BAS1, determined as NADPH oxidation (Figure 2A), was not detected when activity was assayed as hydrogen peroxide reduction (Figure 2B), lending further support to the proposal that it is due to the diaphorase activity of NTRC.

To characterize the biochemical properties of this new enzyme further, the activity of truncated polypeptides containing either the NTR or the Trx-like domain of NTRC was analyzed. Previously, it was shown that both truncated proteins show NTR and thioredoxin activity (Serrato et al., 2004). When assayed in the presence of BAS1, the Trx-like module did not show any activity (Figure 2C). By contrast, the NTR module showed a low rate of NADPH oxidation (Figure 2C). This activity was also detected in the absence of hydrogen peroxide, thus confirming that the diaphorase activity of NTRC is localized in the NTR module of the enzyme, which contains FAD. Activity assays with a mixture of NTR and Trx-like modules showed a higher rate of NADPH oxidation than the NTR module alone, but very low when compared with the activity of the full-length NTRC (Figure 2C). These results show that the native conformation of NTRC with both NTR and Trx domains in a single polypeptide is required for full activity and lends further support to the proposal that NTRC is a high-efficiency system for 2-Cys Prx-mediated hydrogen peroxide reduction.

The Rice 2-Cys Prx, BAS1, Is Efficiently Reduced by NTRC

According to the mechanism of catalysis proposed for 2-Cys Prxs (Dietz, 2003; Konig et al., 2003; Wood et al., 2003b), the results described above suggest that NTRC might be able to reduce the disulfides formed by the Cys residues (Cys-61 and Cys-183) at the active site of BAS1. This possibility was analyzed in two ways. First, a BAS1 mutant was produced, replacing Cys-61 by Ser. In SDS-PAGE analysis under nonreducing conditions, wild-type BAS1 was detected in dimeric form, whereas BAS1(C61S) was detected as a monomer (Figure 3A). No activity was observed when wild-type NTRC was incubated with BAS1(C61S) mutant, the residual oxidation of NADPH being due to the diaphorase activity of NTRC (Figure 3B). Considering that,
as stated before, NTRC acts as an NTR/Trx system, it should be expected that the active site of the NTR and Trx-like modules of the enzyme are involved in the redox interchange with BAS1. To test this possibility, the Cys residues at the active sites of the NTR and Trx-like modules of NTRC were replaced by Ser, producing NTRC mutants C140S and C143S (NTR domain) and C377S and C380S (Trx-like domain). None of these mutants showed activity when incubated with wild-type BAS1 (Figure 3B; data not shown), hence confirming that the active sites of both NTR and Trx-like modules of NTRC are essential for activity.

The second approach consisted in the determination of the direct reduction of BAS1 by NTRC. Given that active site Cys-61 and Cys-183 are the only Cys residues of BAS1, it was possible to distinguish reduced and oxidized BAS1 by 4-acetamido-4'-maleimidylstilbene (AMS) labeling of sulfhydryl groups. AMS did not label the oxidized, dimeric form of BAS1 (Figure 4A, lane 1). A similar result was obtained when NADPH was added in the absence of NTRC (Figure 4A, lane 2) or when NTRC was added in the absence of NADPH (Figure 4A, lane 3). However, when BAS1 was preincubated with the complete system, NTRC and NADPH, BAS1 was partially reduced as shown by the shift of the BAS1 band (Figure 4A, lane 4). BAS1 was also detected in monomeric form, which corresponds to the fully reduced form of the enzyme, suggesting that NTRC is able to use NADPH to reduce the two disulfides of BAS1. The different electrophoretical mobility of NTRC depending on the presence or absence of NADPH should be noted. In the presence of NADPH, most of the enzyme was detected as a band corresponding to the monomer (Figure 4A, lane 4), which was almost undetectable in the absence of NADPH (Figure 4A, lane 3). To test whether the difference of electrophoretical mobility was a direct effect of NADPH, purified NTRC was preincubated in the presence or absence of NADPH and then subjected to electrophoresis. Figure 4B shows that NTRC was detected in monomeric and dimeric forms in the presence of NADPH; however, these forms were almost undetectable in the absence of NADPH because most of the enzyme was detected as a high-molecular band at the upper part of the gel (Figure 4B). These results suggest that NADPH affects the aggregation state of NTRC, but the mechanism of this effect of NADPH is not yet understood.

Figure 3. Effect of Active Site Cys Mutations on NTRC-BAS1 Activity.  
(A) Purified BAS1 wild type and mutant C61S (10 μg of protein) were subjected to SDS-PAGE under nonreducing conditions. Molecular mass markers (kD) are on the left.  
(B) NADPH-dependent reduction of hydrogen peroxide activity was assayed in a reaction mixture containing 100 mM phosphate buffer, pH 7.0, 2 mM EDTA, 0.25 mM NADPH, and 0.5 mM hydrogen peroxide in the presence of 2 μM NTRC plus 6 μM BAS1 (closed squares), 2 μM NTRC plus 6 μM BAS1(C61S) (closed triangles), or 2 μM NTRC(C377S) plus 6 μM BAS1 (open triangles).

Figure 4. Reduction of BAS1 by NTRC.  
(A) Reduction level of BAS1 was determined by AMS labeling. Purified BAS1 (3.5 μg) was incubated with 6.25 mM hydrogen peroxide for oxidation, then the protein was diluted 12.5-fold with 100 mM phosphate buffer, pH 7.0, and 2 mM EDTA and incubated in the presence of 0.45 mM NADPH and/or 7.5 μM NTRC as indicated. Molecular mass markers (kD) are on the left.  
(B) Purified NTRC (4.5 μg) was preincubated in the presence or absence of 1 mM NADPH for 4 min at room temperature and subjected to SDS-PAGE. Samples were not boiled before loading. d, dimer; m, monomer; o, oligomer.
NTRC Is the Most Efficient Reductant of BAS1

Previous reports described two plastidial thioredoxins, CDSP32 and Trx x, as electron donors to the 2-Cys Prx, and it was proposed that the source of reducing power for this system is photosynthetically reduced ferredoxin and FTR (Broin et al., 2002; Collin et al., 2003). The activity of NTRC as an NTR/Trx system able to use NADPH to reduce the 2-Cys Prx BAS1 suggests that this may be an alternative system to transfer reducing power from NADPH to the chloroplast detoxification system. We therefore established which of the systems is more efficient as a reductant of 2-Cys Prx. Since both Trx x and CDSP32 were assayed in the presence of DTT (Broin et al., 2002; Collin et al., 2003), we determined the DTT-dependent activity of NTRC, corresponding to the Trx-like module (Serrato et al., 2004), to allow a direct comparison. Under these assay conditions, NTRC was the most efficient electron donor to BAS1 (Figure 5). In addition, these assays confirm that Trx x was a more efficient reductant of BAS1 than CDSP32, which showed almost undetectable activity. In the presence of NADPH, its physiological electron donor, NTRC proved to be by far the most efficient reductant (Figure 5). The kinetic parameters confirmed the high efficiency of NTRC, which showed higher affinity for BAS1 than Trx x, as deduced of the lower $K_m$ value (Table 1). Similarly, the catalytic efficiency ($K_{cat}/K_m$) of NTRC was $\sim$100-fold higher (Table 1). Although Trx x kinetic parameters were determined with a nonphysiological assay using DTT, these results highly suggest that the presence of both NTR and Trx activity in a single polypeptide increases the efficiency of the system.

An Arabidopsis NTRC Knockout Mutant Shows Damage of the Photosynthetic Apparatus

The in vitro assay results shown above suggest that NTRC is an alternative system able to divert reducing power (NADPH) to the 2-Cys Prx (BAS1) involved in chloroplast detoxification. To test the functional relevance of this system, we characterized the photosynthetic phenotype of an Arabidopsis knockout mutant deficient in NTRC (Serrato et al., 2004). The content of photosynthetic pigments, chlorophylls a and b, and carotenoids was significantly lower in the ntrc knockout plants (Table 2). The rate of electron transfer, determined in vitro as oxygen production by thylakoid fractions in the presence of ferricyanide, was severely reduced in the knockout mutant (Table 2). In addition, we analyzed the rate of photosynthesis in vivo, as CO$_2$ fixation, at different light intensities. As expected, the ntrc mutant showed a lower rate of CO$_2$ fixation, the difference being highly dependent on light intensity (Figure 6). Indeed, at low intensity (100 μmol m$^{-2}$ s$^{-1}$), the rate of photosynthesis of the ntrc mutant was negative, meaning that it was lower than the rate of respiration, whereas at higher intensity (500 to 1500 μmol m$^{-2}$ s$^{-1}$), the ntrc mutant presented slightly lower CO$_2$ fixation than the wild type. So, NTRC deficiency seems to exert a stronger effect on CO$_2$ fixation at lower light intensity.

To test that the phenotype of the knockout mutant was due to NTRC deficiency, the wild-type NTRC gene was expressed under the control of the cauliflower mosaic virus 35S promoter in ntrc knockout and wild-type Arabidopsis plants. Two transgenic lines showing a high expression of the NTRC gene, as detected by RT-PCR analysis (Figure 7A), were analyzed in detail. Despite the higher accumulation of NTRC transcripts in both transgenic lines, the amount of NTRC polypeptide was similar in leaves from wild-type and transgenic lines (Figure 7B), which suggests a posttranscriptional regulation of the amount of NTRC enzyme in vivo. The transgenic mutant plants expressing wild-type NTRC, KO;P$_{35S}$NTRC, showed a growth indistinguishable from the wild-type plants or the wild-type plants expressing the NTRC gene, WT;P$_{35S}$NTRC (Figure 7C).

Mesophyll cells of the ntrc knockout mutant show an irregular shape and a variable content of chloroplasts compared with the wild-type cells (Figures 8A and 8B). Moreover, NTRC deficiency caused abnormal chloroplast structure, which affected the content and organization of thylakoids (Figures 8E and 8F) compared with chloroplasts from wild-type cells (Figure 8D). Accordingly, mesophyll cells (Figure 8C) and chloroplast structure (Figure 8G) of KO;P$_{35S}$NTRC transgenic plants showed wild-type morphology. Moreover, KO;P$_{35S}$NTRC and WT;P$_{35S}$NTRC transgenic plants showed a wild-type level of photosynthetic pigments and rate of oxygen production by isolated thylakoids (Table 2), as well as rate of photosynthesis determined as CO$_2$ fixation (Figure 6). Therefore, these results show that the mutant phenotype was rescued by expression of wild-type NTRC.

### Table 1. Kinetic Parameters of Rice NTRC and Trx x with BAS1

<table>
<thead>
<tr>
<th></th>
<th>$V_{max}$ (μmol min$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$K_{cat}$ (s$^{-1}$)</th>
<th>$K_{cat}/K_m$ (μM$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTRC</td>
<td>277.77</td>
<td>21.8</td>
<td>4.62</td>
<td>0.2100</td>
</tr>
<tr>
<td>Trx x</td>
<td>62.89</td>
<td>77.4</td>
<td>0.14</td>
<td>0.0018</td>
</tr>
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</table>

Reactions were performed at a fixed concentration of NTRC (1 μM) and variable concentrations of BAS1 (2 to 10 μM) in the presence of NADPH (0.25 mM). For determination of the kinetic parameters of Trx x, a fixed concentration of Trx x (7.5 μM) was used with 10 to 25 μM BAS1 in the presence of DTT (0.5 mM).
Effect of Environmental Stresses on *Arabidopsis* Wild-Type and *ntrc* Knockout Plants

To analyze in more detail the function of NTRC, *Arabidopsis* wild-type and *ntrc* knockout plants were subjected to different environmental stresses, and the photosystem II (PSII) photochemical efficiency, determined as $F_v/F_m$, was used to determine the plant response to stress treatments. The PSII photochemical efficiency of the *ntrc* knockout was slightly lower than the wild-type plants under standard growth conditions (Table 3). Previously, we have shown that treatments with salt, drought, or methyl viologen caused growth inhibition on the *ntrc* mutant (Serrato et al., 2004); however, these treatments, as well as cold, produced a low effect on the *ntrc* photochemical efficiency in the mutant (Table 3). Since the rate of CO$_2$ fixation of the *ntrc* knockout was slightly lower than the wild-type plants under standard growth conditions (Table 3). Previously, we have shown that treatments with salt, drought, or methyl viologen caused growth inhibition on the *ntrc* mutant (Serrato et al., 2004); however, these treatments, as well as cold, produced a low effect on the *ntrc* photochemical efficiency in the mutant (Table 3). Since the rate of CO$_2$ fixation of the *ntrc* knockout was slightly lower than the wild-type plants under standard growth conditions (Table 3).

The amount of NTRC was not significantly altered by the darkness treatment in any of the plant lines analyzed but was increased in the mutant plants after 3 d under standard growth conditions (Figures 9B and 9C, D-1-LD treatment). Therefore, stress symptoms in leaves were detected when mutant plants were again illuminated after the prolonged darkness treatment.

NTRC Deficiency Causes Hypersensitivity to Prolonged Darkness

During darkness, when there is no photosynthetic electron transport, NADPH in chloroplasts is still produced by the initial reactions of the oxidative pentose phosphate pathway (Neuhaus and Emes, 2000). So, NTRC, which uses NADPH to reduce the 2-Cys Prx, may become a key reductant system during the night. In this regard, it should be mentioned that the phenotype of the NTRC-deficient plants depends on light intensity and photoperiod showing more severe growth retardation when grown under short-day conditions, with a 16-h-dark/8-h-light photoperiod (see Supplemental Figure 1 online).

To characterize the function of NTRC further, we analyzed in more detail the effect of continuous darkness on wild-type, transgenic, and knockout plants. A treatment of 72 h in continuous darkness produced pronounced symptoms of leaf bleaching in the knockout plants (Figure 9A, D treatment). When plants were incubated under normal growth conditions following dark treatment (16 h light/8 h dark) for 3 d (D-1-LD), the wild-type and transgenic plants resumed growth, although showing more symptoms of leaf senescence than control (LD-1-LD) plants (Figure 9A). However, the NTRC-deficient plants presented accelerated leaf senescence (Figure 9A, D-1-LD treatment). The level of peroxides (Figure 9B) and lipid peroxidation (Figure 9C) in leaves, which were slightly higher in the *ntrc* mutant, were not significantly affected by a treatment of continuous darkness in any of the plant lines analyzed but were increased in the mutant plants after 3 d under standard growth conditions (Figures 9B and 9C, D-1-LD treatment). Therefore, stress symptoms in leaves were detected when mutant plants were again illuminated after the prolonged darkness treatment.

![Figure 6. Light-Dependent CO$_2$ Fixation Response Curves of Wild-Type, Knockout, and Transgenic Plants.](image)

Wild-type, NTRC-deficient *Arabidopsis*, and transgenic lines expressing NTRC under the control of the 35S promoter as indicated were grown for 30 d. Six determinations were performed per light intensity with leaves from different plants, and the mean values ± SD are represented. PAR, photosynthesis active radiation.

### Table 2. Effect of NTRC Deficiency on the Content of Photosynthetic Pigments and the Rate of Photosynthesis

<table>
<thead>
<tr>
<th>Plant Line</th>
<th>Photosynthetic Pigments (µg mg$^{-1}$ Fresh Weight)</th>
<th>Photosynthesis Rate (nmol min$^{-1}$ mg Chlorophyll$^{-1}$)</th>
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<tbody>
<tr>
<td></td>
<td>Total Chlorophyll</td>
<td>Chlorophyll a</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.57 ± 0.12</td>
<td>0.42 ± 0.10</td>
</tr>
<tr>
<td>NTRC-KO</td>
<td>0.43 ± 0.05</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>KO:P35SNTRC</td>
<td>0.67 ± 0.06</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>WT:P35SNTRC</td>
<td>0.57 ± 0.05</td>
<td>0.44 ± 0.05</td>
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</table>

Pigments were determined on 38-d-old plants. Data are mean ± SD of four independent determinations. Photosynthesis rate was determined on chloroplasts isolated from two independent sets of plants, and a representative result is shown.
BAS1 as a double band corresponding to the dimeric forms of the protein, the amount of dimeric form not being significantly affected by the darkness treatment in the wild-type (Figure 10B) and transgenic lines (Figure 10C). A progressive decrease of BAS1 dimers was observed in dark-treated mutant plants, BAS1 dimers being almost undetectable after reillumination (Figure 10B). Under nonreducing conditions, part of BAS1 was detected in monomeric form, which corresponds to fully reduced BAS1, in wild-type and transgenic lines (Figures 10B and 10C). By contrast, in NTRC-deficient plants, the fully reduced, monomeric form of BAS1 was almost undetectable (Figure 10B). These results show that NTRC deficiency causes unbalance of the redox state of BAS1 and lowers its capacity to form dimers in dark-treated plants.

DISCUSSION

Oxygenic photosynthesis is the primary source of organic matter in the biosphere but is inevitably accompanied by the production of ROS, such as hydrogen peroxide. Given the dual role of hydrogen peroxide in signaling (Kwak et al., 2003; Overmyer et al., 2003; Vandenabeele et al., 2003; Foreman et al., 2004) and as a potentially harmful molecule (Mittler et al., 2004), the mechanism to control its production by the chloroplast is of great relevance. Previous studies identified a 2-Cys Prx, termed BAS1, as an important component for chloroplast peroxide detoxification (Konig et al., 2002). This Prx is efficiently reduced by Trx (Collin et al., 2003) and interacts in vitro and in vivo with CDS32, a plastid protein containing two Trx folds (Broin et al., 2002; Rey et al., 2005). Based on these results, the current model for chloroplast peroxide detoxification is based on the transfer of photosynthetic reducing power to ferredoxin, which in a reaction catalyzed by FTR would transfer reducing power to Trx and/or CDS32 and then to the 2-Cys Prx. Though no in vitro reconstitution of the FTR/Trx and CDS32/BAS1 system has been reported, the hypersensitivity of Arabidopsis FTR-deficient mutants to oxidative stress (Keryer et al., 2004) shows that this redox system plays an important role in chloroplast detoxification.

In this report, we show that rice NTRC, a novel type of NTR recently discovered in oxygenic photosynthetic organisms (Serrato et al., 2004), is able to transfer reducing power from NADPH to the 2-Cys Prx BAS1. This result suggests that NADPH,
which is produced in the photosynthetic electron chain during the light period (Nelson and Ben-Shem, 2004), but also from the oxidative pentose phosphate pathway during the night (Neuhaus and Emes, 2000), may act as an alternative source of reducing power for chloroplast detoxification.

The catalytic mechanism of typical 2-Cys Prx involves two Cys residues: stated peroxidatic and resolving (Wood et al., 2003b). In the case of rice BAS1, the peroxidatic Cys residue Cys-61 is essential for activity and participates in the formation of the double disulfide, Cys-61–Cys-183, linking the oxidized, homo-dimeric form of BAS1 (Figure 3). AMS-labeling experiments clearly demonstrate that NTRC is able to reduce both disulfides using NADPH as source of reducing power (Figure 4A). Prokaryotes and eukaryotes seem to have evolved different mechanisms for 2-Cys Prx disulfide reduction. In eukaryotes, reduction is catalyzed by thioredoxins, which in turn are reduced by NTR (Schüermann and Jacquot, 2000). In prokaryotes, the more deeply characterized 2-Cys Prx, AhpC, is reduced by an NADH:peroxidin reductase, AhpF (Poole et al., 2000a), a bimodular flavoenzyme composed of an NTR module and two Trx-like folds, with only one containing a redox-active disulfide (Poole et al., 2000b). Like AhpF, NTRC is a bimodular flavoenzyme and catalyzes the same reaction, that is, the transfer of electrons to the 2-Cys Prx BAS1. However, both enzymes show important differences: AhpF contains a double Trx-like fold at the N terminus, whereas NTRC contains a single Trx-like module at the C terminus, and, most importantly, AhpF is NADH dependent (Poole et al., 2000a; Wood et al., 2003b), whereas NTRC uses NADPH as source of reducing power (Figure 2).

The transfer of reducing power from NADPH to the 2-Cys Prx catalyzed by NTRC means that NTRC is a novel type of enzyme conjugating the activity of a NTR/Trx system in a single polypeptide. The lack of activity of any of the NTRC active sites mutants (C140S, C143S, C377S, and C380S) reveals that both modules, NTR and Trx-like, are essential for NTRC activity. Moreover, it is absolutely required that both activities form part of a single polypeptide as shown by the low activity obtained when mixtures of NTR and Trx-like truncated polypeptides were assayed compared with the full-length protein (Figure 2C).

Prxs are considered peroxidases of low enzymatic efficiency based on standard activity assays using NTR and Trx (Dietz, 2003). In agreement with this view, when the 2-Cys Prx BAS1 was assayed with wheat NTR and Trx hA or truncated NTR and Trx-like modules from NTRC (Figure 2C), the enzyme showed low catalytic efficiency. Remarkably, the activity of NTRC as a NTR-Trx system produces a drastic increase of the catalytic efficiency of the 2-Cys Prx, implying that the NTRC-BAS1 system is a high-efficiency redox system for peroxide reduction. The fact that NTRC genes are found only in cyanobacteria and plants (Serrato et al., 2004) suggests that this high-efficiency mechanism of detoxification is exclusive of oxygenic photosynthesis.

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<thead>
<tr>
<th>Treatment</th>
<th>Fv/Fm Wild Type</th>
<th>Fv/Fm ntrc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.822 ± 0.011</td>
<td>0.811 ± 0.005</td>
</tr>
<tr>
<td>Drought</td>
<td>0.812 ± 0.003</td>
<td>0.798 ± 0.013</td>
</tr>
<tr>
<td>Salt</td>
<td>0.819 ± 0.003</td>
<td>0.785 ± 0.015</td>
</tr>
<tr>
<td>Cold</td>
<td>0.813 ± 0.010</td>
<td>0.770 ± 0.041</td>
</tr>
<tr>
<td>Darkness (48 h)</td>
<td>0.579 ± 0.059</td>
<td>0.385 ± 0.069</td>
</tr>
</tbody>
</table>

Fv/Fm was determined on six leaves from different plants subjected to the indicated stress treatments as described in Methods. Values are mean ± SD. Stress treatment experiments were repeated twice.

Figure 9. Effect of Continuous Darkness and Reillumination on Wild-Type and NTRC-Deficient Arabidopsis Plants.

(A) Arabidopsis wild-type, ntrc knockout, and transgenic plants, as indicated, grown for 28 d in a growth chamber in a 16-h-light/8-h-dark cycle were incubated for 3 d under the same light/dark cycle (LD) or with continuous darkness (D). Both control (LD + LD) and dark-treated (D + LD) were then incubated for an additional 3 d under a 16-h-light/8-h-dark cycle. A representative plant is shown.

(B) and (C) Plants treated as indicated were collected, and the level of hydrogen peroxide (B) or lipid peroxidation as malonyldialdehyde (MDA) (C) in leaves was determined. Values represent means ± sd of at least three determinations per sample. FW, fresh weight.
probably because of the large amount of hydrogen peroxide produced by this process. In rice and *Arabidopsis*, we have shown that NTRC is localized to chloroplasts (Serrato et al., 2004). However, the rice gene undergoes several alternative splicing events (J.M. Pérez-Ruiz and F.J. Cejudo, unpublished data), which might produce versions of NTRC localized in other cell compartments not yet identified. So, the possibility of high-efficiency detoxification systems in cell compartments other than the chloroplast cannot be ruled out and deserves future investigation.

Based on the results reported here, we propose the existence of two pathways for transferring reducing power to the 2-Cys Prx involved in chloroplast detoxification (Figure 11). One of the pathways is formed by NTRC and uses NADPH as a source of reducing power; the other one is formed by FTR and Trx and/or CDSP32 and uses reduced ferredoxin as a source of reducing power (Broin et al., 2002; Collin et al., 2003; Rey et al., 2005). Based on kinetic parameters, we conclude that NTRC is more efficient than the FTR-Trx pathway. Moreover, our results with the rice enzymes show that Trx is much more efficient than CDSP32 to reduce BAS1 (Figure 5), in agreement with previous reports with the *Arabidopsis* enzymes (Broin et al., 2002; Collin et al., 2003). Given the low efficiency of CDSP32 as reductant of BAS1, compared with Trx and NTRC, the function of this protein in peroxide detoxification is not clear. It should be taken into account that CDSP32 is induced during severe drought and oxidative stress (Rey et al., 1998; Broin et al., 2000). Therefore, CDSP32 interaction with BAS1 may have a function more relevant under stress conditions that is yet to be determined.

According to the proposed model (Figure 11), it would be expected that NTRC has an important function in plant growth and development as well as in response to environmental conditions. We have addressed the function of NTRC by analyzing an *Arabidopsis* knockout mutant. A previous description of this mutant showed retarded growth, light-green leaf color, and...
hypothesis to abiotic stress (Serrato et al., 2004). Here, we show that NTRC deficiency severely affects leaf photosynthetic cells (Figure 8), most of the effects altered chloroplast structure, lower content of photosynthetic pigments, and lower rate of photosynthesis being directly related with the photosynthetic machinery. These results provide an explanation for the retarded growth of the mutant and are in agreement with the effect of decreased levels of 2-Cys Prx, the target of NTRC, in transgenic Arabidopsis plants, which showed impaired photosynthesis and increased oxidative damage (Baier and Dietz, 1999; Baier et al., 2000).

The low rate of CO2 fixation of the NTRC-deficient plants (Figure 6) is an expected effect of the altered photosynthetic machinery of these plants. However, it is remarkable that this effect was light dependent, being negative at low light intensity and recovering to levels slightly lower than the wild type at higher light intensity. The strong effect of continuous darkness on the NTRC-deficient plants determined by the Fv/Fm ratio (Table 3) and leaf bleaching (Figure 9) and the severe retardation of growth when plants were grown under short-day conditions (see Supplementary Figure 1 online) lends further support to the proposal that NTRC plays an important role during the night.

The proposed model of NTRC function in chloroplast detoxification provides a possible explanation for the severe effect of darkness/reillumination treatment on the mutant plants. Under standard long-day growth conditions during the light period, both sources of reducing power for chloroplast detoxification, NADPH and reduced ferredoxin, are produced. So, NTRC deficiency might be partially compensated for by the light-dependent FTR/Trx pathway. However, during darkness, ferredoxin cannot be reduced by the photosynthetic electron chain, the only source of reduced ferredoxin being the reversal of the reaction catalyzed by ferredoxin oxidoreductase (Emes and Neuhau, 1997); therefore, the FTR/Trx pathway for BAS1 reduction is probably less operative. The fact that this treatment is not lethal for the wild-type and transgenic plants suggests that NTRC is still able to transfer reducing power from NADPH produced in the oxidative pentose phosphate pathway to the 2-Cys Prx. The analysis of BAS1 under nonreducing conditions shows that most of the protein is detected in dimeric form (Figures 10B and 10C). However, in wild-type and transgenic plants, part of BAS1 appeared in monomeric form, indicating the amount of BAS1 fully reduced in vivo. The scarce detection of monomeric BAS1 in the mutant plants shows that NTRC deficiency causes unbalance of the redox state of BAS1, so that the fully reduced form of the enzyme is severely decreased. It should be noted that no monomeric BAS1 was detected in mutant plants incubated under control conditions (Figure 10B, LD), suggesting that the NTRC pathway is more relevant for BAS1 reduction than the FTR/Trx pathway even during the light period. These results are in agreement with the in vitro data showing that NTRC is able to reduce the two disulfides of BAS1 to produce the fully reduced monomeric form of the enzyme (Figure 4A) and the higher catalytic efficiency of NTRC compared with the Trx x or CDSP32 system (Figure 5). Though the amount of BAS1 was not altered, as shown by the analysis in reducing conditions, prolonged darkness treatment progressively reduced the capacity of BAS1 to form dimers in the NTRC-deficient mutant (Figure 10B). These results show that the redox status of BAS1 is severely altered in the mutant plants and suggest that BAS1 is very sensitive to darkness treatment in the absence of NTRC.

In summary, we describe a novel enzyme, NTRC, conjugating the activities of the NTR/Trx system in a single polypeptide and show that it is a high-efficiency reductant of the 2-Cys Prx BAS1. In vitro kinetic data and the unbalance of the redox state of BAS1 in the NTRC-deficient mutant suggest that the NTRC pathway plays a predominant role for BAS1 reduction in vivo. Whether the severe effect of NTRC deficiency on the photosynthetic machinery described here is due exclusively to the suppression of the NTRC-BAS1 detoxification pathway or to additional processes in which NTRC might be involved is not yet known. To address this point, the identification of additional targets of NTRC in the chloroplast is now underway.

METHODS

Plant Material

Rice (Oryza sativa ssp japonica cv Nipponbare) grains were sterilized and germinated at 25°C on filter paper soaked with water. Arabidopsis thaliana wild-type (ecotype Columbia) and the T-DNA insertion mutant SALK_012208 were grown in moist vermiculite supplemented with Hoagland medium in culture chambers at 22°C during the light and 20°C during darkness. The light intensity was set at 140 μmol m–2 s–1. Long-day conditions consisted of 16 h light/8 h darkness, whereas short-day conditions consisted of 14 h darkness/10 h light. For production of Arabidopsis transgenic lines, full-length NTRC cDNA (DNA stock U-14278), obtained from the ABRC, was digested with KpnI and SalI and inserted into the binary vector pBIB-A7 (Becker, 1990). The construct was integrated into Arabidopsis wild-type and T-DNA insertion mutant SALK_012208 by Agrobacterium tumefaciens (C58pMP90)–mediated transformation using the floral dip method (Clough and Bent, 1998). Transformants were selected on Murashige and Skoog plates containing 50 μg mL–1 hygromycin. Transgenic lines with a single insertion, and homozygous for the transgene, were selected for further characterization.

Stress Treatments

Stress treatments were applied to Arabidopsis plants grown for 24 to 28 d. For salt treatment, plants were irrigated during 5 d with nutritive solution supplemented with 200 mM NaCl at days 1, 3, and 5. For drought treatment, plants were not irrigated during 5 d. Methyl viologen was sprayed on rosette leaves at days 1 and 3 of treatment. For cold treatment, plants were incubated during 5 d at 10°C constant temperature under the same day/night cycle, and continuous darkness was applied for periods of up to 3 d in the growth chamber at 22 to 20°C. When indicated, plants were incubated with standard growth conditions following darkness treatment (16 h light, 22°C/8 h darkness, 20°C).

Cloning of BAS1, CDSP32, and Trx x cDNAs from Rice: Expression and Purification of the Recombinant Proteins

The sequences of the rice homologs of BAS1, CDSP32, and Trx x genes were obtained from the Torrey Mesa Research Institute (Goff et al., 2002), and their putative gene structure was established using the GeneScan program. Rice BAS1 and Trx x cDNAs were cloned by RT-PCR from 1 μg total RNA of 5-d-old rice seedling shoots, which was reverse transcribed in the presence of oligo(dT) and 200 units of reverse transcriptase (Invitrogen). For rice BAS1 cloning, an aliquot (1 μL) was used as template.
in a PCR reaction with oligonucleotides 5'-AACGCTCAAGAGCGC-3' and 5'-ATCAGAGCGGAGCTG-3'. A 0.96-kb fragment was obtained, cloned in pGEM vector (Promega), and sequenced in both strands. To express rice BAS1 in Escherichia coli, the coding sequence, excluding the putative signal peptide (53 residues), was amplified from the full-length cDNA with oligonucleotides 5'-AGAGGATCCGCCGAGCGGAGCTG-3' and 5'-GAGTAGCTTAATTTAACTCAGAAGGAGCAGTACT-3', which added BamHI and HindIII sites (underlined) at the 5' and 3' ends, respectively. The cDNA encoding the putative mature form of rice Trx (excluding 65 residues at the N terminus), was directly produced by PCR using as template 1 μL of the above-described reverse transcriptase reaction and oligonucleotides 5'-TTTTTGATCCGAGCGGAGCGTGAAGTT-3' and 5'-GGGGAAGCTTTAATTAAGCAGAAGGAGCAGTACT-3', which added BamHI and HindIII sites (underlined) at the 5' and 3' ends, respectively. Rice CDSP32 is apparently an intronless gene in rice, so the full-length DNA for CDSP32, except the putative signal peptide (47 residues), was directly amplified from rice genomic DNA using oligonucleotides 5'-GAGAAGCTTCACTGCAGCCATCAGTTG-3' and 5'-GAGAAGCTTTAATTTAACTCAGAAGGAGCAGTAC-3', adding BamHI and HindIII sites (underlined) at the 5' and 3' ends, respectively. The truncated polypeptide containing exclusively the C-terminal Trx fold of CDSP32 was produced by the same approach using as template full-length CDSP32 cDNA and the pair of oligonucleotides 5'-AAAGAAGCTCCAAGGAGGCACCACTCAGCTG-3' and 5'-GAGAAGCTTTAATTTAACTCAGAAGGAGCAGTAC-3', adding BamHI and HindIII sites. All cDNAs were sequenced in both strands. The PCR fragments were digested with BamHI and HindIII, subcloned into the pQE-30 expression vector (Qiagen), and introduced into E. coli XL1-Blue. Overexpressed proteins contained a His-tag at the N terminus and were purified by NTA affinity chromatography in prepacked HiTrap affinity columns (Amersham Biosciences).

Mutants of rice BAS1 (C61S) and NTRC (C140S, C143S, C377S, and C380S) were obtained by site-directed mutagenesis on the corresponding wild-type cDNAs cloned in the expression vector pQE30, according to the method previously described (Serrato et al., 2001). Pairs of oligonucleotides for each mutant were as follows: BAS1-C61S (5'-CCTCAGCTTCCCTGCTGAGCCACC-3') and 5'-GTAATCTCGGTCGGGACTGAT-3'); BAS1-C140S (5'-AGGTATCCGTGACTCGATATGTTGATG-3') and 5'-CCTCAGCTTCCCTGCTGAGCCACC-3'); NTRC-C143S (5'-AGGTATCCGTGACTCGATATGTTGATG-3') and 5'-CCTCAGCTTCCCTGCTGAGCCACC-3'); NTRC-C377S (5'-AGGTATCCGTGACTCGATATGTTGATG-3') and 5'-CCTCAGCTTCCCTGCTGAGCCACC-3'); NTRC-C380S (5'-AGGTATCCGTGACTCGATATGTTGATG-3') and 5'-CCTCAGCTTCCCTGCTGAGCCACC-3').

Relative RT-PCR, Production of Anti-BAS1 Polyclonal Antibodies, and Protein Gel Blot Analysis

RT-PCR analysis was performed as previously described (Sánchez and Cejudo, 2003) with gene-specific oligonucleotides for NTRC (5'-GAAGGATCCGAGCGGAGCTG-3' and 5'-GTTTTGATCCGCCGAGCGGAGCTG-3'). Anti-BAS1 antibodies were raised by immunizing rabbits with purified His-tagged rice BAS1 at the Service for Animal Production (University of Seville, Spain). Protein gel blot analysis was performed as previously described (Serrato et al., 2002) using anti-NTRC as probe (Serrato et al., 2004). The anti-Rubisco polyclonal antibodies were purchased from Agrisera. Protein gel blot analysis of BAS1 was performed under reducing and nonreducing conditions. For nonreducing conditions, frozen leaves were ground with extraction buffer (100 mM Tris HCl, pH 7.9, 10% [v/v] glycerol, 1% [v/v] protease inhibitor cocktail [Sigma-Aldrich], 1 mM PMSE, 1 mM EDTA, and 100 mM MgCl2), and protein extracts were subjected to SDS-PAGE (15% polyacrylamide) in 16 × 20-cm gels overnight. For reducing conditions, the extraction buffer was supplemented with 10 mM DTT and 1.25% [v/v] β-mercaptoethanol, and samples were boiled before loading.

Thioredoxin and Peroxiredoxin Activity Assays

Peroxiredoxin activity was determined as oxidation of NADPH following absorbance at 340 nm in a reaction mixture containing 100 mM phosphate buffer, pH 7.0, 2 mM EDTA, 0.25 mM NADPH, 0.5 mM hydrogen peroxide, and purified enzymes at the concentrations indicated in the figure legends. Alternatively, disappearance of hydrogen peroxide or tBOOH was followed exclusively at 560 nm in the same reaction mixture without EDTA with the Peroxoxuant reagent (Perbio Science). Thioredoxin activity was determined by the DTT-dependent reduction of insulin as described by Holmgren and Björnstedt (1995).

 Determination of Photosynthetic Pigments

Photosynthetic pigments (chlorophyll a and b and carotenoids) were extracted with 80% (v/v) ice-cold acetone from 0.5 g (fresh weight) of leaf fragments. Chlorophyll determinations were performed according to Lichtenthaler and Wellburn (1983). Chloroplasts were isolated with the chloroplast isolation kit (Sigma-Aldrich) according to manufacturer’s instructions.

 Determination of Lipid Peroxidation

Measurements of lipid peroxidation in leaves were performed with the thiobarbituric acid (TBA) test, which determines MDA as an end product of lipid peroxidation (Heath and Parker, 1968). Briefly, 200 mg of leaves were homogenized in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA) solution on ice. The homogenate was centrifuged at 12,000g for 15 min, and 0.4 mL of the supernatant was added to 0.8 mL of 0.5% (w/v) TBA in 20% TCA. The mixture was incubated in boiling water for 30 min, and the reaction was stopped by incubation in ice. Samples were then centrifuged at 10,000g for 5 min, and the absorbance of the supernatant was measured at 532 nm, subtracting the value for nonspecific absorption at 600 nm. The amount of MDA-TBA complex was calculated from the extinction coefficient 155 mm⁻¹ cm⁻¹.

 Determination of Hydrogen Peroxide in Leaf Extracts

Hydrogen peroxide was determined according to Velikova et al. (2000). Leaf tissues (200 mg) were homogenized in 2 mL of 0.1% (w/v) TCA solution on ice. The homogenate was centrifuged at 12,000g for 15 min, and 0.4 mL of the supernatant was added to 0.4 mL of 10 mM potassium phosphate buffer, pH 7.0, and 0.8 mL of 1 M KI. The absorbance of the supernatant was measured at 390 nm. The content of H₂O₂ was calculated by comparison with a standard calibration curve previously made using different concentrations of H₂O₂.

 Determination of the Photosynthesis Rate, and PSII Photochemical Efficiency

In vitro photosynthetic rate of electron transport was determined as oxygen evolution at 25°C in a Hansatech oxygen electrode using osmotic shock-treated chloroplasts and 5 mM potassium ferricyanide as electron acceptor. Response curves of CO₂ fixation to different light intensities (photosynthesis active radiation) were made with the portable photosynthesis system (LI-6400; LiCor), which allows environmental conditions inside the cuvette to be precisely controlled. Air temperature in the growth chamber was set at 25°C, and humidity was maintained at 50%. Six response curves were measured, each on a leaf from a different plant.

The change in chlorophyll fluorescence was measured at 22°C with the chlorophyll fluorometer PAM 2000 (Walz). The maximal quantum yield of
PSII (Fv/Fm) was determined from the following equation: Fv/Fm = (Fm – Fo)/Fm, where Fo is the initial minimal fluorescence on dark-adapted leaves for 15 min and Fm is maximal dark-adapted fluorescence.

In Vitro Determination of the Reduced State of BAS1

The redox state of BAS1 was determined by labeling Cys sulfhydryl groups with AMS (Hosoyoga-Matsuda et al., 2005). BAS1 incubated under different conditions, as indicated in the figure legends, was precipitated with 10% (v/v) TCA and collected by centrifugation. Precipitated protein was then washed with ice-cold acetone and dissolved in freshly prepared 50 mM Tris-HCl, pH 7.5, 1% (v/v) SDS, and 10 mM AMS. Labeled proteins were subjected to nonreducing SDS-PAGE (10% polyacrylamide).

Optical and Electron Microscopy Studies

For morphological analysis, small fragments of leaves from 29-d-old Arabidopsis wild-type, ntrc knockout mutant, and KO:P35SNTRC transgenic plants were fixed in 4% (v/v) glutaraldelyde prepared in 0.1 M cacodylate buffer, pH 7.2, for 3 h at 4°C and postfixed in 1% OsO4 for 2 h at 4°C. Samples were dehydrated in an acetone series and embedded in Epon (epoxy embedding medium). Toluidine blue–stained semithin sections (0.5–μm thick) were viewed in a Leitz (Aristoplan) light microscope. The images were captured by means of a digital camera (Leica DC-100). For electron microscopy, thin sections (60- to 80-nm thick) were stained with uranyl acetate and lead citrate and examined in a Philips CM-10 microscope. The images were captured by means of a digital camera (Megaview 3).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AM039889 (BAS1), AM039890 (CDSP32), and AM183298 (Trx x).

Supplemental Data

The following material is available in the online version of this article.

Supplemental Figure 1. Effect of Short-Day Conditions on ntrc Mutant Growth.

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Rice NTRC Is a High-Efficiency Redox System for Chloroplast Protection against Oxidative Damage
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