A Comparative Analysis of the NADPH Thioredoxin Reductase C-2-Cys Peroxiredoxin System from Plants and Cyanobacteria

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Redox regulation based on disulfide-dithiol conversion catalyzed by thioredoxins is an important component of chloroplast function. The reducing power is provided by ferredoxin reduced by the photosynthetic electron transport chain. In addition, chloroplasts are equipped with a peculiar NADPH-dependent thioredoxin reductase, termed NTRC, with a joint thioredoxin domain at the carboxyl terminus. Because NADPH can be produced by the oxidative pentose phosphate pathway during the night, NTRC is important to maintain the chloroplast redox homeostasis under light limitation. NTRC is exclusive for photosynthetic organisms such as plants, algae, and some, but not all, cyanobacteria. Phylogenetic analysis suggests that chloroplast NTRC originated from an ancestral cyanobacterial enzyme. While the biochemical properties of plant NTRCs are well documented, little is known about the cyanobacterial enzyme. With the aim of comparing cyanobacterial and plant NTRCs, we have expressed the full-length enzyme from the cyanobacterium *Anabaena* species PCC 7120 as well as site-directed mutant variants and truncated polypeptides containing the NTR or the thioredoxin domains of the protein. Immunological and kinetic analysis showed a high similarity between NTRCs from plants and cyanobacteria. Both enzymes efficiently reduced 2-Cys peroxiredoxins from plants and from *Anabaena* but not from the cyanobacterium *Synechocystis*. Arabidopsis (*Arabidopsis thaliana*) NTRC knockout plants were transformed with the *Anabaena* NTRC gene. Despite a lower content of NTRC than in wild-type plants, the transgenic plants showed significant recovery of growth and pigmentation. Therefore, the *Anabaena* enzyme fulfills functions of the plant enzyme in vivo, further emphasizing the similarity between cyanobacterial and plant NTRCs.

Hydrogen peroxide is a by-product of aerobic metabolism that, when accumulated at high levels, may cause oxidative damage to the cell. Despite this potential toxic effect, hydrogen peroxide is also an important second messenger, in particular in eukaryotic organisms (Veal et al., 2007; Toledano et al., 2010). Therefore, in order to balance the toxic and signaling effects, the intracellular hydrogen peroxide concentration needs to be tightly controlled. For that purpose, cells are equipped with different enzymatic systems for hydrogen peroxide reduction, including peroxiredoxins (Prxs), which are thiol-based peroxidases able to detoxify hydrogen peroxide, organic peroxides and peroxynitrite (Poole et al., 2004). Based on structural and catalytic properties, Prxs are classified into three types, 1-Cys Prxs, typical 2-Cys Prxs, and atypical 2-Cys Prxs (Wood et al., 2003b). In multicellular organisms, Prxs are encoded by small gene families. For example, mammals are equipped with six Prxs distributed in different cell compartments that include cytosol, endoplasmic reticulum, mitochondria, and peroxisomes (Rhee et al., 2005). In plants, the gene family encoding Prxs is even more complex, since it is formed by 10 genes in Arabidopsis (*Arabidopsis thaliana*) and at least eight genes in rice (*Oryza sativa*; Dietz, 2003). Prxs are also distributed in different cell compartments in plant cells: PrxII A through D are localized to the cytosol; PrxII F to the mitochondria; and 1-Cys Prx is also cytosolic (Dietz et al., 2006) but accumulates to high levels in the nucleus (Stacy et al., 1999; Pulido et al., 2009). It is remarkable that the chloroplast is the plant organelle with the highest content of Prxs. The Arabidopsis chloroplast is equipped with three types of Prxs: two almost identical typical 2-Cys Prxs, termed 2-Cys Prx A and B; PrxII E; and Prx Q (Dietz et al., 2006; Kirchsteiger et al., 2009).

Following each catalytic cycle of peroxide decomposition, a disulfide-reducing catalyst must regenerate the Prxs. Although typical and atypical 2-Cys Prxs can be recycled by cyclophilins and glutaredoxins, the most common reductant for Prxs are thioredoxins (Trxs; Dietz, 2003). The reducing power of Trxs is normally provided by NADPH in a reaction catalyzed by the NADPH thioredoxin reductase (NTRC).
by NADPH-dependent thioredoxin reductase (NTR; Florencio et al., 1988). Thus, the pathway of reducing power required to maintain the Prx-dependent detoxifying activity depends on NADPH and is formed by a two-component system, NTR and Trx. However, there are two remarkable exceptions. In bacteria, such as Salmonella typhimurium, the abundant typical 2-Cys Prx, termed AhpC, is recycled by a bimolecular enzyme, AhpF, composed of a double Trx fold and an NTR domain (Poole et al., 2000). This enzyme uses NADH, not NADPH, as a source of reducing power (Poole et al., 2000; Reynolds and Poole, 2000). The other exception is a peculiar NTR with a joint Trx domain at the C terminus, termed NTRC, which is found exclusively in oxygenic photosynthetic organisms (Serrato et al., 2004). NTRC is able to conjugate NTR and Trx activities to reduce 2-Cys Prx with a high catalytic efficiency (Moon et al., 2006; Pérez-Ruiz et al., 2006; Alkhalfioui et al., 2007; Pérez-Ruiz and Cejudo, 2009), thus serving as an antioxidant system of the chloroplast.

While all plant and algal genomes so far sequenced contain a single gene encoding NTRC, the distribution of NTRC in cyanobacteria is uneven. Besides cyanobacteria that contain a gene encoding NTRC, there are other cyanobacteria that lack this gene (Florencio et al., 2006; Pascual et al., 2010). Interestingly, in the cyanobacterium Thermosynechococcus elongatus, NTRC was identified as a component of a protein complex showing NAD(P)H oxidase activity. The other component of this complex, which is induced by oxidative stress, was identified as the 2-Cys Prx (Sueoka et al., 2009). The tight association of NTRC with 2-Cys Prx in this cyanobacterium suggests that, as occurs in plant chloroplasts, NTRC acts as an efficient reductant of 2-Cys Prx, thus serving as an antioxidant system.

Eukaryotic 2-Cys Prxs become reversibly inactivated at elevated peroxide concentrations by overoxidation of the peroxidatic catalytic Cys residue to sulfenic acid. This is believed to facilitate the signaling of hydrogen peroxide in eukaryotes (Wood et al., 2003a). Recently, it was shown that cyanobacterial 2-Cys Prx also could undergo overoxidation (Pascual et al., 2010). The degree of overoxidation depends on the intracellular peroxide concentration but also on the efficiency of reduction of the 2-Cys Prx, since the disulfide-bonded form should be inert to peroxides. Interestingly, the 2-Cys Prx from Anabaena, a cyanobacterium harboring NTRC, is more sensitive to overoxidation than the enzyme from Synechocystis, which lacks NTRC. These cyanobacteria seem to have developed different strategies to cope with hydrogen peroxide. Anabaena, which is equipped with the NTRC-2-Cys Prx system but has low catalase activity, is more sensitive to hydrogen peroxide than Synechocystis, which lacks NTRC but has high catalase activity (Pascual et al., 2010).

As plant chloroplasts are equipped with NTRC and sensitive-2-Cys Prxs, but not with catalase, it was proposed that this antioxidant system evolved from the system present in cyanobacterial strains, such as Anabaena (Pascual et al., 2010). Indeed, the plant NTRC is involved in several functions that are associated with 2-Cys Prx reduction (Pérez-Ruiz et al., 2006; Stenbaek et al., 2008). However, the different phenotype of the NTRC knockout mutant of Arabidopsis, as compared with a 2-Cys Prx double mutant, suggested additional functions for NTRC unrelated to reduction of 2-Cys Prxs (Pulido et al., 2010). These functions include the redox regulation of starch synthesis (Michalska et al., 2009) and the metabolism of aromatic amino acids (Lepistö et al., 2009). These findings suggest that NTRC has evolved to adopt a wide variety of functions in plant chloroplasts, which might imply different properties of the enzymes from cyanobacteria and plants. However, our knowledge of NTRC from cyanobacteria is still very scarce. The objective of this work was to characterize the biochemical properties of a cyanobacterial NTRC and to perform a comparative analysis with the plant enzyme. For that purpose, we expressed in Escherichia coli and purified the NTRC from the cyanobacterium Anabaena and analyzed the interaction with 2-Cys Prxs from either cyanobacterial or plant origin. Furthermore, the ability of cyanobacterial NTRC to complement the phenotype of the NTRC-deficient mutant of Arabidopsis was analyzed.

RESULTS

NTRC from Anabaena Is a Bimodular Enzyme with NTR and Trx Activity

In plants, NTRC is encoded by a single gene, which produces a bimodular enzyme composed of an NTR domain at the N terminus, a Trx domain at the C terminus, and an N-terminal sequence serving as transit peptide to target the enzyme to the chloroplast (Serrato et al., 2004). With the aim of characterizing a cyanobacterial NTRC, we focused on the gene all0737 of Anabaena sp. PCC 7120, which encodes a protein showing 60.1% identity with the rice NTRC (OsNTRC). The deduced amino acid sequence of the Anabaena putative NTRC (Serrato et al., 2004), herein denoted AnabNTRC, showed that, as the plant enzyme, it is composed of NTR and Trx domains. Moreover, the characteristic motifs of the NTR domain, the FAD and NADPH binding sites, and the active sites of the NTR and Trx domains are highly conserved in the cyanobacterial enzyme (Serrato et al., 2004).

The coding sequence of the putative NTRC from Anabaena was cloned into the expression vector pQE30 so that the recombinant protein was produced in E. coli as an N-terminally His-tagged protein, following the strategy previously described for the rice enzyme, which was included in this study for comparative purposes (Fig. 1A, lanes 1 and 2). To test the functionality of the two domains, NTR and Trx, of the cyanobacterial enzyme, these were also produced as truncated His-tagged polypeptides (Fig. 1A, lanes 3 and 4). The full-length protein, AnabNTRC, and the rice enzyme

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were both efficiently detected by an anti-NTR antibody raised against wheat (*Triticum aestivum*) NTR (Serrato et al., 2002). As expected, this antibody cross-reacted with the truncated polypeptide containing the NTR domain but not the Trx domain of the cyanobacterial enzyme (Fig. 1B). The anti-OsNTRC antibody, which was raised against the Trx domain of the rice enzyme, detected both the full-length enzyme and the Trx domain from *Anabaena* but not the NTR domain. However, this antibody detected the cyanobacterial NTRC less efficiently than the rice enzyme (Fig. 1C). Therefore, the immunological analysis confirmed that *Anabaena* NTRC, like the plant enzyme, is made up of two distinct domains, NTR and Trx.

In order to characterize biochemically the cyanobacterial NTRC, the NTR and Trx activities of the full-length enzyme and the truncated versions were analyzed and compared with the previously reported rice enzyme (Serrato et al., 2004). The full-length AnabNTRC showed Trx activity, as determined by the dithiothreitol (DTT)-dependent insulin reduction assay, which was slightly lower than the activity of the rice enzyme (Fig. 2A). This activity was due to the Trx domain of the enzyme, which alone also showed DTT-dependent insulin reduction activity (Fig. 2A). Similarly, the full-length AnabNTRC showed NTR activity, assayed as NADPH-dependent reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), at a higher rate than the rice enzyme (Fig. 2B). As expected, this activity was due to the NTR domain of the enzyme, which showed NADPH-dependent reduction of DTNB, although at a lower rate than the full-length enzyme (Fig. 2B). Therefore, the biochemical analysis confirmed that the cyanobacterial NTRC, as the plant enzyme, is made up of two functional domains with the expected NTR and Trx activities, thus emphasizing the high similarity of the NTRCs from plant and cyanobacteria.

**Cyanobacterial and Plant NTRCs Are Efficient Reductants of 2-Cys Prxs from Plant and *Anabaena* But Not from *Synechocystis* **

Comparative analyses of the reactivity of plant and cyanobacterial NTRCs were carried out with 2-Cys Prxs from either plant or cyanobacteria. To that end, 2-Cys Prxs from the cyanobacterial strains *Anabaena* and *Synechocystis* as well as from rice were expressed in *E. coli* as N-terminally His-tagged polypeptides (Pérez-Ruiz et al., 2006; Pascual et al., 2010). *Anabaena* NTRC was an efficient reductant of the 2-Cys Prx from *Anabaena* and, to a somewhat lower extent, of the 2-Cys Prx from rice. However, it failed to reduce the 2-Cys Prx from *Synechocystis* (Fig. 3A). Similarly, the rice NTRC efficiently reduced 2-Cys Prx from rice and *Anabaena* but not from *Synechocystis* (Fig. 3B). The kinetic analysis of AnabNTRC with 2-Cys Prxs from plants (rice and Arabidopsis) or *Anabaena* showed slightly lower *Km* and higher *kcat* for 2-Cys Prx from *Anabaena* as compared with the values obtained with the plant 2-Cys Prxs (Table I). As a consequence, AnabNTRC showed better catalytic efficiency in terms of *kcat*/*Km* with the

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**Figure 1.** Immunological characterization of recombinant AnabNTRC and NTR and Trx domains. **A,** Purified His-tagged (1 μg of protein) OsNTRC (lane 1), AnabNTRC (lane 2), NTR domain (lane 3), or Trx domain (lane 4) of AnabNTRC were subjected to SDS-PAGE under reducing conditions. Proteins were stained with Coomassie Brilliant Blue R-250. Molecular markers were loaded, and their molecular mass, in kDa, is indicated on the left. **B** and **C,** Replicates of this gel but loaded with 50 ng of the purified proteins were electrotransferred to nitrocellulose membranes and probed with anti-NTRB (B) and anti-OsNTRC (C) antibodies, as indicated.

**Figure 2.** Trx and NTR activity of recombinant AnabNTRC and the NTR and Trx domains. **A,** Insulin reduction catalyzed by the AnabNTRC polypeptide was performed in an incubation mixture containing 2 μM AnabNTRC (squares), 2 μM OsNTRC (diamonds), and 2 μM Trx domain truncated polypeptide (triangles) supplemented with 0.5 mM DTT. **B,** NADPH-dependent reduction of DTNB was assayed at room temperature in a buffer containing 0.1 μM AnabNTRC (squares), 0.1 μM OsNTRC (diamonds), 0.5 μM NTR domain truncated polypeptide (circles), or 1.0 μM Trx domain truncated polypeptide (triangles) in 100 mM potassium phosphate buffer, pH 7.0, 2 mM EDTA, 5 mM DTNB, and 150 μM NADPH. A negative control in the absence of enzymes was performed (solid line) for both panels. Assays were performed at least three times with similar results, and representative results are shown.
2-Cys Prx from *Anabaena* (Table I). Therefore, these results underscore the similarity of the rice and *Anabaena* NTRC. Furthermore, the results display a clear difference between the 2-Cys Prx from *Anabaena*, which behaves as a plant enzyme, and the *Synechocystis* 2-Cys Prx, which shows no reactivity with NTRC from either source.

To further characterize the biochemical properties of the *Anabaena* NTRC, mutant variants of the enzyme were produced by a Cys-to-Ser mutation at the active site of both domains: C170S mutant in the NTR domain, and C411S mutant in the Trx domain. Neither of these mutants showed significant activity when assayed in the presence of the *Anabaena* 2-Cys Prx (Fig. 4). In addition, the wild-type *AnabNTRC* showed almost negligible activity when assayed with NADH as electron donor (Fig. 4).

**The *Synechocystis* 2-Cys Prx Quaternary Structure Is Different from That of *Anabaena* and Plant Enzymes**

Finally, the quaternary structure of the components of the NTRC-2-Cys Prx system was analyzed by exclusion gel chromatography. In contrast to NTRC from rice, which oligomerized in the absence of NADPH (Pérez-Ruiz et al., 2009), the *Anabaena* enzyme eluted as a dimer regardless of the presence of NADPH (Fig. 5A). Concerning 2-Cys Prxs, the enzyme from *Anabaena* showed an elution profile almost identical to the rice 2-Cys Prx, the most abundant form of the protein eluting at a volume consistent with an octamer or a decamer (Fig. 5B). In contrast, the elution profile of the *Synechocystis* 2-Cys Prx was remarkably different. The enzyme eluted predominantly as a dimer and showed different intermediary forms, including the decamer, all of which were present in lower amounts. Thus, despite the high sequence similarity between *Anabaena* and *Synechocystis* 2-Cys Prxs (Pascual et al., 2010), both the quaternary structure and the reactivity with NTRC indicate clearly different properties of these enzymes.

Structure modeling of the 2-Cys Prxs from *Synechocystis* and *Anabaena* predicted a very similar structure for both enzymes (Fig. 6). However, there is one significant difference affecting the location of the peroxidatic Cys residue in the reduced monomeric form, which is predicted to be more exposed in the *Anabaena* than in the *Synechocystis* 2-Cys Prx (Fig. 6). Interestingly, the model of the rice 2-Cys Prx predicts a structure highly similar to the *Anabaena* enzyme, with the peroxidatic Cys residue more exposed. In contrast, the structure deduced for the 2-Cys Prx from *S. typhimurium*, a prokaryotic enzyme insensitive to overoxidation (Wood et al., 2003a), predicted a more buried position of the peroxidatic Cys residue, resembling the structure of the *Synechocystis* enzyme (Fig. 6). Therefore, the modeling of the 2-Cys Prxs from these different sources suggests that the structural determinants around the peroxidatic Cys may be critical to the

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**Table 1.** *Kinetic parameters of the interaction of Anabaena NTRC with 2-Cys Prx from different sources*

Reactions were performed at a fixed concentration of AnabNTRC (2 μM) and variable concentrations of the 2-Cys Prxs in the presence of 0.25 mM NADPH. Data are means ± SD of three determinations.

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<th>Source</th>
<th><em>V</em><del>max</del> (μmol min⁻¹)</th>
<th><em>K</em><del>m</del> (μM)</th>
<th><em>k</em><del>cat</del> (s⁻¹)</th>
<th><em>K</em><del>cat</del>/<em>K</em><del>m</del> (μM⁻¹ s⁻¹)</th>
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<td>Anabaena 2-Cys Prx</td>
<td>169 ± 6.4</td>
<td>2.84 ± 0.3</td>
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<td>Rice 2-Cys Prx</td>
<td>170.1 ± 6.98</td>
<td>7.9 ± 0.4</td>
<td>1.42 ± 0.06</td>
<td>0.18</td>
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<tr>
<td>Arabidopsis 2-Cys Prx A</td>
<td>138 ± 2.9</td>
<td>5.9 ± 0.4</td>
<td>1.15 ± 0.1</td>
<td>0.19</td>
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<tr>
<td>Arabidopsis 2-Cys Prx B</td>
<td>114.3 ± 0.7</td>
<td>6.3 ± 0.2</td>
<td>0.95 ± 0.007</td>
<td>0.15</td>
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properties of these enzymes, apart from previously recognized structural features located at the C terminus (Wood et al., 2003a).

Expression of NTRC from *Anabaena* in the Arabidopsis NTRC Knockout Mutant

As described above, NTRC from *Anabaena* is remarkably similar to the plant enzyme, a notion further supported by the high efficiency of the cyanobacterial enzyme to reduce 2-Cys Prxs of plant origin in vitro. These results prompted us to analyze whether the cyanobacterial NTRC is able to carry out the functions of the plant enzyme in vivo. To test this possibility, the coding sequence of the *Anabaena* NTRC was fused to the putative chloroplast transit peptide of the enzyme from Arabidopsis to target the expressed protein to the chloroplast. The resulting gene was expressed in the Arabidopsis NTRC knockout mutant (*ntrc*) and wild-type plants under the control of the cauliflower mosaic virus 35S promoter. Northern-blot analysis revealed a high expression of the transgene in the wild-type background (Supplemental Fig. S1, WT_AnabNTRC lines) but a much lower expression in the *ntrc* mutant plants (Supplemental Fig. S1, *ntrc*_AnabNTRC lines).

Of the transgenic lines obtained, WT_AnabNTRC lines 3.7.1.3 and 3.7.3.2 and *ntrc*_AnabNTRC lines 3.3.6.1 and 3.3.6.2 were chosen for further analysis. For comparison, transgenic plants previously described (Pérez-Ruiz et al., 2006) expressing the wild-type enzyme from Arabidopsis under the control of the 35S promoter in the wild type (WT_AtNTRC) and the *ntrc* mutant background (*ntrc*_AtNTRC) were included in these studies.

The content of *Anabaena* NTRC in the transgenic plants was examined by western-blot analysis of stromal extracts from isolated chloroplasts probed with anti-NTRC antibodies raised against the rice and the *Anabaena* enzymes (Fig. 7, A and B). As expected, the anti-OsNTRC antibody showed the presence of the endogenous enzyme in stromal fractions from the wild type, transgenic plants in the wild-type background, and the transgenic line (*ntrc*_AtNTRC) expressing the plant enzyme in the mutant background (Fig. 7A). However, this antibody failed to detect the cyanobacterial enzyme in any of the transgenic lines in the mutant background and only detected the *Anabaena* enzyme, with an electrophoretic mobility reflecting a lower $M_r$ in the WT_AnabNTRC line 3.7.1.3 (Fig. 7A), in agreement with the high content of transcripts shown by the northern-blot analysis (Supplemental Fig. S1). The failure of the anti-OsNTRC antibody to detect AnabNTRC in the transgenic lines was not surprising, since this antibody cross-reacted poorly with recombinant purified AnabNTRC (Fig. 1C). To overcome this problem, western blots were probed with an antibody that detected the *Anabaena* enzyme (Fig. 7B). This antibody showed the presence of AnabNTRC in the transgenic lines generated either in the wild type or the *ntrc* mutant background, although the level of AnabNTRC in the *ntrc* background

Figure 4. Effect of mutation of the *Anabaena* NTRC active sites. The activity of the NTRC-2-Cys Prx system was assayed as oxidation of NAD(P)H in a reaction mixture containing 100 mM potassium phosphate buffer, pH 7.0, 2 mM EDTA, 0.5 mM hydrogen peroxide, 8 $\mu$M Anabaena 2-Cys Prx, and 2 $\mu$M wild-type AnabNTRC supplemented with 0.25 mM NADPH (black squares) or 0.25 mM NADH (white squares). The effect of mutations at the active sites of the NTR and Trx domains of the Anabaena NTRC was assayed replacing the wild-type enzyme by mutants AnabNTRC (C170S) (black triangles) and AnabNTRC (C411S) (white triangles), respectively. Assays were performed at least three times with similar results, and representative results are shown.

Figure 5. Analysis of the oligomeric state of AnabNTRC and 2-Cys Prx from rice and cyanobacteria. Purified His-tagged AnabNTRC (0.5 mg; A) and purified His-tagged 2-Cys Prx (0.5 mg; B) from rice (thin line), *Anabaena* (dotted line), and *Synechocystis* (thick line) were subjected to Superdex 200 gel filtration chromatography in 20 mM potassium phosphate buffer, pH 7.4, 150 mM NaCl. Molecular mass markers, in kD, are indicated.
In the transgenic lines expressing the cyanobacterial NTRC, the content of chloroplasts in the Arabidopsis plant and cyanobacterial NTRCs was not fully recovered in the wild-type plants. In agreement with the lower content of photosynthetic pigments, chlorophyll and carotenoids, which are reduced in the wild-type plants (Fig. 8A, lines 3.3.6.1 and 3.3.6.2), these plants showed a partial but significant recovery of the wild-type background (Fig. 8B). Moreover, the content of photosynthetic pigments, chlorophyll and carotenoids, which are reduced in the wild-type plants, were grown under a short-day photoperiod, which causes more severe phenotypic effects in the Arabidopsis ntrc background, although the content of the enzyme is lower than in wild-type plants. In agreement with the lower content of cyanobacterial NTRC, the redox status of the chloroplast 2-Cys Prxs was not fully recovered in the transgenic lines expressing the cyanobacterial NTRC in the ntrc background, as compared with wild-type plants and the transgenic lines expressing the plant enzyme or the cyanobacterial enzyme in the wild-type background (Fig. 7C).

To test whether the cyanobacterial enzyme complements the phenotypic effects caused by the deficiency of NTRC, transgenic plants were grown under a short-day photoperiod, which causes more severe phenotypic effects in the ntrc mutant (Pérez-Ruiz et al., 2006; Lepistó et al., 2009). Despite the low content of cyanobacterial NTRC in the ntrc background transgenic plants (Fig. 8A, lines 3.3.6.1 and 3.3.6.2), these plants showed a partial but significant recovery of the wild-type phenotype, which was confirmed by the fresh weight of the rosette leaves (Fig. 8B). Moreover, the content of photosynthetic pigments, chlorophyll and carotenoids, which are reduced in the ntrc mutant, were fully recovered (Fig. 8, C and D). These results show that the cyanobacterial NTRC is able to carry out, at least partially, the functions of the plant enzyme in vivo.

**DISCUSSION**

NTRC is an enzyme exclusive for photosynthetic organisms encoded by a single gene in all plant and algal genomes so far sequenced. However, it is found in some, but not all, cyanobacteria (Serrato et al., 2004; Pascual et al., 2010). Previously reported analysis showed the close phylogenetic relationship between plant and cyanobacterial NTRCs, thus suggesting a cyanobacterial origin for the plant enzyme (Serrato et al., 2004; Alkhalfioui et al., 2007). If this was the case, a high similarity between NTRC from plants and cyanobacteria should be expected. However, a comparison between these enzymes has hitherto not been possible due to the limited knowledge of cyanobacterial NTRC. The objective of this work was to perform a biochemical characterization of a cyanobacterial NTRC, from *Anabaena* sp. FCC 7120, and to carry out a comparative analysis with a plant enzyme.

The truncated polypeptide containing the NTR domain of the cyanobacterial enzyme was efficiently detected with an anti-NTR antibody raised against NTRB from wheat (Serrato et al., 2002), which showed a similar cross-reactivity with the NTR domain of the plant enzyme (Fig. 1B; Serrato et al., 2004). Similarly, the anti-OsNTRC antibody, which was raised against the Trx domain of the rice enzyme, detected the truncated polypeptide containing the Trx domain of the *Anabaena* enzyme. Therefore, the *Anabaena* NTRC, as the rice enzyme, may be considered as a functional NTR with a joint Trx domain at the C terminus. However, this antibody showed a poor detection of the full-length NTRC from *Anabaena* as compared with the rice enzyme (Fig. 1C). This result suggests a somehow different conformation of AnabNTRC limiting the cross-reactivity with the antibody. In this regard, it should be noted that NTRC from *Anabaena* is a homodimer regardless of the reducing conditions (Fig. 5A). This feature constitutes a remarkable difference with respect to the enzyme from plants, which is a homodimer in its catalytically active form (Pérez-Ruiz and Cejudo, 2009) but shows a high tendency to aggregate under oxidizing conditions (Pérez-Ruiz et al., 2009). The examination of the amino acid sequence of the plant enzymes (Serrato et al., 2004) reveals the presence of three Cys residues (positions 187, 499, and 525 in the Arabidopsis enzyme) that are absent from the *Anabaena* NTRC. To determine whether these Cys residues play any role in the redox-sensitive tendency of the plant enzyme to aggregate requires further study.
Our results from analyses of the recombinant full-length enzyme, as well as truncated polypeptides containing either the NTR or the Trx domains of the *Anabaena* enzyme, clearly show that the cyanobacterial NTRC may be considered as a bimodular protein formed by two domains, both of which show the expected NTR and Trx activities (Fig. 2). Therefore, the cyanobacterial enzyme is highly similar to the eukaryotic counterparts, such as the enzymes from rice (Serrato et al., 2004) or the green alga *Chlorella* (Michida et al., 2007). It should be noted that the full-length *AnabNTRC* showed a higher rate of NADPH-dependent reduction of DTNB than the truncated NTR domain of the enzyme (Fig. 2B); therefore, the Trx domain, which has no NTR activity, does contribute to the NTR activity of the full-length enzyme, most probably because this domain is important for the dimeric conformation of NTRC. Indeed, when truncated NTR and Trx domains of rice NTRC were incubated together, the activity was much lower than that of the full-length enzyme (Pérez-Ruiz et al., 2006). Thus, both domains must be part of a single polypeptide chain to show full activity. This bimodular enzyme, encoded by a single gene, may be evolutionarily advantageous because of its higher catalytic efficiency as compared with the two-component system formed by separate NTR and Trx enzymes, and thus encoded by two genes, which will require coordinated expression.

To our knowledge, the only NTRC so far described from cyanobacteria is the *Thermosynechococcus elongatus* enzyme (Sueoka et al., 2009). In this thermophilic cyanobacterium, NTRC was identified as a component of an NAD(P)H oxidase complex induced by oxidative stress, which also contained 2-Cys Prx. This result indicates that the cyanobacterial enzyme might act as a reductant of 2-Cys Prx and, thus, may function as an antioxidant system, as initially proposed for the enzyme in plant chloroplasts. However, the NTRC from *T. elongatus* seems to have a reaction mechanism different from that of the plant enzyme, as suggested by the fact that a mutant variant at the active site of the Trx domain of this enzyme was active (Sueoka et al., 2009). In contrast with this result, the analysis of mutant variants at the active site of either the NTR or Trx domain of the *Anabaena* enzyme completely lost activity (Fig. 4), thus showing the same behavior as the plant enzyme. Moreover, whereas the *T. elongatus* NTRC was reported to oxidize NADH (Sueoka et al., 2009), the *Anabaena* NTRC shared with the plant enzyme its specificity for NADPH as a source of reducing power (Fig. 4), once again emphasizing its close relationship with the plant enzyme. Based on these results, we propose that the reaction mechanism of the *Anabaena* NTRC is very similar to that previously reported for the plant enzyme. The different properties of the *T. elongatus* enzyme might be indicative of the existence of other forms of NTRC in cyanobacteria, but the clarification of this question awaits the characterization of NTRC from additional cyanobacterial sources.

The kinetic analysis of the interaction of NTRC with 2-Cys Prxs revealed a high catalytic efficiency of the *Anabaena* NTRC as a reductant of 2-Cys Prxs from either cyanobacterial or plant origin (Table I). Surprisingly, neither the *Anabaena* nor the plant NTRC was able to reduce the 2-Cys Prx from *Synechocystis* (Fig. 3). Because *Synechocystis* 2-Cys Prx shows a dimeric conformation and a low tendency to form decamers, a possibility to be taken into account is that NTRC has affinity for decamers rather than for dimers. However, the rice NTRC was shown to be active with the dimeric form of the 2-Cys Prx (Pérez-Ruiz and Cejudo, 2009). Previous analyses have revealed important differences between the 2-Cys Prx from *Anabaena* and *Synechocystis* (i.e. the *Anabaena* enzyme is more sensitive to overoxidation and requires a lower concentration of DTT for its reduction in vitro; Pascual et al., 2010). Thus, the inability of the NTRCs from either cyanobacteria or plants to reduce the *Synechocystis* 2-Cys Prx underscores the different properties of this enzyme as compared with the plant or *Anabaena* counterpart. These results lend further support to the proposal that an ancestral cyanobacterium resembling the modern
Anabaena harbored the original components of the chloroplast hydrogen peroxide detoxification system formed by NTRC, 2-Cys Prx and sulfiredoxin (Deusch et al., 2008; Pascual et al., 2010), and are in agreement with additional phylogenetic analyses of 2-Cys Prxs from cyanobacteria, algae, and plants (Baier and Dietz, 1997; Pitsch et al., 2010). The inability of NTRC to reduce the Synechocystis 2-Cys Prx does not have physiological implications, since this cyanobacterium lacks NTRC. Moreover, it was established that 2-Cys Prx reduction in this cyanobacterium may be catalyzed by the simple-module Trxs m, x, and y (Pérez-Pérez et al., 2009). Despite the high sequence similarity (75% identity) of the 2-Cys Prxs from Anabaena and Synechocystis (Pascual et al., 2010), modeling of their tridimensional structure revealed a remarkable difference between these enzymes affecting the peroxidatic Cys residue, which is predicted to be more exposed to the protein surface in the reduced monomeric form of the Anabaena enzyme (Fig. 6). Interestingly, the model of the Anabaena 2-Cys Prx predicted a high similarity to the rice homolog, whereas the Synechocystis enzyme was predicted to be similar to the prokaryotic enzyme from Salmonella. In agreement with this prediction, the Anabaena and rice 2-Cys Prxs show an almost identical quaternary structure, the enzyme being detected predominantly in oligomeric form (Fig. 5B). The oligomer-dimer transition allows the switch between the two activities of this enzyme: the low-molecular-weight form functions as a peroxidase, the high-molecular-weight form as a chaperone (Jang et al., 2004). Interestingly, the Synechocystis enzyme has a rather poor capacity to oligomerize (Fig. 5B), suggesting that this enzyme is unable to switch between peroxidase and chaperone activity. The crystallization and structure analysis of the 2-Cys Prxs from Anabaena and Synechocystis might help clarify the molecular evolution of these enzymes, which have been proposed to play an important function in signaling in eukaryotic organisms (Wood et al., 2003a; Dietz et al., 2006; Woo et al., 2010).

The similar biochemical properties of the Anabaena and plant NTRCs suggested the possibility that the Anabaena NTRC might carry out the functions of the plant enzyme in vivo. This was addressed by the expression of the Anabaena NTRC in the Arabidopsis ntrc knockout mutant. Although the cyanobacterial enzyme was correctly targeted to the chloroplast in the transgenic plants, for unknown reasons, the transgene was poorly expressed in the ntrc mutant background, in contrast to its high expression in the wild-type background (Supplemental Fig. S1). In agreement with the low expression of the transgene, the content of the Anabaena NTRC in the transgenic plants, based on western-blot analysis with either anti-OsNTRC or anti-AnabNTRC antibody, was lower than that of the endogenous enzyme in wild-type plants (Fig. 7, A and B). Nevertheless, the transgenic plants showed a partial recovery of the redox status of the 2-Cys Prxs as compared with the NTRC knockout plants (Fig. 7C), which is indicative of the activity of the cyanobacterial enzyme in the context of the plant chloroplast. It was previously proposed that NTRC exerts functions associated with its ability to reduce 2-Cys Prx (Pulido et al., 2010), including the protection against oxidative stress of the enzyme magnesium-protoporphyrin...
monomethylcysteine cyclase, which is involved in the synthesis of chlorophylls (Stenbaek et al., 2008). The recovery of the content of photosynthetic pigments in the transgenic plants (Fig. 8, C and D) suggests that the capacity of the cyanobacterial NTRC to reduce the Arabidopsis 2-Cys Prxs, although incomplete, is sufficient to complement this function. However, the transgenic plants did not display a fully recovered phenotype, as shown by the reduced fresh weight and the reversed chlorophylls (Stenbaek et al., 2008). The putative signal peptide of the NTRC cDNA from Arabidopsis (78 N-terminal residues) was amplified by PCR using primers derived from the transgenic plants. The additional functions of NTRC in plants, unrelated to the cyanobacterial enzyme. However, this might also be true, at least in part, to the lower content of this enzyme in the transgenic plants. The additional functions of plastid NTRC include redox regulation of starch synthesis (Michalska et al., 2009) or synthesis of aromatic amino acids and auxin (Lepisto et al., 2009), whose inhibition is expected to reverse the growth phenotype, as shown by the reduced growth and lower fresh weight of leaves as compared with the wild-type plants (Fig. 8, A and B). This result suggests that additional functions of NTRC in plants, unrelated to 2-Cys Prx reduction, might not be efficiently performed by the cyanobacterial enzyme. However, this might also be true, at least in part, to the lower content of this enzyme in the transgenic plants. The additional functions of plastid NTRC include redox regulation of starch synthesis (Michalska et al., 2009) or synthesis of amino acids and auxin (Lepisto et al., 2009), whose inhibition is expected to decrease the growth of leaves as compared with the wild-type plants (Fig. 8, A and B). This result suggests that additional functions of NTRC in plants, unrelated to 2-Cys Prx reduction, might not be efficiently performed by the cyanobacterial enzyme. However, this might also be true, at least in part, to the lower content of this enzyme in the transgenic plants.

MATERIALS AND METHODS

Plant Material

*Anabaena* (Anab*disopsis* *italiana* ecotype Columbia) wild type and the previously reported NTRC knockout mutant, T-DNA insertion line SALK_012208 (Serrato et al., 2004), were grown in soil supplemented with Hoagland medium in culture chambers. Plants were grown under a short-day photo period of 14 h of darkness at 20°C/10 h of light at 22°C. The light intensity was set at 140 μmol m⁻² s⁻¹. For production of Arabidopsis transgenic lines, the gene encoding NTRC of *Anabaena* sp. PCC 7120 (open reading frame all0737) was amplified from genomic DNA by PCR using the oligonucleotides *AnaNTRC* (forward, 5′-GAGATTGCACAAGCCG-3′) and AnaNTRC2 (5′-CCCTCGACCTTAAAGATTACC-3′), which added Xhol and SalI restriction sites (underlined) at the 5′ and 3′ ends, respectively. The putative signal peptide of the NTRC cDNA from Arabidopsis (78 N-terminal amino acids) was directly produced by PCR using primers as template the full-length NTRC cDNA clone from Arabidopsis (DNA stock no. U-14278), obtained from the Arabidopsis Biological Resource Center, with oligonucleotides 5′-TACCATCGCTGCTCTTC-3′ and 5′-AGAATCTGACTGCGTCCACCTGAA-3′, which added KpnI and XhoI restriction sites (underlined) at the 5′ and 3′ ends, respectively. Both fragments were digested, ligated, and cloned into the pGEMt vector (Promega), producing a 1.76-kb fragment, which was sequenced in both strands. The construct was then inserted into the binary vector pBB-A7 (Becker, 1990) and integrated into the Arabidopsis wild type and T-DNA insertion mutant SALK_012208 (ntc) by Agrobacterium tumefaciens (C58pMP90)-mediated transformation using the floral dip method (Clough and Bent, 1998). Transformants were then introduced into plates with Murashige and Skoog medium supplemented with 25 mg L⁻¹ hygromycin. Transgenic lines with a single integration, and homozygous for the transgene, were selected for further analysis by scoring for resistance to hygromycin. Mutant lines were checked by sequencing the final constructs. These plasmids were then introduced into *E. coli* BL21 (DE3), and the expression and purification of the recombinant proteins were performed as described for the wild-type enzyme.

NTR, Trx, and Prx Activity Assays

Prx activity was determined as oxidation of NADPH following the A340 in a reaction mixture containing 100 mM potassium 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. Trx activity was determined by the DTT-dependent reduction of insulin as described by Serrato et al. (2001). The reaction mixture contained 100 mM phosphate buffer, pH 7.0, 2 mM EDTA, 0.5 mM MgCl₂, 1 mM bovine insulin, and 2 μM purified enzymes. The reaction was initiated by the addition of 0.5 mM DTT, and the increase in A290 was monitored. NTR activity was determined by the reduction of DTNB according to the method described by Holmgren and Björnden (1985). The reaction was performed in 100 mM potassium phosphate buffer, pH 7.0, 2 mM EDTA, 5 mM DTNB, 150 μM NADPH, and purified enzymes at the concentrations indicated in the figure legends. The reduction of DTNB was monitored by the increase in A412.

Determination of Photosynthetic Pigments

Photosynthetic pigments (total chlorophyll and carotenoids) were extracted from leaf discs from plants that were grown for 52 d under short-day conditions with 100% methanol, and the content was determined according to Lichtenthaler and Wellburn (1985).

Gel Filtration Chromatography

The oligomeric state of the recombinant proteins was analyzed by gel filtration chromatography in Superdex 200 prep grade columns (Amersham Biosciences). The chromatography was performed with 20 mM potassium phosphate buffer, pH 7.4, supplemented with 0.15 M NaCl at a constant flow rate of 0.5 mL/min.
rate of 0.5 to 1 mL min⁻¹. The elution profile was monitored at 280 nm, and proteins used as standards (Sigma Chemical) were thyroglobulin (669 kD), apoferritin (443 kD), β-amylase (200 kD), alcohol dehydrogenase (150 kD), bovine serum albumin (67 kD), and carbonic anhydrase (29 kD).

Isolation of Intact Chloroplasts
Arabidopsis chloroplasts were isolated from approximately 10 g of leaves using the chloroplast isolation kit (Sigma Chemical). Leaves were homogenized with a mixer in 80 mL of ice-cold chloroplast isolation buffer, provided by the manufacturer, supplemented with 50 mM ascorbic acid. Homogenates were then filtered through two layers of nylon mesh (20 µm) and centrifuged for 7 min at 1,000g. Chloroplasts were purified by centrifugation on a 40% to 80% Percoll gradient, washed with 3 volumes of chloroplast isolation buffer, and lysed by hypotonic shock by resuspension in 62.5 mM Tris-HCl, pH 7.5, 2 mM MgCl₂. After centrifugation at 14,000g, the supernatant was analyzed as the stromal soluble fraction.

SDS-PAGE and Western-Blot Analysis
Proteins were fractionated by SDS-PAGE (10%–12% polyacrylamide gels) and electrotransferred onto nitrocellulose sheets, which were then probed with the following antibodies: anti-OsNTRC (Serrato et al., 2004), anti-NTRB (Serrato et al., 2002), or anti 2-Cys Prx from rice (M. Finkemeier I, T. G. F. Buchanan, B. J. Cejudo, F. J. Cejudo). After centrifugation at 14,000g, the supernatant was used as the stromal soluble fraction.

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

Supplemental Figure S1. Northern-blot analysis of NTRC transcript content in Arabidopsis transgenic lines.

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Pascual et al.

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