A Novel NADPH Thioredoxin Reductase, Localized in the Chloroplast, Which Deficiency Causes Hypersensitivity to Abiotic Stress in Arabidopsis thaliana*

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Plants contain three thioredoxin systems. Chloroplast thioredoxins are reduced by ferredoxin-thioredoxin reductase, whereas the cytosolic and mitochondrial thioredoxins are reduced by NADPH thioredoxin reductase (NTR). There is high similarity among NTRs from plants, lower eukaryotes, and bacteria, which are different from mammal NTR. Here we describe the OsNTRC gene from rice encoding a novel NTR with a thioredoxin-like domain at the C terminus, hence, a putative NTR/thioredoxin system in a single polypeptide. Orthologous genes were found in other plants and cyanobacteria, but not in bacteria, yeast, or mammals. Full-length OsNTRC and constructs with truncated NTR and thioredoxin domains were expressed in Escherichia coli as His-tagged polypeptides, and a polyclonal antibody specifically cross-reacting with the OsNTRC enzyme was raised. An in vitro activity assay showed that OsNTRC is a bifunctional enzyme with both NTR and thioredoxin activity but is not an NTR/thioredoxin system. Although the OsNTRC gene was expressed in roots and shoots of rice seedlings, the protein was exclusively found in shoots and mature leaves. Moreover, fractionation experiments showed that OsNTRC is localized to the chloroplast. An Arabidopsis NTRC knockout mutant showed growth inhibition and hypersensitivity to methyl viologen, drought, and salt stress. These results suggest that the NTRC gene is involved in plant protection against oxidative stress.

Thioredoxins are small 12–13-kDa proteins with a redox-active disulfide bridge and are widely distributed in all types of organisms (1). Because of their disulfide/dithiol interchange activity, thioredoxins interact with target proteins and are involved in the regulation of a large number of cellular processes (2). Plants contain more types of thioredoxin systems than any other organism. The cytosolic system in plants is composed of type f thioredoxins reduced by NADPH in a reaction catalyzed by NADPH thioredoxin reductase (NTR)1 (1). In addition, plants contain other thioredoxin systems localized in organelles. In chloroplasts, the thioredoxin system includes type f, m, and x thioredoxins (3), which are reduced by ferredoxin in a reaction catalyzed by ferredoxin thioredoxin reductase (1). This system is involved in light/dark regulation of chloroplast enzymes (1, 4–6) and in oxidative stress responses (7, 8). A specific mitochondrial thioredoxin system has been described in plants (9) and other organisms (10–12). The plant mitochondrial system is formed by a novel type of thioredoxin (type o), which is reduced by an NADPH-dependent thioredoxin reductase similar to the cytosolic enzyme (9).

Although NTR is universally distributed from bacteria to mammals, two forms of this enzyme have evolved (13): (i) a low molecular mass NTR formed by a homodimer of 35-kDa subunits that is found in bacteria and yeast and is very similar to the plant enzyme (14–16) and (ii) the mammalian NTR, which is also homodimer formed by subunits of 55 kDa with an unusual selenocysteine as the penultimate residue at the C terminus (17).

Taking advantage of the recently sequenced rice genome, we have identified the OsNTRC gene encoding a novel NTR with an extension at the C terminus containing a putative thioredoxin active site. In this paper we report on the cloning of this gene, its expression in Escherichia coli, and the biochemical characterization of the purified recombinant protein as well as truncated polypeptides containing the NTR and Trx domains of the enzyme, showing that it is a bifunctional enzyme with both NTR and thioredoxin activity but not an NTR/thioredoxin system when assayed with insulin as the substrate. Surprisingly, this novel NTR is localized in chloroplasts and, to our knowledge, is the first description of a chloroplast-localized NTR. The identification and characterization of an NTRC knockout mutant of Arabidopsis suggest that this novel NTR is involved in protection against oxidative stress.

EXPERIMENTAL PROCEDURES

Plant Material—Rice (Oryza sativa L. ssp. japonica cv. Nipponbare) grains were sterilized and germinated at 25 °C on filter paper soaked with water. Chloroplasts were isolated as described in (18) from rice leaves of 36-day-old plants. Arabidopsis wild-type and the T-DNA insertion mutant SALK_012208 (19) were grown as reported previously (20). The T-DNA homozygous line was selected after PCR analysis with

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ582621.

1 The abbreviations used are: NTR, NADPH thioredoxin reductase; OsNTR, Oryza sativa NTR; AtNTR, Arabidopsis thaliana NTR; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Trx, thioredoxin.
A Chloroplast-localized NTR

oligonucleotides in the T-DNA (5'-TGGTTCAAGTGGAGGCCTC-3') and the AtNTRC gene (5'-TCACAACTGTTGCCG GCG-3' and 5'-TCTGGTGAAATGGCAGA-3').

Isolation of OsNTRC cDNA—Total RNA (1 μg) from 5-day-old rice seedling shoots was reverse transcribed in the presence of oligo(dT) and 200 units of reverse transcriptase (Invitrogen). An aliquot (1 μl) was used as the template in a PCR reaction with the oligonucleotides 5'-CACCCCTCTCCTCT-3' and 5'-GGTGCAGAACGACGACAA-3'. A 1.5-kb fragment was obtained, cloned in a pGEMt vector (Promega), and sequenced in both strands. The OsNTRC gene sequence was deposited at the EMBL data bank with accession number AJ588261.

Expression and Purification of Recombinant Proteins and the Production of Anti-OsNTR Polyclonal Antibodies—Rice OsNTRC was expressed in E. coli as a His-tagged polypeptide. The coding sequence, excluding the putative signal peptide (36 residues at the N terminus, Fig. 1) was amplified from the full-length cDNA with the oligonucleotides 5'-GAGAGAGCTCGATCTTGGCAAGGGAG-3' and 5'-GAGAAA GCGTTCATTGTGGTACTG-3', which added a SalI site at the 5'-end and a HindIII site at the 3'-end (underlined). The PCR fragment was digested with SalI and HindIII, subcloned into the pQE-30 expression vector (Qiagen), and introduced into E. coli XLI-Blue. The NTR domain of OsNTRC (residues 37 to 359, Fig. 1), was amplified with the oligonucleotides 5'-GAGAGAGCTCGATTTCAACAAAGGGAG-3' and 5'-GAA GGAACTT TTCAACAAAGGGAG-3'. The thioredoxin domain (residues 360 to 488, Fig. 1), was amplified with oligonucleotides 5'-GAG AAGCGTCCACCAACCGGTGGCGG-3' and 5'-GAGAAAGCGTTTGCATGTTACTG-3'. Both of these truncated polypeptides were produced in E. coli as described for full-length OsNTRC. Overexpressed polypeptides were purified by Cu²⁺ affinity chromatography in pre-packed Hi-Trap affinity columns (Amersham Biosciences). Anti-OsNTRC antibodies were raised by immunizing rabbits with purified His-tagged polypeptide.

Functional Characterization of the OsNTRC Polypeptide—To analyze the biochemical properties of the polypeptide encoded by OsNTRC, it was expressed in E. coli without the 36 amino acids at the N terminus (Fig. 3A). Truncated polypeptides containing the NTR- or Trx-like domains of OsNTRC were also produced (Fig. 3A) and purified by Cu²⁺ chromatography (Fig. 3B, lanes 2–4). For comparison, purified His-tagged NTRB (16), the cytosolic form, and TRXαA (22) from wheat were also included (Fig. 3B, lanes 1 and 5, respectively). Western blots of the gel shown in Fig. 3B were probed with anti-NTR antibodies (16), which detected NTR-D (Fig. 3C, lane 3) more efficiently than the full-length OsNTRC (Fig. 3C, lane 2). To obtain polyclonal antibodies specifically cross-reacting with OsNTRC, rabbits were immunized with the purified Trx-D polypeptide found only in OsNTRC. As expected, this antibody specifically cross-reacted with Trx-D (Fig. 3D, lane 4) and OsNTRC (Fig. 3D, lane 2). Furthermore, an antibody against wheat thioredoxin h did not cross-react with either Trx-D or OsNTRC (Fig. 3E).

Because OsNTRC contains both NTR and Trx-like domains, we tested whether this single polypeptide functions as an NTR/thioredoxin system. OsNTRC catalyzes the dithiothreitol-dependent reduction of insulin, but no activity was detected in the presence of NADPH (Fig. 4A). The thioredoxin activity of OsNTRC was due to the Trx-like domain as shown by the insulin reduction activity of the isolated Trx-D polypeptide (Fig. 4B). Both OsNTRC and the NTR domain showed NADPH-dependent reduction of DTNB (Fig. 4C). The Trx-like domain does not affect the NTR activity of OsNTRC (Fig. 4C) and, as expected, did not show any NTR activity. These results show that OsNTRC is a bifunctional enzyme, showing NTR and Trx activity, but it is not an NTR/thioredoxin system when assayed with insulin as the substrate.

As for other NTRs, OsNTRC showed higher affinity for NADPH (Kₘ of 10.4 μM) than for NADH (Kₘ of 1.2 mM). We also tested whether the OsNTRC polypeptide was able to transfer electrons to the Trx-like module, Trx-D, to wheat TRXαA or to thioredoxins f and m from pea. None of them were substrates for OsNTRC (results not shown).

Expression Pattern of the OsNTRC Gene—Relative quantitative reverse transcription PCR was used to analyze the expression of OsNTRC and cytosolic OsNTRB in 4–7-day-old rice seedlings. OsNTRC and OsNTRB transcripts accumulated at similar amount in roots and shoots (Fig. 5A). A similar and invariable amount of the OsNTRC polypeptide was detected in both organs (Fig. 5B) in agreement with the presence of OsNTRC transcripts. Surprisingly, the OsNTRC polypeptide was detected exclusively in shoots (Fig. 5B), showing that NTRC is absent or of low abundance in roots despite the presence of OsNTRC transcripts in this organ.

The fact that the OsNTRC gene is exclusive to oxygenic photosynthetic organisms, along with the presence of the OsNTRC polypeptide in green tissues, suggested its chloroplast localization. To test this possibility, we performed fractionation experiments using chloroplasts purified from rice leaves. Western blot analysis showed that NTRC was more abundant in the chloroplast stroma, although it was also detected in the thylakoid fraction (Fig. 5C). Both the stroma and the thylakoid fractions were free of cytoplasmic or mitochondrial contamination as shown by the lack of signal when Western blots were probed with the anti-NTRC antibody (Fig. 5C).

Characterization of an Arabidopsis NTR Knock-out Mutant—A T-DNA insertion line of the Arabidopsis NTRC gene was identified in the SALK collection (19). PCR-based genotyping identified both heterozygous and homozygous lines for the T-DNA (Fig. 6A), and the homozygous line was further characterized. For unknown reasons, the anti-NTRC antibody detected the
rubulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) large
subunit from Arabidopsis (Fig. 6B, L). Nevertheless, the Western
blot showed the absence of NTRC in the mutant (Fig. 6B),
whereas it contained wild-type amounts of NTRB (Fig. 6B).
In rice seedlings, NTRC was not detected in roots (Fig. 6B,
R), showing that it is either absent or present in very low
amounts in roots. Seeds of the Arabidopsis NTRC
knock-out mutant germinated as efficiently as wild-type seeds,
but mutant plants showed smaller rosette leaves with a pale
green color (Fig. 6C) and clear growth inhibition, which were
evident early after flowering (Fig. 6D) and in adult plants
(Fig. 6E).

This phenotype suggested that standard growth conditions
caused stress to the NTRC-deficient mutant. Because NTRC
polypeptide is localized in the chloroplast, this effect might be
related to chloroplast protection against oxidative damage. To
test this possibility, 29-day-old wild-type and mutant plants
were sprayed with methyl viologen, a photo-oxidizing agent
that causes oxidative stress (26). NTRC-deficient plants
showed a much higher sensitivity to methyl viologen than did
wild-type plants (Fig. 6F), as well as more symptoms of leaf
senescence (Fig. 6F, arrow). Because other environmental
stresses cause oxidative damage, we tested the sensitivity of
the NTRC mutant to salt (Fig. 6G) and drought (Fig. 6H)
stress. Again, the effect of both stresses was more severe in the
mutant than in wild-type plants. For comparison, untreated
wild-type and mutant plants are also shown (Fig. 6I).

**DISCUSSION**

In this paper we describe a gene from rice and Arabidopsis
coding a novel NTR that is found exclusively in oxygenic

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**Fig. 1.** Sequence alignment of NTRs from rice, Arabidopsis, and Nostoc. Protein sequences were compared using the BioEdit sequence alignment editor program (23). The NTRs and their accession numbers, in parentheses, are OsNTRC (AJ582621), AtNTRA (NP_179334.3), AtNTRB (NP_195271.1), AtNTRC (NP_565954.1), and Nostoc (NP_484780). The sequence of OsNTRB was deduced from DNA sequences obtained from Torrey Mesa Research Institute (24) using the GeneScan program. Inverted triangles, FAD binding site; triangles, NADPH binding site; asterisks, Cys residues at the active site; box, putative active site at the thioredoxin domain.
FIG. 2. Phylogenetic tree of NTRs from different sources. The phylogenetic tree was constructed with full-length NTR amino acid sequences using the neighbor-joining method of the program ClustalX, version 1.8 (25). Bootstrap analysis was computed with 1,000 replicates. The NTRs and their accession numbers, in parentheses, are AtNTRA (NP_179334.3), AtNTRB (NP_195271.1), AtNTRC (NP_565954.1), OsNTRC (AJ582621), Schizosaccharomyces pombe (CAA17692), Saccharomyces cerevisiae (1, P29609; 2, AAA64747.1; and mitochondrial, P38816), Neurospora crassa (P51978), Penicillium chrysogenum (P43496), Zymomonas mobilis (AF124757), Haemophilus influenzae (P43788), Yersinia pestis (NP_404967), E. coli (P09625), Mycobacterium leprae (NP_302724), Neisseria meningitidis (NP_284253), Plasmodium falciparum (CA160574), Mus musculus (AAH13688), Homo sapiens (1, XP_049211; 2, AAH07489; 3, S66677; and 4, NP_006431), Nostoc sp. 7120 (NP_484387), Synechococcus sp. WH 8102 (NP_896790), Prochlorococcus marinus (NP_893287), Nostoc punctiforme (NP_00109021), and Thermosynechococcus elongatus (NP_682714).

FIG. 3. Purification and immunological characterization of His-tagged Os-NTRC and NTR and Trx domains. A, scheme showing the polypeptides expressed in E. coli with the corresponding molecular masses in kDa. B, Coomassie Brilliant Blue-stained SDS/PAGE gel loaded with 2 μg of purified wheat NTRB (lane 1), OsNTRC (lane 2), NTR-D (lane 3), Trx-D (lane 4), and wheat TRXhA (lane 5). C–E, replicates of this gel loaded with 50 ng of the purified proteins were probed with anti-NTRB (C), anti-NTRC (D), and anti-TRXhA (E).
photosynthetic organisms. In addition to sequence similarity data, several lines of evidence show that OsNTRC encodes an NTR. The deduced protein, expressed in E. coli, is a flavoenzyme (results not shown) detected by a polyclonal antibody against the cytosolic form of the NTRB enzyme from wheat (16) and shows NADPH-dependent DTNB reduction activity (Fig. 4A). The $K_m$ of the enzyme for NADPH (10.4 $\mu$M) is in the range of values reported for NTRs from different plant sources (14, 16) and, as for other NTRs described to date, it shows a higher affinity for NADPH than for NADH. However, OsNTRC is an unusual NTR due to the presence of a Trx-like domain at the C terminus, which raises the possibility that this enzyme might function as an NTR/thioredoxin system. We demonstrate that OsNTRC shows both NTR and thioredoxin activity due to the NTR and Trx-like domains of the protein; however, OsNTRC was unable to catalyze the NADPH-dependent reduction of insulin. Therefore, we conclude that OsNTRC is a bifunctional enzyme and not an NTR/thioredoxin system, at least under these assay conditions.

Our data show that OsNTRC or the truncated polypeptide containing the NTR domain was unable to transfer electrons to the thioredoxin-like domain of the protein. Furthermore, it was not able to reduce wheat TRX$\alpha$, E. coli thioredoxin, and thioredoxins f and m from pea. Therefore, despite the clear features summarized above indicating that the OsNTRC gene encodes an NTR, none of the plant thioredoxins analyzed are substrates for this enzyme.

According to accessible databases, this novel NTR seems to be exclusive to plants and cyanobacteria. Our phylogenetic analysis shows a close relationship of OsNTRC and AtNTRC with the cyanobacterial NTR genes. The only example of a hybrid protein with NTR and thioredoxin domains was identified in Mycobacterium leprae (27), and, surprisingly, this gene is grouped with the bacterial NTRs and not with NTRC genes (Fig. 2). This Mycobacterium gene seems exceptional, because other mycobacterial strains contain separate genes for NTR and Trx (27). In contrast with the rice OsNTRC protein, the hybrid mycobacterial protein functions as an NTR/thioredoxin system (28).

The analysis of the expression pattern of the OsNTRC gene revealed an unexpected result. The OsNTRC gene was expressed in roots and shoots from growing rice seedlings at the days after imbibition indicated (days 4–7), were analyzed by relative quantitative reverse transcription PCR with OsNTRC and OsNTRB gene-specific primers in the presence of 18S rRNA primers and competimers. B, protein (20 $\mu$g) extracted from rice seedling roots and shoots at the indicated days were subjected to SDS/PAGE, electrotransferred to nitrocellulose sheets, and probed with anti-NTR or anti-NTRC antibodies as indicated. Purified His-tagged NTRB (50 ng) from wheat and His-tagged OsNTRC (100 ng) were loaded. C, chloroplasts were isolated from leaves of 36-day-old rice plants. Protein extracts (10 $\mu$g) from leaves (le) or from stroma (st) and thylakoid fractions (th) were subjected to SDS/PAGE, electrotransferred to nitrocellulose sheets, and probed with anti-NTRC or anti-NTRB antibodies as indicated. Purified His-tagged NTRB (75 ng) from wheat and His-tagged OsNTRC (75 ng) were loaded.

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The analysis of the expression pattern of the OsNTRC gene revealed an unexpected result. The OsNTRC gene was expressed in roots and shoots from rice seedlings (Fig. 5A); however, the OsNTRC polypeptide was predominantly detected in photosynthetic tissues in both rice and Arabidopsis (Figs. 5B and 6B). Indeed, this polypeptide was localized in the chloroplast stroma and, to a lower level, in the thylakoid fraction (Fig. 5C). This finding implies that the extension of the protein at
the N terminus might function as a chloroplast-targeting peptide. Available prediction programs failed to identify this sequence as a chloroplast-targeting signal, but prediction is not always reliable based only on sequence (29). The absence or low abundance of OsNTRC in roots, despite the presence of the corresponding transcripts, suggests that either the transcript is not efficiently translated or that the protein is unstable; but the actual reason for the lack of detection of protein in non-photosynthetic tissues is not yet understood.

The predominant localization of this novel NTR in the chloroplast stroma and its high affinity for NADPH suggests that it may be involved in the use of reducing power from photosynthetic electron transport. Because the chloroplast thioredoxins involved in carbon assimilation are not substrates for NTRC, the possibility that NTRC is involved in redox control of Calvin cycle enzymes may be ruled out. Another likely possibility is that this NTR is involved in transferring reducing power for chloroplast protection against oxidative damage. Several components of the system for chloroplast protection against oxidative damage have been identified, such as the 2-Cys peroxiredoxin BAS1 (30–33). This system requires electrons that are donated by the thioredoxin CDSP32 (34), but to date it is not known how electrons of the photosynthetic flow are diverted to this protective system. The phenotype of the Arabidopsis NTRC knockout mutant suggests the involvement of NTRC in this protective system, but the molecular basis of this mechanism will require the identification of NTRC targets, which is now underway.

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A Chloroplast-localized NTR

43827

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