Unique properties of NADP-thioredoxin reductase C in legumes

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Abstract

NADP-thioredoxin reductases (NTRs) reduce thioredoxins (Trxs), using NADPH as a reductant, together constituting complete redox systems (NTS). Beside NTRA and NTRB targeted to both cytosol and mitochondria of plant cells, there is in chloroplasts an unusual NTR (NTRC) harbouring a Trx domain in a C-terminal extension, as recently reported in Oryza sativa. Although NTRC may constitute a complete NTS, it was described as a bifunctional enzyme. Because the gene is only present in photosynthetic organisms and the protein in green tissues, NTRC was thought to have a role restricted to photosynthetic cells. To determine whether NTRC from dicot plants is a bifunctional enzyme or a complete NTS, as well as to identify its putative target, NTRC from Medicago truncatula was cloned and NTRA was cloned for comparison. Here evidence is presented that MtNTRC (i) acts as an NTS and reduces dithiobisnitrobenzoate (DTNB) with a turnover (0.62 s\(^{-1}\)) similar to that measured with MtNTRA in the presence of a Trxh (0.81 s\(^{-1}\)); (ii) is able to use both NADPH (\(k_M=2.4\ \mu M\)) and NADH (\(k_M=11\ \mu M\)) as cofactors; (iii) efficiently reduces BAS1, a plastidial peroxiredoxin; and (iv) is expressed in both leaves and stems but unexpectedly is even more abundant in cotyledons from dry and germinating seeds. Because BAS1 is also present in both green tissues and seeds, NTRC/BAS1 may be involved in the scavenging of peroxides produced in green tissues during the day or the night and in seeds during germination. These results suggest different roles for NTRC in monocot and dicot plants.

Key words: BAS1, germination, Medicago truncatula, NADP-thioredoxin reductase C, plastids, seed.

Introduction

Thioredoxins (Trxs) are small, powerful disulphide reductases with two close and reactive cysteine residues in a conserved motif: WCG/PPC (Holmgren, 1985; Meyer et al., 2005). They play a post-translational regulatory role by reducing protein targets involved in an ever-increasing number of cellular processes, including metabolism (Calvin cycle and sulphur assimilation), gene expression, seed germination, cell proliferation, or apoptosis (Muller, 1995; Jacquot et al., 1997; Arrigo, 1999; Buchanan and Balmer, 2005). They are also involved in protection from oxidative damage by regeneration of peroxiredoxins and methionine sulfoxide reductases, allowing the detoxification of various peroxides and the repair of proteins (Chae et al., 1994; Arner and Holmgren, 2000). Trx isoforms constitute a particularly important protein family in plants since 22 genes have been detected in the fully sequenced genome of Arabidopsis thaliana (Meyer et al., 2005). In contrast, the genomes of Escherichia coli, Saccharomyces cerevisiae, and humans contain only 2–3 genes coding for a Trx. In plants, Trxs are included in three different redox systems: a chloroplastic ferredoxin–Trx system comprising Trxs f, m, x, or y, and two cytosolic and mitochondrial NADP-Trx systems (NTS). The NTS comprise the NADP-Trx reductases (NTRs) A and B that are highly identical in A. thaliana. Both enzymes have been shown to be localized in either the cytosol or mitochondria (Reichheld et al., 2005). They transfer electrons from NADPH to cytosolic Trxh and mitochondrial Trxs h and o. Plants also possess in chloroplasts an unusual NTR (NTRC) that exhibits a C-terminal extension containing a Trx domain (Serrato et al., 2004). They transfer electrons from NADPH to cytosolic Trxh and mitochondrial Trxs h and o. Plants also possess in chloroplasts an unusual NTR (NTRC) that exhibits a C-terminal extension containing a Trx domain (Serrato et al., 2004). This unusual NTR was also found in cyanobacteria. The deficiency of NTRC in A. thaliana causes severe growth inhibition and hypersensitivity to oxidative stress.

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Abbreviations: DTNB, dithiobisnitrobenzoate; DTT, dithiothreitol; EST, expressed sequence tag; NTR, NADP-thioredoxin reductase; PCR, polymerase chain reaction; Q-PCR, quantitative PCR; RT–PCR, reverse transcription–PCR; ROS, reactive oxygen species; TC, tentative consensus; Trx, thioredoxin.

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indicating a role in protection against oxidative damage (Serrato et al., 2004). The presence of the Trx domain suggests that NTRC may act as a complete NTS. However, the rice enzyme was shown to be a bifunctional protein rather than an NTS, i.e. both domains (NTR and Trx) acting independently (Serrato et al., 2004).

In order to determine whether NTRC from dicot plants is a bifunctional protein or a complete NTS, as well as to identify its putative target, the cDNA coding for NTRC was cloned in the model legume Medicago truncatula and a functional study was performed with the corresponding recombinant protein. An NTR of the A/B type was also cloned for comparison with NTRC. An analysis of the expression of both NTRs was finally performed.

Materials and methods

Biological materials

Surface-sterilized seeds of M. truncatula (genotype Paraggio, Seedco Australia Co-Operative Ltd, Australia) were imbibed on filter paper (Whatman no. 1) in a Petri dish (9 cm diameter) soaked with 3.5 ml of distilled water and allowed to germinate at 20 °C in the dark for 48 h. Seedlings were then transferred to soil, and plants were allowed to grow at 20 °C (16 h of light, 8 h of dark) with regular watering. Roots, stems, and leaves were harvested 13–20 d after the start of seed imbibition.

Rabbit antibodies raised against A. thaliana NTRB and O. sativa NTRC were gifts of Yves Meyer (Unité Mixte de Recherche 5096, Centre National de la Recherche Scientifique, University of Perpignan, France) and Javier Cejudo (Centro de Investigaciones Científicas, Sevilla, Spain), respectively. Recombinant BAS1 as well as antibodies raised against the protein were gifts of Stephane Cuine and Pascal Rey (CEA, Cadarache, France).

Extraction of RNA, cloning of cDNA, and overexpression of recombinant proteins

Total RNAs was extracted from 13-d-old leaves with the RNeasy plant kit (Qiagen) and reverse transcribed using the M-MLV reverse transcriptase (Promega) according to the manufacturer’s instructions. Then, coding regions corresponding to NTRs were amplified by polymerase chain reaction (PCR) with specific primers designed for the amplification of either the full-length coding regions or only the part encoding the putative mature proteins (NTRA+10 sens gac gac gac gac gac aag ATG TCT TCC GAC ACT TCT and NTRA+9 anti gac gac gac gac gac aag ATG ACT GAC ACC ACC ACC and NTRA_mat_sens gac gac gac gac gac aag ATG CTT ACT TAT CTG CTT TCG TGC ACT TCC TCC T and NTRA_mat_anti gac gac gac gac gac aag ATG AAT TAT TGC CTT CTC GAT GAA). The parts of the sequences of the primers that appear in lower case letters were added to allow the cloning of the PCR products into the pRSF2 plasmid of the ligation-independent Ek/LIC cloning kit (Novagen) according to the manufacturer’s instructions. This vector is engineered to express target proteins fused to an N-terminal His tag. PCR was performed using the proof reading KOD HiFi DNA polymerase (Novagen), and an annealing temperature of 52 °C, with a preliminary denaturation step of 4 min at 94 °C, followed by a set of 35 cycles (94 °C×1 min, 52 °C×1 min, and 72 °C×1 min) and then a final elongation step of 10 min at 72 °C. The PCR products were resolved in 1.4% agarose gels and the amplicons of the expected size were excised from the gels, purified with the Qiagel gel extraction kit (Qiagen), and inserted into the vector. Recombinant plasmids were introduced in Nova Blue E. coli for multiplication and sequencing (MWG-Biotech AG ; Ebersberg, Germany) before being transferred into BL21 (pLysS) cells for the production of proteins. Protein purification on Ni2+-chelating Sepharose (Amersham) was carried out according to the manufacturer’s instructions, after a prior purification step on Q-Sepharose for NTRA. In the case of NTRC, the affinity chromatography step was followed by a further purification step involving gel filtration on a Superdex 75 column (Pharmacia; 1.6×56 cm). The column was equilibrated and run in 20 mM potassium phosphate, 200 mM NaCl.

Native molecular weight determination

Native molecular weights of recombinant NTRs were determined by gel filtration on a Superdex 200 column (Pharmacia; 1.6×92 cm) carried out as above. The column was calibrated with thyroglobulin (669 kDa; for void volume determination), apoferritin (444 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa).

Enzymatic assays

NTR activity was measured using dithiobisnitrobenzoate (DTNB) or insulin reduction assays as previously described (Holmgren, 1979; Jacquot et al., 1995) in the presence of NADPH or NADH.

Peroxiredoxin activity of BAS1 was followed by the disappearance of NADPH at 340 nm in a reaction mix containing 20 mM potassium phosphate buffer pH 7.2, 0.16 mM NADPH, 1 mM H2O2, 1–2 μM NTR, and BAS1 varying in the range from 0 μM to 30 μM. In some experiments, NTRC was replaced by 2 μM NTRA+10 μM MtTrxh2, a Trxh from M. truncatula the preparation of which will be described elsewhere.

Quantitative-PCR

Total RNAs were extracted from embryo axes and cotyledons of dry or germinating seeds (imbibed for 14 h and 22 h, i.e. before and after radicle protrusion), roots, leaves, and stems from 13-d-old plants, and reverse transcribed as above after an RQ1 DNase (Promega) treatment. Specific forward and reverse primers for each NTR isoform (NTRA+10 sens AAC AGT CCG CCA AAT TCG G; NTRC-q-AAT AAA AAC CCG TGC AAT CAG A TGA A; NTRA+10 q-Anti CCT TTT TTC TGG TTC G; NTRC-q-Anti GCC CTT TGT TCG CTA CGT A) were designed. Quantitative PCR (Q-PCR) was performed in a total volume of 25 μl containing 2 μl of cDNAs, 0.3 μl of each primer, and 12.5 μl of 2× Sybr Green master mix (Applied; Courtaboeuf, France), with a preliminary step of 5 min at 94 °C, followed by 40 cycles of 94 °C×15 s and 60 °C×1 min (ABI Prism 7000 SDS; Applied Biosystems).

The expression of each gene was determined for each sample and was normalized using the Ms27 transcript expression (Bouton et al., 2005). The results are the average of triplicates of three independent reverse transcriptions.

Protein extraction

Soluble proteins were extracted from the same organs as above using 20 mM potassium acetate buffer, pH 4.5 supplemented with 1 mM phenylmethylsulphonyl fluoride and 1 mM EDTA (5–20 ml g⁻¹ of fresh weight). The resulting homogenate was centrifuged (40 000 g for 30 min at 4 °C) and the pellet was discarded.

SDS PAGE and western blot analyses

Protein extracts or recombinant proteins were resolved by SDS PAGE using 12% (w/v) acrylamide gels under non-reducing or
reducing [100 mM dithiothreitol (DTT)] conditions (Laemmli, 1970). At the end of electrophoresis, proteins were either stained in the gels with colloidal Coomassie blue or transferred onto nitrocellulose membranes (Schleicher & Schull, Dassel, Germany) for immunodetection as previously described (Duval et al., 2002). Membranes were probed with antibodies raised against NTRB from A. thaliana (1/33 000), NTRC from O. sativa (1/5000), or BAS1 from A. thaliana (1/5000). Immunodetection was performed using the alkaline phosphatase assay in the presence of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

Protein content determination

Protein contents of extracts were determined using the Bradford reagent and bovine serum albumin as a standard (Bradford, 1976). The concentrations of pure protein preparations were estimated using their εM at 280 nm.

Results

Identification of NTR sequences in M. truncatula databases

While the sequencing of the M. truncatula Jemalong genome is currently in progress—the release of the complete genome will only be effective in 2007—large public expressed sequence tag (EST) databases are already available (http://www.tigr.org/tdata/mtgi; http://www.medicago.org). MtGI from TIGR notably comprises 226 923 ESTs in 36 978 unique sequences at its last release (19 January 2005). A search in the M. truncatula EST databases allowed four putative tentative consensus sequences (TCs) encoding N- and C-terminal parts of NTRs to be identified (Table 1). These TCs were not overlapping and corresponded to two genes found in the genomic database (http://www.medicago.org). One of the gene sequences was complete; the encoded protein could therefore be easily deduced. Although the second gene sequence was incomplete, the known part overlapped with the two TCs, thus allowing the whole corresponding protein sequence to be deduced. The two protein sequences were aligned with Clustal W and then compared with those of other NTRs from higher plants and photosynthetic micro-organisms in a phylogenetic tree (Fig. 1). On the basis of primary sequence comparison, one of them is an NTR of the A/B type and the other is an NTR of the C type. The full-length gene sequence available in databases corresponds to the NTR of the A/B type. The gene is localized on M. truncatula chromosome 5 and has two exons like the two A. thaliana genes encoding NTRA and NTRB. The protein from M. truncatula, which comprises 376 amino acids, is seemingly more similar to AtNTRA (73% identity) than to AtNTRB (72%) and is thereby named MnNTRA. According to Predotar, chloroP, and TargetP, the putative localization of the M. truncatula protein is the mitochondrion. However, in A. thaliana, both NTRA and NTRB were shown to be targeted to either the cytosol or the mitochondrion, depending on two different sites of transcription initiation (Reichheld et al., 2005). The same process may thus occur in M. truncatula. The incomplete gene sequence present in M. truncatula databases corresponds to NTRC. The chromosomal localization of the gene is still unknown. The deduced protein comprises 514 amino acids and is named hereafter MnNTRC. MnNTRC has ~75% identity with NTRCs from A. thaliana and O. sativa.
and is predicted to be chloroplastic, as has been demonstrated for OsNTRC (Serrato et al., 2004).

**NTRC is expressed in green tissues and even more in cotyledons of dry and germinating seeds**

The pattern of expression of both NTRs was analysed at both the nucleic acid and protein levels in different organs: leaves, roots, stems, and seeds. The expression of NTRs at the nucleic acid level was followed by Q-PCR and compared with that of Msc27, a constitutive gene (Bouton et al., 2005). The results are expressed in arbitrary units corresponding to $2^{-\Delta C_T} \times 10^3$, because the level of NTRs is 1000-fold to 10 000-fold lower than that of the constitutive gene. Figure 2A shows that transcripts of MtNTRC are present in all the organs analysed, although at very different levels. Transcripts are abundant in photosynthetic organs such as leaves and stems. This result is consistent with a chloroplast localization of the protein (Serrato et al., 2004). However, transcripts of MtNTRC are also present in non-green tissues—in both the embryo axes and cotyledons of dry and germinating seeds. While its content remains the same in axes, MtNTRC is accumulated in cotyledons during germination: at 14 h and 22 h of imbibition (i.e. before and after radicle protrusion). It should be noted that *M. truncatula* has an epigeous germination and that cotyledons differentiate in the first leaves after germination. The expression of the messengers in the cotyledons may then prepare the organ to be photosynthetic. Alternatively, MtNTRC may have a role in seeds. On the other hand, MtNTRA is also expressed in roots, but to a lower extent.

In contrast, MtNTRA is almost constitutively expressed in all the organs examined, although its level is low in embryo axes from dry seeds. However, the messenger content increases in this part of the seed during germination to reach a level similar to that found in other organs.

The expression of NTRs at the protein level was analysed by western blotting (Fig. 2B). Antibodies raised against OsNTRC were used to follow the expression of MtNTRC. As expected, MtNTRC was found to be present in leaves and stems. However, surprisingly, the protein is also present in dry and germinating seeds. Although MtNTRC is barely detectable in axes, it is very abundant in cotyledons, even more abundant than in green tissues. During germination, the content of MtNTRC decreases in axes, such that the protein is not further detected at 22 h of imbibition. In contrast, MtNTRC content remains constant in cotyledons throughout this period. These results contrast with those obtained by Q-PCR showing a higher content of transcripts in leaves and stems than in seeds. On the other hand, MtNTRC is not detectable in roots, although low amounts of messenger were found in these organs.

To estimate the amounts of NTRC in the extracts, the signals corresponding to the extracts (on the left of Fig. 2B) were compared with those obtained under the same conditions with different amounts of recombinant NTRC (on the right); the production of recombinant NTRs is described below. The amount of NTRC in cotyledon extracts is $\sim 50 \, \text{ng} \, 20 \, \mu \text{g}^{-1}$ protein, while that in leaves or stem extracts is $\sim 30 \, \text{ng} \, 20 \, \mu \text{g}^{-1}$. This latter value is similar to that reported for NTRC in leaves of rice (Serrato et al., 2004).

Antibodies raised against AtNTRB were used to follow the expression of MtNTRA (Fig. 2B). Because orthologues of NTRA and NTRB found in *A. thaliana* may also exist in *M. truncatula*, anti-AtNTRB could react with both isoforms of the A/B type in *M. truncatula*. Figure 2B shows that MtNTRs of the A/B type are present in all organs analysed but they are more abundant in embryo axes, roots, and stems where they are present in quantities $>100 \, \text{ng} \, 20 \, \mu \text{g}^{-1}$ protein. They are only slightly detectable in cotyledons from dry seeds, but their level increases in this part of the seed during germination. The results obtained by western blot analyses are consistent with those obtained by Q-PCR. They are also in accordance with those reported for cytosolic NTRs from rice and pea (Serrato et al., 2002; Montrichard et al., 2003).
MtNTRC is a homotrimeric flavoenzyme

To determine the structural and enzymatic properties of the two NTRs, full coding regions corresponding to NTRC (DQ822469) and NTRA (DQ822468) from M. truncatula Paraggio were first cloned in the expression vector pRSF2. This vector allows the expression of the recombinant protein fused to an N-terminal His tag. The deduced protein sequences are aligned (Fig. 3). It should be noted that, because seeds germinate more synchronously, the genotype Paraggio that is different from Jemalong, the genomic model of M. truncatula present in databases, was used for the study. Few differences were found in primary sequences of the proteins deduced from the two genotypes. They are indicated in the legend of Fig. 3.

Parts of the sequences that are presented in Fig. 3 are underlined: the sites of FAD (site 1) and NADPH binding (site 2) as well as the catalytic site (site 3) with the two reactive cysteines (Scrutton et al., 1990; Jacquot et al., 1994; Serrato et al., 2004). The putative active site of the Trx domain only present in the C-terminal extension of NTRC is also underlined (site 5).

An attempt was made to overexpress the recombinant proteins in E. coli. However, neither of the full-length proteins was soluble. Thus, the parts of the coding regions encoding putative mature MtNTRC (without the first 51 amino acids) and MtNTRA (without the first 37 amino acids) were finally subcloned into the same expression vector. Mature MtNTRC was found to be poorly expressed and rather unstable. Several attempts were necessary to define which purification steps did not lead to loss of activity. Finally, it was purified in an active state by using two chromatography steps, first Ni2+-chelating Sepharose and then gel filtration on Superdex 75, in which active NTRC was rapidly eluted in the void volume. In contrast, NTRA was purified on Ni2+-chelating Sepharose without loss of activity. An analysis by SDS–PAGE under reducing conditions (Fig. 4A) and with western blots (Fig. 4B) shows that recombinant MtNTRC (52 kDa with the His
tag, lane 1) and MtNTRA (37 kDa with the tag, lane 3) were apparently pure and reacted with antibodies raised against OsNTRC and AtNTRA, respectively.

In non-reducing SDS–PAGE, MtNTRC appears as a single band of ~150 kDa corresponding to a polymer that was highly reactive with anti-OsNTRC (Fig. 4A, B, lane 2). Anti-OsNTRC also revealed a band of 52 kDa that may correspond to the monomer present in low quantity in the fraction. In contrast, MtNTRA (Fig. 4A, B, lane 4) appears as three bands of proteins of different intensity with apparent mol. wts of 35, 67, and ~105 kDa, all detected with anti-AtNTRA. The two lower bands that are much more intense than the upper band correspond to the monomer and the homodimer that A/B type NTRs have been shown to form (Dai et al., 1996). The upper 105 kDa band may correspond to a polymer that comprises more than two subunits and is not stable in non-reducing SDS–PAGE.

To determine more accurately their native molecular weights, the recombinant NTRs were subjected to gel filtration on Superdex 200. Under these conditions, MtNTRC was eluted with a mol. wt of 148 kDa (data not shown), a value similar to that determined in non-reducing SDS–PAGE. Thus, native MtNTRC seems to be a polymer probably comprising three subunits linked by disulphide bridges. This result contrasts with that reported for OsNTRC which is a dimer. As far as MtNTRA is concerned, a native mol. wt of 120 kDa was unexpectedly determined by gel filtration. This suggests that native MtNTRA is not a dimer but rather a tetramer. This result is not in accordance with that previously obtained in crystallographic studies showing that AtNTRA is a dimer (Dai et al., 1996).

NTRs are flavoenzymes that catalyse the transfer of electrons from NADPH to Trx via FAD. As expected, three absorption maxima were observed at 270, 380, and 455 nm that are typical of flavoproteins, when the absorption spectra were obtained with purified recombinant NTRs from M. truncatula (Fig. 5). Based on an extinction coefficient at 454 nm of 11 300 M⁻¹ cm⁻¹ for bound FAD (Jacquot et al., 1994), about one FAD prosthetic group was determined per subunit of each NTR.

**NTRC is a complete NTS that uses either NADPH or NADH as cofactor**

Purified recombinant NTRs were used to perform functional studies. In NTS, the rate of electrons that flow from NADPH to Trx can be determined by using DTNB (NADPH,NTR,Trx,DTNB). Electrons can also flow directly from NTR to DTNB (NADPH,NTR,DTNB) but with a much lower rate than in the presence of Trx. This can be well illustrated with NTRA. For that purpose, the rate of reduction of DTNB initiated by NTRA and NADPH was first measured in the absence or in the presence of MtTrxh2 (the preparation of which will be described elsewhere). As expected, in the absence of Trx, the turnover of the reaction is very low: 0.0046 ± 0.0002 mol DTNB reduced s⁻¹ (mol NTRA)⁻¹ (Table 2). In contrast, in the presence of Trx, the turnover (using NADPH as a cofactor) is 180-fold higher (0.81 ± 0.06 s⁻¹). Because NTRC contains both NTR and Trx domains, it may act as a complete NTS and have a high rate of DTNB reduction. To test this hypothesis, the rate of DTNB reduction initiated by NTRC was measured in the presence of NADPH. The turnover measured for NTRC (0.62 ± 0.02 s⁻¹; Table 2) is indeed similar to that obtained in the presence of both NTRA and Trxh2, suggesting that the flux of electrons in NTRC passes through the Trx domain. Thus, NTRC seems to behave as a complete NTS.
As NTRs are generally highly specific for NADPH, the $K_m$ for NADPH was then determined for each NTR and the specificity for this nucleotide was compared with that for NADH. In the case of NTRA, $K_m$ determination was done in the presence of MtTrxh2. Both enzymes have a high affinity for NADPH (Table 2). However, NTRA can hardly use NADH as a cofactor ($K_m=1.08±0.06$ mM) whereas NTRC has a much greater affinity for this nucleotide with a $K_m$ of $11.0±1.5$ μM (Table 2; Fig. 6). In addition, the turnover of NTRC measured in the presence of NADH is the same as that measured with NADPH (Fig. 6).

**BAS1 may be a target of NTRC**

Because NTRC seemed to function as an NTS, its capacity to reduce insulin, a target that is widely used, was tested. No insulin reduction was observed in the presence of NADPH even at the highest concentration of MtNTRC tested. The same result was obtained with NTRC from rice (Serrato et al., 2004). In contrast, experiments performed with MtNTRA and MtTrxh2 led to an insulin reduction (data not shown). It should be noted that the insulin assay can fail with certain Trxs, as in the case of Trxm3 from *A. thaliana* (Collin et al., 2003) or CDSP32, a 32 kDa Trx-like protein from potato (Broin et al., 2002)—two proteins with a well-established disulphide reductase activity on other substrates.

NTRC from rice was proposed to protect chloroplasts against oxidative damage because a deficiency of NTRC in an *A. thaliana* mutant was associated with hypersensitivity to oxidative stress. On the other hand, BAS1, a chloroplastic 2-Cys peroxiredoxin (Prx), that reduces various peroxides was found to be required for normal leaf development and photosynthetic function (Baier and Dietz, 1999). The actual mode of regeneration of this Prx is, however, still unknown even though certain chloroplastic Trxs or CDSP32 were shown to be able to reduce the protein with various degrees of efficiency (Broin et al., 2002; Konig et al., 2002; Collin et al., 2003). Nevertheless, experiments realized with isolated chloroplasts have indicated that electrons may be given to BAS1 by various NADH- and NADPH-dependent oxidoreductases or via an

#### Table 2. Kinetic parameters of purified recombinant MtNTRA and MtNTRC

<table>
<thead>
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<th>$K_{cat}$ (s$^{-1}$) (NADPH)</th>
<th>$K_m$ (μM or mM)</th>
</tr>
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<tbody>
<tr>
<td>MtNTRA</td>
<td>0.0046±0.0002</td>
<td>NADPH NADH</td>
</tr>
<tr>
<td>MtNTRA+MtTrxh2</td>
<td>0.81±0.06</td>
<td>0.51±0.10 μM 1.08±0.06 mM</td>
</tr>
<tr>
<td>MtNTRC</td>
<td>.62±0.02</td>
<td>2.40±0.30 μM 11.0±1.5 μM</td>
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To determine whether BAS1 could be a target of NTRC in *M. truncatula in vivo*, a western blot analysis of BAS1 expression was performed using antibodies raised against AtBAS1. BAS1 was found to be present in all the tissues analysed in amounts >100 ng 20 μl$^{-1}$ protein (Fig. 2). However, it is much more abundant in green tissues, i.e. leaves and stems. Because NTRC and BAS1 are co-expressed in leaves, stems, and seeds of *M. truncatula*, the redox system NTRC/BAS1 may serve as an efficient scavenger of various peroxides in these organs.

### Discussion

In this report, the structural and biochemical properties of two NTRs from *M. truncatula* Paraggio were described. MtNTRA and MtNTRC are highly similar to their respective orthologues in *A. thaliana* and *O. sativa*, sharing with them >70% identity at the protein level. The sequences of the catalytic site as well as those of the binding sites for FAD and pyridine nucleotides are notably totally conserved between them.

MtNTRA has properties similar to NTR of the A/B type already reported (Jacquot et al., 1994; Serrato et al., 2002;
Montrichard et al., 2003), but it has a different native structure. Like other A/B-type NTRs, MtNTRA is constitutively expressed and can only use NADPH as a cofactor. In *A. thaliana*, each of the two NTRs of this type was shown to be targeted to both cytosol and mitochondria (Reichheld et al., 2003), but it has a different native structure. Like other A/B-type NTRs, MtNTRA is constitutively expressed and can only use NADPH as a cofactor. In *A. thaliana*, each of the two NTRs of this type was shown to be targeted to both cytosol and mitochondria (Reichheld et al., 2003). Because MtNTRA is highly specific for NADPH, it may be mainly targeted to the cytosol where NADPH can be produced by the pentose phosphate pathway. In contrast, the NADPH concentration may be low in mitochondria. However, whereas AtNTRA was reported to be a dimer (Dai et al., 1996), MtNTRA is a tetramer. This apparent discrepancy between the level of polymerization of MtNTRA and AtNTRA may rely on the different methods used to determine the native state of each protein, gel filtration or crystallization. AtNTRA may also be a tetramer, a structure that may not be stable upon crystallization. Concerning MtNTRA, the unique form found upon gel filtration is the tetramer while the main forms visible in non-reducing SDS-PAGE are the dimer and the monomer. Thus, the present results suggest that two dimers, in which the monomers are linked by a disulphide bridge, interact non-covalently to form a tetramer. It is noted that almost all the sequences of NTRs of the A/B type present a cysteine residue in their C-terminal part which is conserved in plants, yeast, and bacteria. This residue is also conserved in NTRC sequences (Fig. 3, site 4). Because in the dimer of AtNTRA, this part of the molecule forms a helix which is in contact with the corresponding helix of the partner molecule (Dai et al., 1996), this residue may be involved in the dimer formation. The possible disulphide-linked dimeric state of NTRs of the A/B type will be an interesting hypothesis to be tested later by mutating the conserved cysteine residue.

MtNTRC was found to act as an NTS as demonstrated with the reduction of both DTNB and BAS1. In contrast, OsNTRC was originally described as a bifunctional enzyme. However, during the preparation of this manuscript, two independent studies showing that NTRCs from rice and *A. thaliana* were able to reduce BAS1 were published (Moon et al., 2006; Pérez-Ruiz et al., 2006). Thus, the ability to act as an NTS and to reduce BAS1 is a common feature of NTRC from monocot and dicot plants.

Nevertheless, NTRC in *M. truncatula* differs from its well-characterized counterpart in *O. sativa* with respect to native structure, specificity, and pattern of tissue expression. First, recombinant MtNTRC is a homotrimer whereas recombinant OsNTRC was described as a homodimer. This is surprising because, in most cases, oligomeric protein complexes show the same oligomer composition irrespective of the species origin. Further study is required to resolve this discrepancy. In the case of MtNTRC, the polymerization seems to rely on the presence of disulphide bridges. Two conserved cysteine residues that are present in plant NTRC sequences may be involved in such bridges: the one that is common to NTRs of the A/B type (Fig. 3, site 4), and another that is situated in the C-terminal part of the Trx domain (site 6). Nonetheless, as in the case of MtNTRA, further experiments are needed to confirm this hypothesis. Secondly, MtNTRC can use either NADPH or NADH as cofactors, while OsNTRC is specific for NADPH (Serrato et al., 2004). The binding site for pyridine nucleotides is highly conserved in NAD(P)(H)-dependent enzymes (Fig. 3, site 3). The motif GxGxxA is common to many NADPH-dependent enzymes, the motif GxGxxG being in contrast indicative of specificity for NADH (Scrutton et al., 1990). Although MtNTRC does have the motif common to many NADPH-dependent enzymes as all the NTRs mentioned in Fig. 1, it can use both NADPH and NADH as cofactors. The motif GxGxxA is indeed not incompatible with the use of NADH, as already shown with Cp34, an unusual member of the Trx reductase family found in *Clostridium pasteurianum* whose activity is dependent on NADH (Reynolds et al., 2002). In its case, it was demonstrated that other amino acid residues played a role in the specificity. Nevertheless, MtNTRC is able to use both types of pyridine nucleotides; determination of the amino acids responsible for this little preference for reduced pyridine nucleotides will require further study. Thirdly, in contrast to OsNTRC which was thought to be expressed only in green tissues (Serrato et al., 2004), MtNTRC was found to be expressed not only in leaves and stems as expected, but also in non-green tissues, i.e. cotyledons from dry and germinating seeds, where it is more abundant than in green tissues.

An interesting feature of the NTRC described here and also in the two papers that have just been published (Moon et al., 2006; Pérez-Ruiz et al., 2006) is its ability to reduce BAS1 efficiently. When compared with Trx, Trx, and CDSP32 (Dietz et al., 2006), NTRC is a more powerful reductant and may be the actual reductant of BAS1 *in vivo*. This hypothesis is strongly supported by.

**Fig. 7.** Peroxiredoxin activity of AtBAS1 in the presence of MtNTRC and NADPH. Peroxiredoxin activity was followed by the decrease of NADPH absorbance at 340 nm in a reaction mix containing 100 μM H2O2, 1.5 μM NTRC, 160 μM NADPH, and different concentrations of recombinant AtBAS1 in the range of 0–10 μM as indicated.
the finding that BAS1 reduction can be achieved in isolated chloroplasts fed with substrates of NAD(P)H reductases (Konig et al., 2002). Interestingly, BAS1 is constitutively expressed in *M. truncatula* as in potato (Broin and Rey, 2003), and a dual role of BAS1 in both green and non-green tissues has already been suggested (Konig et al., 2002). Because NTRC and BAS1 are co-expressed in green tissues and seeds of *M. truncatula*, the redox system NTRC/BAS1 may function in these tissues to scavenge various peroxides. Indeed, both chloroplast function and germination generate reactive oxygen species (ROS). ROS are produced in chloroplasts by photosynthesis during the day when some of the electrons from reduced ferredoxin are transferred to O₂ instead to CO₂. This occurs particularly when CO₂ is in short supply. ROS are also produced during the night by starch and fatty acid metabolism, and chlororespiration. Germination which is associated with a strong resumption of metabolism and an increase in respiratory activity also produces ROS (Puntarulo et al., 1991; Cakmak et al., 1993; Leprince et al., 1994; Aalen, 1999; Bailly, 2004). The ability of *M. truncatula* NTRC to use NADH with an efficiency almost equal to that of NADPH could explain the role of the enzyme in removing ROS not only in chloroplasts but also in germinating legume seeds. In green tissues, during the day BAS1 may be mainly reduced by NTRC, although chloroplastic Trxs (reduced by ferredoxin) may also contribute to its regeneration. However, in low light intensity or during the night, when chloroplastic Trxs are oxidized, the activity of NTRC may be the only way to reduce BAS1. During the day, NTRC would use NADPH produced by photosynthesis, while during the night it would use NADH produced by respiration in mitochondria and exported to the chloroplast by the malate shuttle. Similarly, NTRC would use NADH produced by respiration to reduce BAS1 in seeds during germination. BAS1 would then contribute to eliminate peroxides produced in plastids as well as H₂O₂ that has diffused from the rest of the cell into plastids.

NTRC may actually play an important role in plant chloroplasts during the night and under low light intensity because the mutant of *O. sativa* that is deficient in NTRC was shown to have a severe growth retardation under short-day conditions (Pérez-Ruiz et al., 2006). Beside this function, NTRC has an additional role in seeds of legumes during germination indicating that NTRC may have different roles in monocot and dicot plants. It would be interesting to identify all its targets in green tissues and seeds from legumes.

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