Identification of a Novel Transporter for Dicarboxylates and Tricarboxylates in Plant Mitochondria

BACTERIAL EXPRESSION, RECONSTITUTION, FUNCTIONAL CHARACTERIZATION, AND TISSUE DISTRIBUTION*

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A cDNA from Arabidopsis thaliana and four related cDNAs from Nicotiana tabacum that we have isolated encode a hitherto unidentified member of the mitochondrial carrier family. These proteins have been overexpressed in bacteria and reconstituted into phospholipid vesicles. Their transport properties demonstrate that they are orthologs/isoforms of a novel mitochondrial carrier capable of transporting both dicarboxylates (such as malate, oxaloacetate, oxoglutarate, and maleate) and tricarboxylates (such as citrate, isocitrate, cis-aconitate, and trans-aconitate). The newly identified dicarboxylate-tricarboxylate carrier accepts only the trans-aconitate, and (such as malate, oxaloacetate, oxoglutarate, and maleate) and tricarboxylates (such as citrate, isocitrate, cis-aconitate, and trans-aconitate). The newly identified dicarboxylate-tricarboxylate carrier accepts only the single protonated form of citrate (H-citrate\(^{2-}\)) and the unprotonated form of malate (malate\(^{2-}\)) and catalyzes obligatory, electroneutral exchanges. Oxoglutarate, citrate, malate and malate are mutually competitive inhibitors, showing \(K_c\) close to the respective \(K_m\). The carrier is expressed in all plant tissues examined and is largely spread in the plant kingdom. Furthermore, nitrate supply to nitrogen-starved tobacco plants leads to an increase in its mRNA in roots and leaves. The dicarboxylate-tricarboxylate carrier may play a role in important plant metabolic functions requiring organic acid flux to or from the mitochondria, such as nitrogen assimilation, export of reducing equivalents from the mitochondria, and fatty acid elongation.

The transport of metabolites in and out of mitochondria is mediated by a family of related carrier proteins that span the lipid bilayer of the inner membrane (1). The polypeptide sequences of members of this family are made up of three related domains of about 100 amino acids repeated in tandem, each probably being folded into two trans-membrane \(\alpha\)-helices connected by an extensive hydrophilic loop. The repeats in the various family members are all related, and various sequence features are conserved (1, 2). So far 14 mitochondrial carriers have been identified and sequenced from yeast and animals (see Ref. 3 and references therein). The functions of many other family members found in genomic sequences are unknown.

In plants, many transport activities observed in mitochondria and/or in liposomes reconstituted with more or less purified protein fractions await to be associated with specific protein sequences (for a review see Ref. 4). These include the transport of specific dicarboxylates and tricarboxylates, which is required in several metabolic processes such as primary amino acid synthesis (e.g. nitrate/ammonium assimilation), export of reducing equivalents (e.g. for photosynthesis), fatty acid metabolism (e.g. lipid mobilization and fatty acid elongation), gluconeogenesis, and isoprenoid biosynthesis (5–14). However, protein (gene) sequences are known for only a few plant mitochondrial carriers. Indeed, cDNAs encoding the adenosine nucleotide carrier, the uncoupling protein, and the phosphate carrier have been isolated from various plants based on their high homology with their orthologs in other organisms (15–17). The latter two carriers have been identified also from their transport properties upon expression in Escherichia coli and reconstitution into liposomes (18, 19). Finally, a malate translocator from Panicum miliaceum has been overexpressed, reconstituted, and inferred to be the plant ortholog of the bovine oxoglutarate/malate carrier, although the identity between them is low (32%) (20, 21).

In this paper, the identification and characterization of the plant mitochondrial dicarboxylate-tricarboxylate carrier (DTC)\(^3\) is described. It is based on the identification of a cDNA from Arabidopsis thaliana and four cDNAs from Nicotiana tabacum encoding proteins related to the bovine oxoglutarate-malate carrier (22). These proteins have the characteristic features of the mitochondrial carrier family and are 83–84% identical in their sequences. They were overexpressed in bacteria, purified, and reconstituted into phospholipid vesicles, where they transport dicarboxylates (such as oxoglutarate, oxaloacetate, malate, and succinate) and tricarboxylates (such as citrate, isocitrate, cis-aconitate, and trans-aconitate) by a counter exchange mechanism. From their transport properties and

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1 The abbreviations used are: DTC, dicarboxylate-tricarboxylate carrier protein; PIFES, piperezine-N,N’-bis(2-ethylamino sulfonic) acid; OGC, oxoglutarate-malate carrier; DCA, dicarboxylate carrier; ATP, ATP-dependent carrier; SFC, succinate-fumarate carrier; UCP, uncoupling protein.
phylogenetic analysis, it is concluded that these proteins are isoforms/orthologs of a novel mitochondrial transporter named DTC.

**EXPERIMENTAL PROCEDURES**

**Plant Material and Growth Conditions—**A. thaliana (ecotype Columbia), N. tabacam cv. xanthi, Nicotiana tomentosiformis, and Nicotiana sylvestris were grown in a greenhouse under long day conditions (16 h of light/8 h of dark). Natural light was supplemented with white fluorescent light to provide 200 μmol of photons s⁻¹ m⁻². For nitrogen supply experiments, N. tabacam were grown aerophytically as described in Ref. 23.

**cDNA Library Screening—**A N. tabacam cDNA library was screened as described in Ref. 24 using the SacII/AccI fragment of NdDTC as probe. Hybridization and washing conditions were as described in Ref. 23. Standard DNA manipulations were as described in Ref. 25.

Southern and Northern Blot Analyses—**Genomic DNA was prepared from leaves according to Ref. 26. Total RNA was isolated from various tissues of A. thaliana and N. tabacam using TRIzol (Invitrogen). For nitrogen supply experiments, total RNA was extracted from N. tabacam tissues as described in Ref. 23. Southern and Northern blot analyses were carried out as described in Ref. 24. RNA loading was checked either with ethidium bromide or a 2.7-kbp marker sizes (in kbp) are marked on the left side.

**RESULTS**

Isolation and Characterization of DTC cDNA—The protein sequence of the bovine 2-oxoglutarate/malate transporter (22) was used to search data bases for homologous plant sequences. In this way an A. thaliana EST (GenBank™ accession number Z26469), named AdDTC, was identified. Sequencing showed that it contained a 897-bp open reading frame, encoding a polypeptide of 298 amino acids with a calculated molecular mass of 31.9 kDa. AtDTC was used to screen a N. tabacam cDNA library, and four different cDNAs were identified (named NdDTC1 to NdDTC4). They were classed into two groups containing NdDTC1 with NdDTC2 and NdDTC3 with NdDTC4. Analysis of NdDTC1 showed an 894-bp open reading frame that encoded a 297-amino acid polypeptide with a calculated molecular mass of 31.7 kDa. The NdDTC2 cDNA was not complete, lacking six nucleotides after the start codon as well as a 5' untranslated region. There was 99% amino acid identity between NdDTC1 and NdDTC2. In the second group, NdDTC3 gave a 903-bp open reading frame that encoded a 300-amino acid polypeptide with a calculated molecular mass of 32.1 kDa. NdDTC4 was not a complete cDNA, containing only a 682-bp coding region. There was 98% amino acid identity between NdDTC3 and NdDTC4. When the two groups of tobacco DTCs were compared, an amino acid identity of 91% was found. NdDTC1/2 and NdDTC3 exhibited 84 and 85% identity with AtDTC, respectively. Both AtDTC and the NdDTCs exhibit the hydrophobic profile, the tripartite structure, and the sequence motifs that are characteristic of the mitochondrial carrier family (1).

Number of Genes Coding for the DTC in A. thaliana and N. tabacam—An analysis of the Arabidopsis nucleotide sequence data base showed the presence of a single AtDTC gene located on chromosome V (GenBank™ accession number AF296838). The restriction enzymes EcoRI, BamHI, and HindIII, which do not have recognition sites within this gene, were used to digest A. thaliana genomic DNA. These digests pro-duced a single fragment that hybridized with the AtDTC probe (Fig. 1A), thus confirming that the Arabidopsis genome contains a single copy of the AtDTC gene. The four NdDTCs were isolated from N. tabacam, an amphidiploid species derived from ancestors that are closely related to the present-day species N. sylvestris and N. tomentosiformis. Therefore, Southern analyses were performed using genomic DNA from N. tabacam, N. sylvestris, and N. tomentosiformis. A number of hybridizing bands were detected in the different tobacco genomes when

![Fig. 1. Southern blot analysis of genomic DNA. A. DNA (15 μg) from A. thaliana was digested with EcoRI (E), HindIII (H), and BamHI (B) and hybridized with the SacII/AccI fragment of AdDTC. B. DNA (30 μg) from N. tabacam (N. tab), N. sylvestris (N. syl), and N. tomentosiformis (N. tom) were digested with SacI (S), EcoRI (E), and HindIII (H) and hybridized with the EcoRI/SacI fragment of NdDTC2. The molecular marker sizes (in kbp) are marked on the left side of each panel.](http://www.jbc.org/)

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using a nonspecific NtDTC probe, thus confirming that several distinct genes encode DTC in tobacco (Fig. 1B). Indeed, it appeared that N. sylvestris and N. tomentosiformis contained at least two DTC genes, whereas the N. tabacum pattern was the sum of these two genomes. Southern blotting with a specific NtDTC4 probe and PCR analyses carried out on genomic DNA using gene-specific oligonucleotide primers revealed that Nt-DTC2/3 and NtDTC1/4 were encoded by the N. sylvestris and N. tomentosiformis genomes, respectively (data not shown).

Molecular and Phylogenetic Relationship of AtDTC and Nt-DTC Proteins with Other Mitochondrial Transporters—Amino acid sequence comparisons revealed that AtDTC and the Nt-DTCs showed the highest degree of similarity with other plant proteins. For example, NtDTC1 exhibited 94% identity with a putative potato oxoglutarate-malate carrier (GenBank™ accession number X99853) and 81% identity with the millet malate transporter (20). Among mitochondrial transporters from other organisms, the tobacco DTCs showed the highest homology with the mitochondrial oxoglutarate-malate carriers sharing 41–42% identical amino acids with this carrier from bovine (22), human (31), rat (32), and Caenorhabditis elegans (GenBank™ accession number P90992). A phylogenetic analysis carried out using AtDTC, NtDTC1–4, and the sequences of four groups of mitochondrial transporters (i.e. the oxoglutarate-malate carrier (OGC), dicarboxylate (DIC), and tricarboxylate (CTP) carriers, and the uncoupling (UCP) proteins) revealed that AtDTC and the NtDTCs were more closely related to the OGC group (Fig. 2). However, DTCs and OGCs form two clearly distinct clusters. Furthermore, the phylogenetic analysis confirmed the presence of two distinct DTC subgroups in tobacco (made up of the homologous DTC proteins from each of the two N. tabacum genomes) that appear to have originated from a recent gene duplication event.

Expression of DTC in Various Plant Tissues—The expression of DTC in A. thaliana and N. tabacum was examined in different tissues by Northern blot analysis. The presence of DTC transcripts was found in all plant tissues examined, although at different levels (Fig. 3). In Arabidopsis, the flower bud showed the highest DTC transcript levels, whereas the root had the lowest (Fig. 3A). In N. tabacum DTC expression in flower bud and root appeared to be comparable; the highest transcript levels were detected in sepal, petal, male, and female tissues, whereas somewhat lower amounts were found in leaf and stem (Fig. 3B). Although we were unable to distinguish between the different tobacco DTCs with the probe used in Fig. 3, reverse transcription-PCR experiments using gene-specific primers indicated that each tobacco DTC gene was expressed in the different organs/tissues analyzed (data not shown).

Effect of Nitrate Supply on NtDTC Steady-state mRNA Levels—Organic acids produced in the mitochondria are required for ammonium assimilation via the glutamine synthetase/glutamate synthase pathway located in the plastids (33). Previous work has shown a coordinated expression of certain genes (NAD-dependent isocitric dehydrogenase, nitrate reductase, and glutamine synthetase) when nitrate is supplied to nitrate-starved tobacco plants (23). It was therefore interesting to investigate whether DTC expression varied under the same conditions. The effect of nitrate supply to tobacco plants that were nitrogen-starved for 4 days was investigated in roots (Fig. 4A) and devided leaves (Fig. 4B) by Northern blot analysis as described in Ref. 23. In both tissues, nitrate resupply led to an increase in NtDTC mRNA steady-state levels (Fig. 4).

DTC Is a Mitochondrial Membrane Protein—To study whether the DTC protein is located in the membranes of plant mitochondria, a His6-tagged NtDTC2 protein was purified by affinity chromatography (Fig. 5, lane 1) and used to raise polyclonal antibodies in rabbits. The antibodies recognized the recombinant DTC protein as shown by Western blot analysis (Fig. 5, lane 5). DTC could not be detected in crude tobacco leaf extracts (Fig. 5, lanes 2 and 6). In contrast, a prominent immunoreactive band of about 32 kDa was observed using purified mitochondria from potato tubers. To examine the subcellular localization of DTC, integral membrane proteins were separated from soluble and peripheral proteins of potato mitochondria by carbonate treatment (Fig. 5, lanes 3 and 4, respectively). The 32-kDa band was found only in the membrane fraction (Fig. 5, lanes 3 and 7). Therefore, DTC is an integral mitochondrial membrane protein. The antiserum cross-reacted with another protein of about 60 kDa. However, this protein is not related to DTC because it was still recognized when the immunoreaction was carried out in the presence of soluble recombinant NtDTC2 (not shown).

Functional Characterization of the Recombinant DTC Proteins—To study their functional properties, NtDTC1–3 and AtDTC proteins were expressed at high levels in E. coli C0214 (DE3). They accumulated as inclusion bodies and were purified...
by centrifugation and washing with a yield of 80–100 mg of purified protein/liter of bacterial culture. Neither protein was detected in bacteria harvested immediately before induction of expression or in control (with only vector) cells harvested after induction (not shown). The identity of the expressed proteins was confirmed by N-terminal sequencing and Western blotting. Neither protein was detected in bacteria harvested immediately before induction of expression. Furthermore, no reconstitution of sarkosyl-solubilized material from bacterial cells either lacking the DTC expression vector or harvested immediately before induction of expression. Furthermore, no uptake was observed of external [14C]oxoglutarate into liposomes that did not contain internal oxoglutarate, indicating that reconstituted DTCs do not catalyze unidirectional transport (uniport).

The substrate specificity of the tobacco and *A. thaliana* reconstituted proteins was investigated in detail by measuring the uptake of [14C]oxoglutarate into proteoliposomes that had been preloaded with potential substrates. As shown in Table I, the highest activities were detected in the presence of internal trans-aconitate, and sulfate were exchanged for external 2-oxoglutarate, although to a slightly lower extent than the dicarboxylates. No significant exchange was detected with internal fumarate, phosphoenolpyruvate, phosphate, pyruvate, glutamate (Table I), aspartate, glutamine, carnitine, ornithine, or ADP (not shown). Similar results were obtained with NtDTC2 (data not shown).

The ability of nonradioactive substrates (at a concentration, 20 mM) to replace the uptake of [14C]oxoglutarate and terminated after 20 s. Similar results were obtained in four independent experiments. The protein extracts were subjected to 10% SDS-PAGE and analyzed by Coomassie Blue staining and Western blotting with antibodies raised against the NtDTC2-His<sub>6</sub> protein (lanes 5–8). Lanes 1 and 5, purified recombinant NtDTC2-His<sub>6</sub> protein; lanes 2 and 6, crude extract from tobacco leaves; lanes 3 and 7, membrane proteins from *S. tuberosum* mitochondria; lanes 4 and 8, soluble proteins from *S. tuberosum* mitochondria; lane M, molecular weight marker proteins.

### Table I

| Internal substrate | NtDTC1 | NtDTC3 | AtDTC |
|--------------------|--------|--------|-------|
| None (Cl<sup>-</sup> present) | 0.04 | 0.01 | 0.02 |
| Oxoglutarate | 4.12 | 0.25 | 1.18 |
| Oxoadipate | 1.47 | 0.03 | 0.58 |
| l-Malate | 5.05 | 0.22 | 1.63 |
| Succinate | 3.42 | 0.27 | 1.26 |
| Maleate | 4.96 | 0.37 | 1.29 |
| Fumarate | 0.06 | 0.01 | 0.02 |
| Oxaloacetate | 3.75 | 0.30 | 1.90 |
| Malonate | 5.05 | 0.28 | 1.93 |
| Citrate | 3.08 | 0.18 | 1.07 |
| l-Isocitrate | 2.39 | 0.10 | 0.53 |
| cis-Aconitate | 2.37 | 0.11 | 0.65 |
| trans-Aconitate | 3.95 | 0.15 | 0.73 |
| Phosphoenolpyruvate | 0.03 | 0.00 | 0.03 |
| Sulfate | 2.45 | 0.08 | 0.46 |
| Phosphate | 0.01 | 0.02 | 0.04 |
| Pyruvate | 0.04 | 0.00 | 0.01 |
| Glutamate | 0.13 | 0.00 | 0.02 |

The uptake of [14C]oxoglutarate into proteoliposomes that had been preloaded with potential substrates. As shown in Table I, the highest activities were detected in the presence of internal 2-oxoglutarate, malate, maleate, oxaloacetate, succinate, or malonate. Interestingly, citrate, isocitrate, cis-aconitate, trans-aconitate, and sulfate were exchanged for external 2-oxoglutarate, although to a slightly lower extent than the dicarboxylates. No significant exchange was detected with internal fumarate, phosphoenolpyruvate, phosphate, pyruvate, glutamate (Table I), aspartate, glutamine, carnitine, ornithine, or ADP (not shown). Similar results were obtained with NtDTC2 (data not shown).

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a very low inhibitory effect (<20%), indicating that the reconstituted DTC proteins have a very low affinity for sulfate, at least at the external surface of the proteoliposomes. Phosphoenolpyruvate, fumarate, phosphate, pyruvate, glutamate, ornithine, carnitine, and ADP had a very limited inhibitory effect, if any. Therefore, the substrate specificity of the plant DTC proteins appears to be broader than that of known oxoglutarate-malate carriers, because DTC accepts both dicarboxylates and tricarboxylates.

The [14C]oxoglutarate/oxoglutarate exchange in proteoliposomes reconstituted with the tobacco and A. thaliana DTC proteins was inhibited strongly (61–98%) by organic mercurials, such as mersalyl (0.1 mM) and p-chloromercuriphenylsulfonate (0.1 mM), and by bathophenanthroline (1 mM) (inhibitors of several mitochondrial carriers). The impermeable dicarboxylate butyrlmalonate (2 mM), an inhibitor of the dicarboxylate and oxoglutarate/malate carriers (34, 35), and especially phthalonate (2 mM), an inhibitor of oxoglutarate and oxaloacetate transport in mitochondria (11, 13), inhibited (60–99%) the reconstituted transport activity. Furthermore, the tricarboxylate analog 1,2,3-benzenetricarboxylate (2 mM), a specific inhibitor of the tricarboxylate carrier (36), had a considerable inhibitory effect (61–65%), as did pyridoxal 5’-phosphate (1 mM, 45–60% inhibition). In contrast, N-ethylmaleimide (1 mM), α-cyanoisocinnamate (0.1 mM), and carboxylattractyloside (0.02 mM), known inhibitors of the phosphate, pyruvate, and ADP/ATP carriers, respectively, had no effect on the activities of the reconstituted DTC proteins.

**Effect of pH on DTC-catalyzed Transport**—The DTC-mediated oxoglutarate and citrate homoexchanges were found to be strongly dependent on pH (Fig. 6). With both NtDTC1 and AtDTC (Fig. 6), the oxoglutarate/oxoglutarate and citrate/citrate exchanges increased on decreasing the pH from 8.0 to 5.5. Below this pH value transport was inhibited, possibly because of carrier inactivation. Three additional points are noteworthy. First, oxoglutarate transport catalyzed by AtDTC (Fig. 6B) increased slightly on decreasing the pH from 7.0 to 5.5 as compared with oxoglutarate transport catalyzed by NtDTC1 (Fig. 6A). Second, citrate transport was very low above pH 7.0 with both reconstituted NtDTC1 and AtDTC (Fig. 6). Third, reconstituted NtDTC2 gave virtually the same results as those for NtDTC1 (Fig. 6A), whereas reconstituted NtDTC3 gave results qualitatively similar to those for AtDTC (Fig. 6B), although NtDTC3 transport rates were lower (data not shown).

**Kinetic Characteristics of the Recombinant DTC Proteins**—The kinetic constants of reconstituted DTC from tobacco and Arabidopsis were determined at pH 6 and 7 by measuring the initial transport rate at various external [14C]oxoglutarate, [14C]malate, or [14C]citrate concentrations in the presence of a constant saturating internal concentration of the same substrate (homoexchange). The mean $K_m$ and $V_{max}$ values and their standard errors are shown in Table II. For all DTC proteins tested at pH 6, the maximum rates ($V_{max}$) of malate transport were higher than those of oxoglutarate and citrate, whereas oxoglutarate transport was intermediate. The apparent transport affinities ($K_m$) for citrate and oxoglutarate were virtually the same, whereas those for malate were higher (about 5-fold with NtDTC1 and about 2.5-fold with AtDTC). At pH 7, the $K_m$ for oxoglutarate and malate as well as the $V_{max}$ of oxoglutarate, malate, and citrate transport were only slightly different from those measured at pH 6. On the other hand, the $K_m$ values for citrate were about 6-fold higher at pH 7 than at pH 6.

Citrate and malate were found to be competitive inhibitors of NtDTC1-mediated oxoglutarate transport because they increased the apparent $K_m$ without changing the $V_{max}$ of [14C]oxoglutarate/oxoglutarate exchange. Similarly, oxoglutarate and malate were found to act as competitive inhibitors of the NtDTC1-catalyzed [14C]citrate/citrate exchange. The inhibition constants ($K_i$) at pH 6 were 0.30 ± 0.11 mM for oxoglutarate, 1.21 ± 0.36 mM for malate, and 0.29 ± 0.06 mM for citrate (means ± S.E. of eight experiments). In three other experiments, citrate and malate were found to inhibit the AtDTC-mediated oxoglutarate homoexchange in a competitive manner.

**Substrate Species Transported by the DTC Proteins**—Citrate has $pK_a$ values of 3.14, 4.77, and 5.40; between pH 6 and 7 citrate$^{3−}$ is the predominant species. As shown in Table II, in the pH range between 6 and 7, the $V_{max}$ of citrate transport varied only slightly, whereas the apparent transport affinity decreased considerably with increasing pH. In principle, this pH dependence of citrate transport may be caused by a pH effect on the protein and/or by the availability of the transported substrate species. To investigate these possibilities, the $K_m$ values for the different citrate species were calculated. Table III reports the data obtained for the reconstituted NtDTC1. It is clear that the $K_m$ values for H-citrate$^{2−}$ at pH 6 and 7 were similar (as is the maximal rate of citrate transport). In
The data were obtained from Lineweaver-Burk plots of the rate of the homoexchange versus the concentration of external substrate. The concentration of the internal substrate was 20 mM. The exchange rates were measured at the pH indicated, which was the same inside and outside the proteoliposomes. The values given in the table are the means ± S.E. of at least four independent experiments. ND, not determined.

| Carrier | Substrate | pH 6 | V_{max} | pH 7 | V_{max} |
|---------|-----------|------|---------|------|---------|
|         |           | mM   | mmol/min/mg protein | mM   | mmol/min/mg protein |
| NiDTC1  | Oxoglutarate | 0.25 ± 0.06 | 11.9 ± 3.8 | 0.34 ± 0.03 | 7.3 ± 2.3 |
| NiDTC1  | Malate     | 1.21 ± 0.39 | 20.1 ± 6.4 | 1.45 ± 0.41 | 16.2 ± 5.0 |
| NiDTC3  | Oxoglutarate | 0.30 ± 0.09 | 0.84 ± 0.27 | 0.30 ± 0.06 | 0.51 ± 0.23 |
| NiDTC3  | Citrate    | 0.31 ± 0.07 | 0.38 ± 0.11 | 1.91 ± 1.20 | 0.31 ± 0.12 |
| AtDTC   | Oxoglutarate | 0.14 ± 0.03 | 2.9 ± 1.4 | 0.24 ± 0.07 | 2.8 ± 1.1 |
| AtDTC   | Malate     | 0.36 ± 0.05 | 4.2 ± 1.7 | ND         | ND         |
| AtDTC   | Citrate    | 0.15 ± 0.04 | 1.7 ± 0.9 | 0.95 ± 0.13 | 1.3 ± 0.6 |

### DISCUSSION

In this work overexpression in *E. coli* of hitherto unidentified mitochondrial carriers and reconstitution of recombinant proteins in liposomes have been employed to study the transport properties and kinetic parameters of plant DTCs. The results demonstrate that the *Arabidopsis* and tobacco DTC proteins are isofoms/orthologs of a novel mitochondrial carrier that is capable of transporting both dicarboxylates (such as oxoglutarate, oxaloacetate, malate, succinate, maleate, and malonate) and tricarboxylates (such as citrate, isocitrate, cis-aconitate, and trans-aconitate). The specificity of DTC for dicarboxylates and tricarboxylates is demonstrated by the dependence of [14C]oxoglutarate transport on countersubstrates and by the sensitivity of this transport to externally added substrates and to impermeable analogs of dicarboxylates and tricarboxylates. DTC appears to share a common substrate-binding site for oxoglutarate, malate, and citrate, because these substrates are mutually competitive inhibitors and because their $K_m$ values are close to the respective $K_m$ values. Furthermore, DTC accepts only the single protonated form of citrate (H-citrate$^-$) and the unprotonated form of malate (malate$^{2-}$) and catalyzes electroneutral exchanges, for example of a dicarboxylate for a tricarboxylate + $H^+$. The more pronounced pH dependence from 7.0 to 5.5 of oxoglutarate transport catalyzed by NiDTC1 and NiDTC2, as compared with that of NiDTC3 and AtDTC, may be explained by a pH effect on one or more nonconserved residues between the DTC isofoms/orthologs, possibly histidine 145 of NiDTC1 and NiDTC2.

The substrate specificity of DTC is distinct from that of any other mitochondrial carrier of known sequence and function, including those for either dicarboxydrates or tricarboxylates, although with these there is some degree of substrate overlap. The substrate specificity of DTC differs from that of its closest sequence homolog, the oxoglutarate/malate carrier (22), which does not transport citrate, isocitrate, cis-aconitate, trans-aconitate, and sulfate and transports oxaloacetate only very inefficiently. DTC differs from dicarboxylate (7, 39), succinate/fumarate (40), oxaloacetate (41), and oxodicarboxylate (42) carriers.
the principal substrates of which are malate and phosphate, succinate and fumarate, oxaloacetate and sulfate, and oxoacidate and oxoglutarate, respectively) because DTC transports dicarboxylates efficiently, transports oxoacidate to a low extent, and does not transport phosphate and fumarate. It also differs from the tricarboxylate carrier (43, 44) because the latter transports only dicarboxylates (but not trans-aconitate), dicarboxylates (but not oxoglutarate and oxaloacetate), and phosphoenolpyruvate. Furthermore the DTC proteins share only 15 and 21% amino acid identity with the yeast (43) and the rat (44) tricarboxylate carriers, respectively.

The higher relatedness of DTC with oxoglutarate-malate carriers (41–42% identity), as compared with any other characterized carrier (Fig. 2), suggests that these transporters originated from a common ancestor. This ancestor duplicated in some organisms, for example in animals, giving rise to the oxoglutarate-malate carrier and the tricarboxylate carrier, whereas in plants it evolved into DTC, which exhibits the combined characteristics of both the oxoglutarate-malate and the tricarboxylate carriers. Several available plant protein sequences show a 81–94% identity with our DTC proteins, including the millet malate translocator (20) and a putative potato oxoglutarate/malate carrier (GenBank™ accession number X99853) as well as many partial sequences, including AF101583 from Oryza sativa, BG444303 from Gossypium arboreum, BG645594 from Medicago truncatula, BG320815 from Zea mays, BE421905 from Hordeum vulgare, BF480205 from Mesembryanthemum crystallinum, BM437521 from Vitis vinifera, BF011098 from Beta vulgaris, BG526291 from Stevia rebaudiana, and BM411548 from Lycopersicon esculentum. In view of the high degree of identity between these two proteins and DTC, we conclude that they are all dicarboxylic acid carriers.

It is interesting to note that the transport characteristics of the recombinant DTC from Arabidopsis and tobacco (two C3-type plants) closely resemble those of a 31-kDa mitochondrial protein purified from maize (a C4-type plant) (14). Indeed, this protein, when reconstituted into liposomes, transports citrate, isocitrate, cis-aconitate, oxoglutarate, oxaloacetate, malate, succinate, and malonate by an exchange mechanism. Furthermore, oxoglutarate and malate are competitive inhibitors with respect to citrate. Their inhibition constants (0.5 mM for oxoglutarate, 2.4 mM for malate) are very close to the $K_i$ values determined in this work with the recombinant DTC. In the light of our findings it is likely that the purified 31-kDa protein from maize represents the DTC as both catalyze an active exchange of dicarboxylates and tricarboxylates. This hypothesis is supported by the observation that antibodies against the recombinant N’DTC2 cross-react with the purified maize protein (not shown), but more conclusive evidence is lacking because the N terminus of the protein is blocked, and insufficient amounts are available for mass spectrometry of digested peptides. In addition, proteoliposomes reconstituted with mitochondrial extracts from potato tuber were shown to catalyze a phthalonate-sensitive exchange of oxaloacetate for dicarboxylates (oxoglutarate, malate, and succinate) as well as for citrate (13). It was concluded that potato mitochondria contain an oxaloacetate translocator different from all other known mitochondrial transporters. We suggest that the above-mentioned transport activity is explained by DTC, which is present in potato (Fig. 5) and is largely spread in the plant kingdom (see above).

Because DTC transports a broad spectrum of dicarboxylates and tricarboxylates, this carrier may play a role in a number of important plant metabolic functions that require organic acid flux to or from the mitochondria. For example, the citrate exported from the mitochondria to the cytosol in exchange for oxaloacetate can be cleaved by citrate lyase to acetyl-CoA and oxaloacetate and used for fatty acid elongation and isoprenoid synthesis. The malate/oxaloacetate exchange catalyzed by DTC can enable the export of redox equivalents from the mitochondrial matrix that can act as reductants for the production of glyceraldehyde during photorespiration in photosynthesizing cells. In agreement with a central role in cell metabolism, the expression pattern of DTC transcripts (Fig. 4) shows that it is present in all of the plant tissues/organ systems analyzed in this work. On the other hand, in P. miliaceum, a NAD-malic enzyme-type C4 plant, the DTC (“malate transporter”) gene was found to be specifically expressed in the bundle sheath cells of leaves where its expression increased with light and during greening in a manner similar to that of photosynthetic genes (44). These observations are in agreement with the involvement of DTC in the C4 pathway of photosynthesis. A further significance for DTC is the very likely possibility that it is involved in nitrogen assimilation, because 2-oxoglutarate is required for the assimilation of ammonium into amino acids by the glutamine synthetase/glutamate synthase pathway (33). To date, the exact enzymatic origin of this key organic acid for plant ammonium assimilation is still unknown (33). Two pathways have been proposed for the production of 2-oxoglutarate for ammonium assimilation, both requiring the export of organic acids from the mitochondria. In the first case, citrate is used to produce oxoglutarate in the cytosol by the concerted action of the cytosolic aconitase and NADP-dependent isocitrate dehydrogenase. In the second case, oxoglutarate is produced in the mitochondria by the NAD-isocitrate dehydrogenase. The observed increase in steady-state N’DTC transcript levels by the addition of nitrate to nitrogen-starved tobacco plants (Fig. 4) suggests a role of DTC in nitrogen assimilation. Under the same experimental conditions Lancien et al. (23) previously found an induction in the expression of NAD-isocitrate dehydrogenase, as well as citrate synthase, aconitate, nitrate reductase, and glutamine synthetase. The coordinated response of DTC and NAD-isocitrate dehydrogenase expression indicates that DTC is directly involved in the export of oxoglutarate from the mitochondria for nitrogen assimilation.

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Identification of a Novel Transporter for Dicarboxylates and Tricarboxylates in Plant Mitochondria: BACTERIAL EXPRESSION, RECONSTITUTION, FUNCTIONAL CHARACTERIZATION, AND TISSUE DISTRIBUTION
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