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Review

Plant mitochondrial carriers: an overview

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Abstract. In the two last decades, biochemical studies using mitochondrial swelling experiments or direct solute uptake in isolated mitochondria have lead to the identification of different transport systems at the level of the plant mitochondrial inner membrane. Although most of them have been found to have similar features to those identified in animal mitochondria, some differences have been observed between plant and animal transporters. More recently, molecular biology studies have revealed that most of the mitochondrial exchanges are performed by nuclear encoded proteins, which form a superfamily. Members of this family have been reported in animals, yeast as well as plants. This review attemps to give an overview of the present knowledge concerning the biochemical and molecular characterisation of plant members of the mitochondrial carrier family and, when possible, a comparison with carriers from other organisms.

Key words. Plant mitochondrial transporters; phosphate carrier; adenine nucleotide translocator; uncoupling protein; monocarboxylate carrier; dicarboxylate carrier; tricarboxylate carrier; metabolite transport.

Introduction

Respiration, which takes place in mitochondria – ubiquitous organelles in animal and plant cells – produces not only Adenosine triphosphate (ATP) as energy source for the cell but also carbon compounds necessary for many biosynthetic pathways. In plant cells, the functional significance of mitochondrial activity during photosynthesis has been debated for a long time. However, an increasing number of observations provide the evidence that photosynthesis and respiration are mutually beneficial and that respiration maintains a high photosynthesis rate necessary for maximal growth [1–4]. Plant respiration also takes part in other processes such as photorespiration (involving three different organelles: chloroplast, mitochondria and peroxisome), lipid-sugar transformation during seed germination and nitrate assimilation in the cytosol. During photorespiration, mitochondria reduce the glycine produced in the peroxisome and export-reducing equivalents for the reduction of hydroxypyruvate in the peroxisome. When lipid-sugar transformation occurs in germinating lipid-storing seeds, mitochondria import succinate from the glyoxysome, leading to gluconeogenesis. Finally, nitrate assimilation in the cytosol requires reducing equivalents, which are mostly exported from the mitochondria [5]. The biochemical link between respiration and these processes is the rapid exchange of metabolites between the different organelles. Furthermore, these exchanges are fundamental for the maintenance of efficient mitochondrial function.

The transport of metabolites across the inner mitochondrial membrane is catalysed by specific carriers that span the lipid bilayer. All these carriers have a similar structure, a molecular mass of around 30 kDa and a high isoelectric point. They are characterised by a tripartite structure, made up of related tandem domains of

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about 100 amino acids. Each domain consists of two transmembrane α-helices separated by a hydrophilic extra-membrane loop (fig. 1). Furthermore, a consensus sequence called the mitochondrial energy signature (PS00215) with the following sequence P-x-[DE]-x-[LIVAT]-[RK]-x-[LRH]-[LIVMFY]-[QMAIGV] is found in all carriers in one, two or three copies. The similarity observed between these transporters indicates that they are all members of a mitochondrial carrier superfamily [6–10].

Numerous studies have been performed on mammalian carriers; however, the data available from plants are less abundant. Plant mitochondrial transporters have been identified via biochemical studies performed on isolated mitochondria either by swelling experiments or direct substrates uptake. They have been differentiated from each other on the basis of their substrate specificity and sensitivity to inhibitors. Some transporters are involved in oxidative phosphorylation, others in the transport of Tricarboxylic acid (TCA) cycle intermediates, amino acids or cofactors [11]. The aim of this review is to summarise the knowledge related to plant mitochondrial carriers with an emphasis on new data arising from molecular biology investigations.

Transporters involved in ATP production

Oxidative phosphorylation, which leads to the formation of ATP in the matrix of mitochondria, is dependent on import of phosphate and ADP. Phosphate is taken up via a phosphate carrier (PiC) and Adenosine diphosphate (ADP) is exchanged with the produced ATP, via the adenine nucleotide translocator (ANT). Furthermore, the uncoupling protein (UCP), a transporter recently discovered in plants, might be involved in the regulation of oxidative phosphorylation by decreasing the proton electrochemical potential difference across the inner mitochondrial membrane (fig. 2).

Phosphate carrier

The PiC is responsible for a fast uptake of phosphate, which serves as substrate for phosphorylation of ADP. It catalyses the phosphate ($H_2PO_4^-$)/proton symport or phosphate/hydroxyl ion antiport as well as the exchange of matrix and cytosolic phosphate with each other [12–15]. This transport, which is electroneutral, is driven by the pH difference (ΔpH) maintained across the membrane by the mitochondrial electron transport. In animals and yeast, this carrier has been described to be also responsible for an uncoupled and unidirectional Pi transport after modification with $HgCl_2$. The transporter is in fact able to shift between the coupled and uncoupled Pi transport mode in a reversible manner [16, 17]. Recently, a fourth transport mode has been described in yeast using heterologous expression. The carrier displays Cl^- channel behaviour and thus has an anion channel activity [18]. Finally, thiol reagents such as *N*-ethylmaleimide (NEM) and mersalyl have been described to inhibit this carrier

Biochemical characterisation in plants. The existence of a phosphate translocator in plant mitochondria has been suggested via experiments performed on isolated mitochondria from various plant tissues, such as maize shoots [19], potato tubers [20] and hypocotyls of etiolated bean [21]. Plant mitochondria swell spontaneously when suspended in a solution of ammonium phosphate. *N*-ethylmaleimide, which was known to inhibit phosphate hydroxyl antiport of animal mitochondria, inhibits this swelling by more than 80% [20]. If the ΔpH is collapsed with nigericin, which catalyses a K^+/H^+

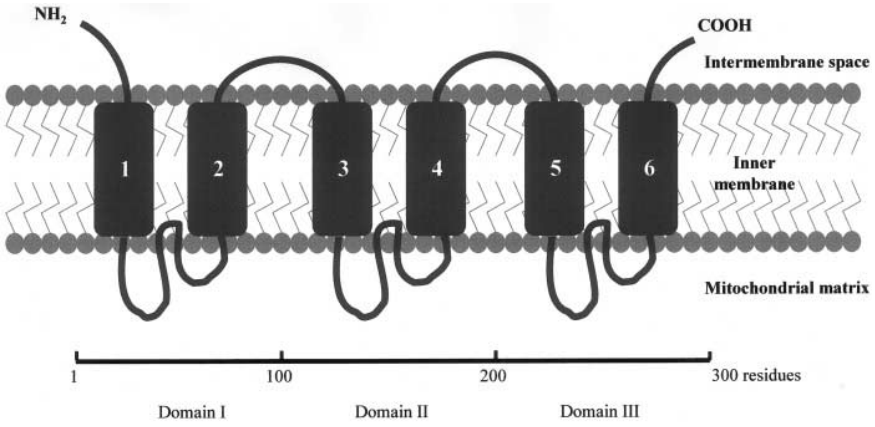

Figure 1. Mitochondrial carrier protein domain structure.

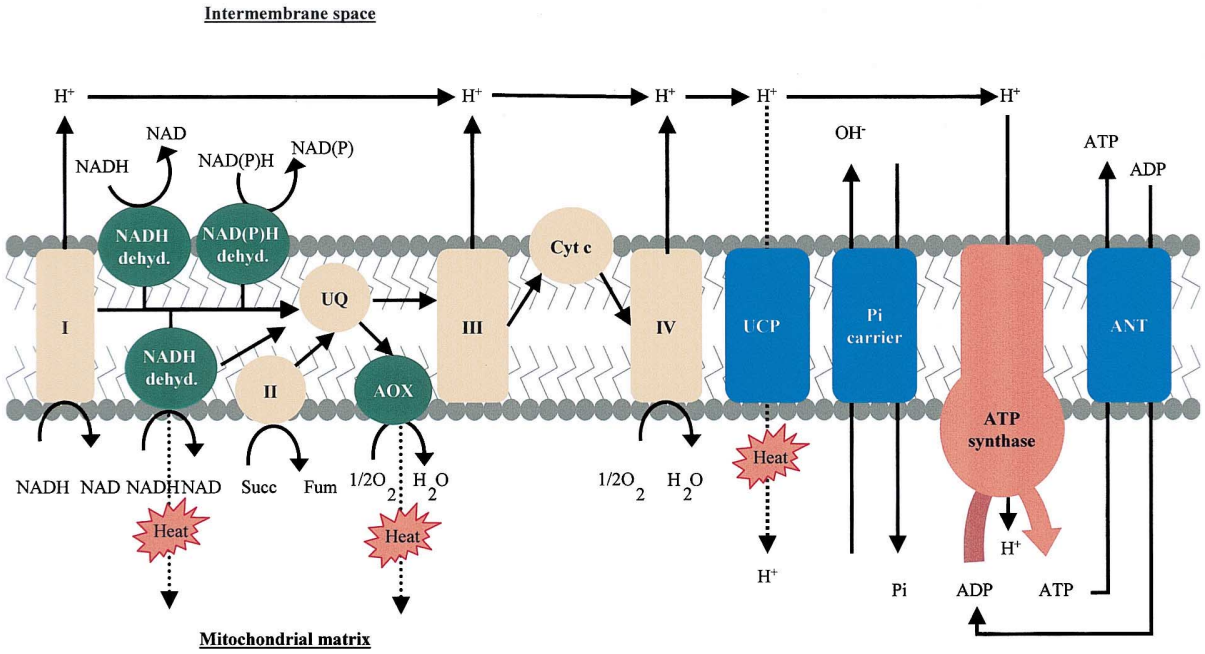

Figure 2. Schematic representation of the plant inner mitochondrial membrane showing proteins involved in the electron transfer common to plants and animals in orange, plant-specific proteins in green and mitochondrial carriers involved in oxidative phosphorylations in blue. I, Complex I or NADH dehydrogenase complex; II, complex II or succinate; III, complex III or cytochrome *c* reductase; IV, complex IV or cytochrome *c* oxidase; dehyd., deydrogenase; Succ, succinate; Fum, fumarate; AOX, alternative oxidase; Cyt c, cytochrome c.

exchange, a rapid passive efflux of Pi, which can be inhibited by mersalyl, occurs [22]. These results indicate that a phosphate carrier similar to that of animal mitochondria is present in plant mitochondria and that a high matrix-phosphate content is maintained by the ΔpH.

Hydroxylapatite chromatography with solubilised pea mitochondrial membranes gave a transporter-enriched fraction with no apparent loss in the phosphate transport activity. The major protein bands of this fraction have apparent molecular masses of 33 and 35 kDa [23]. After reconstitution into liposomes, this fraction was responsible for phosphate/phosphate exchange and phosphate/proton symport (table 1). Phosphate/phosphate exchange occurs with an apparent K_m of 1.6 mM and a V_{max} of 209 nmol/min/mg protein. This K_m value is in the same range than those obtained for mammalian ($K_m = 1.5–2.5$ mM) [16, 24–26] and yeast phosphate carriers (2.2 mM) [27]. Douce et al. [11], using isolated potato mitochondria, have reported a K_m of 0.6 mM and a V_{max} of 5.6 µmol Pi/min/mg protein. Hanson et al. [28] have determined a K_m of 0.2 mM. Even if the differences observed between these measurements can be easily explained by the use of different techniques or plants, we cannot exclude the detection of different PiC. Rebeille et al. [29], using ^{31}P-Nuclear magnetic resonance (NMR), determined a phosphate concentration in sycamore cells of $\sim$5–6 mM and 3 mM when cells were grown on phosphate-free medium. Under these conditions, the phosphate carrier is saturated and is not a limiting factor for Pi transport and respiration. In reconstituted vesicles, *p*-hydroxyphenylglyoxal, diethyl pyrocarbonate and sulfhydryl reagents such as *p*-hydroxymercuribenzoate, pyridoxal 5-phosphate and dansyl chloride inhibited exchange of phosphate. However, as for the yeast phosphate transporter and in contrary to bovine and rat carriers, NEM does not inhibit the pea mitochondrial phosphate carrier, indicating that pea PiC may be more related to yeast than mammalian carriers (see next paragraph). Carboxyatractyloside (CATR), α-cyano-4-hydroxycinnamate and *n*-butylmalonate did not inhibit phosphate transport, indicating that this carrier is different from the adenylate, monocarboxylate or dicarboxylate transporters.

Primary structure of mitochondrial phosphate carrier. A partial primary sequence of phosphate carriers was first determined in porcine and bovine heart mitochondria by direct protein sequence analysis [30, 31]. This sequence information was used to screen a bovine complementary DNA (cDNA) library with oligonucleotides

and led to the determination of the complete sequence of a bovine heart phosphate carrier [32]. Other mitochondrial *PiCs* have been cloned from rat liver [33], human heart [34], *Caenorhabditis elegans* and yeast [35]. Only recently, cDNAs with high similarity to these carriers have been described in plants. A strongly ozone-inducible transcript from *Betula pendula* has been isolated by differential display [36]. The screening under low-stringency conditions of a cDNA library of root tips of *Bradyrhizobium*-uninfected soybeans with a maize cDNA having similarity with early nodulin led to the isolation of a phosphate carrier cDNA from this species. Subsequently, maize, rice and *Arabidopsis* cDNAs were isolated with the soybean cDNA as a probe [37]. Finally, using an *Arabidopsis* Expressed sequence tag (EST) having similarity with animal phosphate carriers, we have isolated a potato phosphate carrier [Koßmann J., Laloi H., Riesmeier J.W., unpublished data]. All phosphate carrier cDNAs encode proteins which show a very high sequence similarity (30–91% identity) to one another and have the tripartite structure characteristic for the mitochondrial transporter family. They contain one mitochondrial transporter signature in the first 100-amino acid domain (table 2), and the hydrophobicity analysis has revealed the presence of six transmembrane regions. Plant phosphate transporters, like their animal counterparts, contain a putative targeting sequence at their N-terminal ends. In order to analyse the role of this presequence, Pratt et al. [38] have constructed a truncated rat liver PiC lacking 35 amino acid residues of the presequence. This truncated transporter has only little capacity for import into mitochondria, leading to the conclusion that optimal import of mitochondrial phosphate carrier is dependent on the presence of the presequence. However, a mature PiC lacking the entire presequence was imported with an efficiency of about 50% as compared with the precursor, demonstrating that the presequence was not essential [39]. Furthermore, the presequence by itself was not able to target a passenger protein into the mitochondria

Table 1. Biochemical characterisation of plant mitochondrial transporters via reconstitution in proteoliposomes.

Carrier	Plant	kDa	Substrates	Inhibitors	Ref
PiC	pea	33-35 H	Pi/Pi, Pi/H^+symport	sulfhydryl reagents, not NEM	[22]
	soybean	32 R	Pi/Pi	NEM	[35]
ANT	maize	32 H	ATP, ADP, GDP, GTP	ATR, CATR, BGK, Mersalyl	[51]
	pea	T	ATP/ADP	CATR	[54]
UCP	potato	32 H	fatty acid-mediated H^+ transport	ATP-GTP	[111]
PUMP				linoleic acid (activator)	
PTP	*Ricinus*	12-74 H	pyruvate/pyruvate	NEM, CHCA	[144]
	pea	19 H+A	pyruvate/pyruvate	CHCA	[145]
CTP	pea	14-35 H+A	citrate^T, succinate^T, malate^T, malonate^T, pyruvate^t, oxoglutarate^t, PEP^t, phosphate^t, isocitrate^t	*n*-butylmalonate, PCMB, 1,2,3-benzene-tricarboxylate, *p*-hydroxyglyoxal	[169]
DTP	pea	26 H+A	malate^T, succinate^T, phosphate^T, citrate^t, oxoglutarate^t	butylmalonate	[157]
OMT	corn	12–70 H	oxoglutarate^T, malate^T, malonate^T, oxaloacetate^T succinate^T, aspartate^t, glutamate^t, fumarate^t, phosphate^t, sulfate^t, citrate^t	phthalonate	[182]
	Panicum	32 R	malate^T, oxoglutarate^T, citrate^T, phosphate^t, glutamate^t, fumarate^t	n.d.	[183]
OAT	potato	T	oxaloacetate^T, malate^T, succinate^T, oxoglutarate^T, citrate^T, aspartate^T	phthalonate DIDS, pCMBS	[195]
Asp/Glu	pea	21 H+A	glutamate/aspartate	n.d.	[187]

PiC, phosphate carrier; ANT, adenine nucleotide translocase; UCP, uncoupling protein; PTP, pyruvate transport protein; CTP, citrate transport protein; DTP, dicarboxylate transport protein; OMT, oxoglutarate malate translocator; OAT, oxaloacetate transporter; Asp/Glu, glutamate-aspartate transporter; H, protein purification with hydroxylapatite column; A, identified protein with antibody; R, recombinant protein; T, total mitochondrial membrane proteins; ^{T}, transported substrates; ^{t}, substrates not transported; n.d., not determined.

Table 2. Cloned plant mitochondrial carriers with accession numbers in GenBank, number of amino acid residues, calculated molecular weight, calculated pI, mitochondrial signature and presence of presequence.

Carriers*	Accession no.	AA	kDa	pI	Signatures†	Preseq.	Ref.
AtPiC	AB016066(p)	288			PFEAVKVRVQ	1–?	[35]
BpPiC	Y08499	364	39	9.40	-M--------	1–?	[34]
GmPiC	AB016063	375	39.7	9.35	----------	1–?	[35]
OsPiC	AB016065	368	38.9	9.36	----------	1–?	[35]
StPiC		361	38.7	9.22	----------	1–?	
ZmPiC	AB016064	366	38.6	9.29	----------	1–?	[35]
AtANT1	X65549	379	41.3	9.87	PIERVKLLIQ PIDTVRRRMM	1–68	[63]
AtANT2	X68592	385	41.8	9.83	id	1–74	[64]
GhANT	AF006489	386	42.1	9.88	id	1–76	[65]
LaANT	AJ003197	388	42.1	9.76	id	1–?	[67]
LeANT	U89839	386	42	9.75	id	1–?	
OsANT	D12637	382	41.5	9.79	id	1–71	[66]
StANT1	X57557	386	41.8	9.74	id	1–77	[62]
StANT2	X62123	386	42	9.79	id	1–76	[61]
TtANT1	X95863	331	35.9	9.80	id	1–?	[60]
TtANT2	X95864	331	35.8	9.78	id	1–?	[60]
ZmANT1	X57556	387	42.4	9.85	id	1–77	[59]
ZmANT2	X59086	387	42.3	9.85	id	1–77	[59]
AtUCP1 or AtPUMP	AJ001264 AJ223983	306	32.7	9.62	PLDTAKVRLQ PTDLVKVRLQ PVDVVKSRMM ----------	no	[115]
AtUCP2	AB021706	305	33.4	9.05	---------- -I-------- ----------	no	
StUCP	Y11220	306	32.4	9.4	---------- ----------	no	[114]
OsOMT	AF010583(p)	218			no signature		
PmOMT	D45073	302	32.2	9.48	no signature	no	[183]
StOMT	X99853	297	31.7	9.46	PIDMIKVRIQ	no	
Ribes nigrum	AJ00758	289	30.4	7.61	PVDTLKTRIQ PMDVVKQRLQ	no	[200]

(p), partial sequence; id, identical. * See table 1 and figure 4 for abbreviations. † Mitochondrial signatures are written only for the first carriers; for the others only the differences are indicated. "-" indicates identical amino acids.

with a high efficiency, suggesting that the mature PiC contains information necessary for import. Finally, the deletion of the presequence was responsible for a more efficient heterologous import in yeast. These results indicate that the presequence has a stimulatory effect and determines the import specificity. Contrary to animal carriers, no information is available concerning the role of this cleavable presequence in plants.

NEM has been demonstrated to react only with one single cysteine residue, which is surrounded by two basic amino acids (Lys-Cys^{42}-Arg), in the animal phosphate carrier [40]. These basic residues lower the pK_a of the cysteine residue, consequently making it more reactive than the other cysteines toward alkylating agents. In the yeast carrier, which is insensitive to NEM, this cysteine residue is replaced by a threonine [27, 35]. The yeast carrier is, however, still sensitive to mersalyl, which might react with another cysteine residue. The replacement in the yeast PiC of the threonine by a cysteine [41] produced a Pi transport system with a cysteine residue flanked by two basic amino acids, resembling the bovine heart PiC. The reactivity of the new cysteine residue was greatly increased by the adjacent basic residues, and the yeast PiC-T^{43}C acquired NEM sensitivity. In plant PiC, the presence of Cys^{42} might indicate that they are NEM-sensitive. However, an asparagine, and not an arginine residue, follows this residue. Takabatake et al. [37] have measured phosphate uptake by proteoliposomes containing a solubilised and reconstituted soybean PiC purified after overexpression in *Escherichia coli*. Uptake experiments with such a system have shown that the soybean protein was responsible for a Pi/Pi exchange, which was almost completely inhibited by 0.5 mM NEM. These results disagree with the data from McIntosh and Oliver [23] obtained after reconstitution of a purified pea mitochondrial phosphate transporter. The pea carrier in those conditions was not inhibited by NEM. The au-

thors postulated that the pea carrier might be more similar to the yeast protein and lack the reactive cysteine residue. This hypothesis can only be confirmed with the pea amino acid sequence.

The replacement of each of the three cysteine residues (Cys^{28}, Cys^{134}, Cys^{300}) present in yeast transporter by a serine [17] reveals that Cys^{300} does not seem to be relevant for transport activity, that Cys^{134} is the major target for SH reagents like mersalyl and p-chloromercuribenzenesulfonic acid (PCMBS) and that Cys^{28} is involved in the reversible conversion between coupled antiport and uncoupled uniport of the PiC. The Cys^{300} is only present in yeast. In plant and animal proteins, it is replaced by a leucine. This might argue for a diminished role in transport activity. In contrast, Cys^{28} seems to be essential for the transport mode, being common to all PiC cloned in animals and in plants. Like the yeast PiC, animal PiCs have been described as able to switch between coupled and uncoupled transport modes [16]. Considering the presence of Cys^{28} in plant carriers, it is tempting to postulate that these proteins can also switch their transport mode even if no evidence for this is available. Cys^{134} is common to yeast and plant carriers but is replaced in animals by an alanine. Animal carriers are also sensitive to SH reagents, which might in this case react with another residue. Furthermore, plant and animal carriers have four other Cys residues in common which are not present in the yeast protein. Finally, three residues (His^{32}, Glu^{126}, Glu^{137}) which are conserved in yeast and animal phosphate carrier proteins [42] have been described to be critical for transport since their replacement in the yeast phosphate carrier blocks the transport activity. Phelps et al. [42] proposed, by comparison with the transport pathway for protons in bacteriorhodopsin, that these residues form a proton cotransport pathway which is somehow coupled to a phosphate transport path. Interestingly, these residues are conserved in plant phosphate carriers, and are not present in other mitochondrial transporters. Thus, plant PiCs might also use the proton pathway, which seems to be a general feature for all mitochondrial phosphate carriers.

Genes and gene expression. In human and bovine genomes the presence of a single gene encoding a phosphate carrier has been reported, but both animal genes contain evidence for alternative splicing [43]. Two isoforms, A and B, can be generated. Isoform A is highly expressed in heart, skeletal muscle and diaphragm mitochondria, whereas isoform B is ubiquitously expressed [44, 45]. The two isoforms have the same substrate specificity and inhibitor sensitivities, but isoform A has a higher affinity for Pi. These results lead to the hypothesis that isoform B is responsible for the basic energy requirements for all tissues, and isoform A becomes operative to accommodate higher-energy demands associated with contraction of muscle fibres. In plants, Kiiskinen et al. [36] have suggested that the birch genome contains a single copy of the mitochondrial transporter. However, Takabatake et al. [37] suggested the presence of multiple copies of phosphate transporter genes in plants, which is in contrast to the cases of mammalian and yeast carriers.

Northern blot analysis performed with soybean [37] indicates that the phosphate carrier is expressed at a high level in meristematic tissues such as root tips and shoot apices. The messenger RNA (mRNA) level of developing root nodules was high, but it decreased with nodule maturation and the beginning of nitrogen fixation. Kiiskinen et al. [36] found that birch phosphate carrier expression increases slowly with the expansion of young leaves, that the expression in roots is moderate and that the highest expression occurs in young shoots. In our laboratory, also using Northern analysis, we observed that the phosphate carrier from potato was strongly expressed in roots, small tubers and buds. The expression was lower in sink leaves, petiole, shoots and stolons [Kossmann J., Laloi M., Riesmeier J.D. unpublished data]. Finally, Kiiskinen et al. [36] have isolated a birch cDNA encoding a protein with similarity to a PiC by screening a cDNA library prepared from birch leaves after 2 h of ozone exposure. They confirmed the induction of the expression by Northern analysis and could point out an accumulation of transcripts during oxidative stress imposed by ozone 2 h after the beginning of ozone exposure. This accumulation was maximal at 12 h. Considering that ozone-induced responses in plants resemble pathogen-induced responses, the authors postulate that the increase of PiC expression by ozone could be related to an increase in mitochondrial activity associated with oxidative stress.

In conclusion, the mitochondrial phosphate carrier seems to be highly expressed in developing organs where tissues contain dividing cells requiring a high energy level. This expression indicates that, together with the adenine nucleotide translocator, the phosphate carrier plays an important physiological role in the energy supply for the cells.

Adenine nucleotide translocator

The ANT, the most abundant carrier in mitochondria, catalyses the exchange of ATP^{4-} synthesised by oxidative phosphorylation in the mitochondria matrix with cytosolic ADP^{3-}. In animals, the transport is limited to ATP and ADP only; Adenosine monophosphate (AMP) and the guanine, cytidine and uracil nucleotides are transported at very low rates [46]. The driving force for the transport is the membrane potential across the inner membrane (outside positive) generated by electron transport. Three specific inhibitors can inhibit the

adenine nucleotide transport: atractyloside (ATR), carboxyatractyloside (CATR) and bongkrekic acid (BGK). The transporter is able to bind ATR and BGK in an asymmetric manner. The ATR binds to the carrier on the cytosolic surface of the inner membrane and BGK on the matrix-facing surface. Finally, it has been demonstrated that the adenine nucleotide translocator can take two conformations, named C and M. ATR and CATR stabilise the C conformation and BGK the M conformation [47].

Biochemical features of plant adenine nucleotide transporter. In plant mitochondria the presence of an ANT was first suggested by the observation that ATR inhibits the state 3 respiration initiated by ADP in intact cauliflower mitochondria [48]. Direct uptake of ADP and ATP by isolated mitochondria from Jerusalem artichoke has been shown to be inhibited by BGK but not by ATR [49]. In contrast, ATR has been described to inhibit this exchange for corn mitochondria [50]. Furthermore, CATR appears to be a competitive inhibitor of the adenine translocation in bean hypocotyl mitochondria [51] and inhibits direct uptake of ATP and ADP in sycamore mitochondria [52]. All together, these studies have demonstrated that ATP/ADP transport in plant mitochondria involves an ANT similar to the mammalian one, being inhibited by BGK, CATR and ATR, although the inhibitory effect of the latter was less pronounced than in mammalian mitochondria.

The ANT has been successfully purified from maize shoot mitochondria using hydroxylapatite chromatography followed by Matrex Gel Blue B chromatography [53] (table 1). This procedure led to the isolation of a single polypeptide with an apparent molecular mass of 32 kDa. Partial amino acid sequencing revealed that the purified protein was the product of *ANT-G1* gene [54] (see below). When reconstituted into liposomes, this protein catalyses ATP/ATP and ATP/ADP exchange. K_m and V_{max} values for ATP transport were 17.8 ($\pm$ 2.9) μM and 2.15 ($\pm$ 0.53) μmol/min/mg, respectively. The specific exchange of ATP and ADP was strongly inhibited by ATP, ADP, ATR, CATR, BGK and mersalyl. Thus, the substrate specificity and the inhibitor sensitivity are very similar to those observed for adenine nucleotide transport in animal and yeast mitochondria. However, a difference was observed between animal and plant ANT: maize ANT was also able to transport GDP and GTP at a lower efficiency. Brustovesky and Klingenberg [55] have demonstrated that GDP and GTP bind to the ADP/ATP translocator of bovine heart but are not transported. This finding has led Genchi et al. [53] to suggest that maize and animal carriers do not differ in substrate binding but rather in the translocation channel.

In plants, ADP/ATP transport occurs not only in mitochondria but also in plastids. The kinetic constants and substrate specificities of these two transport systems have been compared. Envelope membranes from pea root plastids and spinach chloroplasts and membranes from pea leaf mitochondria were solubilised and reconstituted in liposomes [56]. The K_m values for the uptake of ATP into liposomes containing envelope membranes from pea root plastids and spinach chloroplasts were 30 and 70 μM, respectively. Those for the uptake of ATP and ADP into liposomes containing mitochondrial membrane proteins were 53 and 94 μM. The highest uptake rate for both translocators was found upon preloading with ATP or ADP. CAT inhibited both translocators; however, the mitochondrial ADP/ATP translocator was more strongly inhibited than the ADP/ATP translocator from chloroplast and pea root plastids. Furthermore, Western blot analysis performed with an antiserum directed against the ANT from *Neurospora crassa* has clearly shown that this antibody reacts with a 32-kDa polypeptide in the mitochondrial membrane but not with the proteins in envelope membranes of chloroplasts and pea root plastids. Based on these results, the authors have postulated that plastid and mitochondrial ADP/ATP translocators are proteins which might derive from different ancestors [56]. The sequence of two plastidic ANTs from *Arabidopsis* [57] confirms this hypothesis. Plastidic ANTs are highly hydrophobic membrane proteins (12 potential transmembrane helices), which have some similarities with bacterial adenylate transporter. This structure strongly differs from mitochondrial carrier structure, indicating that two different carrier families solve the transport of nucleotides in mitochondria and plastids.

Primary structure and gene expression. The primary structure of the mitochondrial ANT is now known in many organisms, and at least 31 sequences are reported in databases. The genomes of *Schizosaccharomyces pombe* [58] and *N. crassa* [59] are described as containing only one gene encoding a mitochondrial ANT. In contrast, three genes are reported in human and *Saccharomyces cerevisiae*. Plants seem to contain at least two genes, as described for maize [54, 60, 61], wheat [62], potato [63, 64], *Arabidopsis* [65, 66] and cotton [67]. The primary sequences for individual mitochondrial ANTs is also known in other plants such as rice [68], *Lupinus albus* [69] and tomato. The maize genes are very similar to one another and contain two introns which split the coding sequence into three approximately equal exons. The *Arabidopsis* genes contain three introns, one located in the 5′ region of the gene and two located in positions identical to the situation in the maize genes.

The deduced amino acid sequences of all these transporters are highly conserved and present the tripartite structure of mitochondrial carriers (table 2). They contain two mitochondrial signatures which are completely

identical in all the plant ANTs. Adenine nucleotide translocators from higher plants differ from their animal and yeast counterparts in the fact that they are synthesised with an N-terminal extension, which is cleaved upon import into the mitochondria. In vitro experiments performed with the maize ANT1 indicate that the mature part of the protein contains sufficient information for mitochondrial import, although the presequence may carry some targeting information as well [64]. To investigate whether the N-terminal extension is needed for correct intracellular sorting in vivo, Mozo et al. [70] have, in tobacco, expressed potato ANT-β-glucuronidase fusion proteins in the presence or absence of the ANT presequence. The analysis of these different transgenic lines has shown that the plant translocator presequence is not necessary for the correct localisation of the ANT to the mitochondria and that the mature part of the protein contains sufficient information for mitochondrial targeting. The information contained in the mature part of the protein is also sufficient to overcome the presence of a chloroplast targeting sequence as demonstrated by the highest β-glucuronidase (GUS) activity in mitochondria from plants expressing the *ANT* gene fused with the presequence of the chloroplastic triosephosphate/phosphate translocator. Transgenic plants expressing the *GUS* gene fused behind the ANT presequence exhibited the majority of GUS activity in the cytosol, demonstrating that the presequence alone is not able to target a passenger protein to mitochondria in vivo. Finally, the *ANT-GUS* construct was expressed in yeast. The plant ANT protein was located in the yeast mitochondria, and the absence or the presence of the presequence had no effect on targeting. All these results demonstrate that the plant ANT presequence has no influence on the efficiency of import into mitochondria.

Little information is available concerning the expression of *ANT* in plants. Potato *ANT* is expressed at a very high level in roots, anthers, tubers, stems and leaves [63]. Both maize genes are expressed in dark-grown coleoptile tissue. An active transcription of the translocator takes place in the basal meristematic, nonphotosynthetically active region of the leaf and decreases as the tissue becomes photosynthetically active [54]. This expression pattern raises the possibility that active mitochondrial biogenesis occurs in the basal meristem, which contains the actively dividing cells. Finally, rice *ANT* expression in suspension culture cells is four-fold induced by exposure to low temperature (10 °C for 5 days) or salt stress (2% NaCl for 24 h). Also, sucrose induced the transcription two-fold [68]. In all cases, it appears that ANT proteins are the most abundant nuclear-encoded mitochondrial carriers and that the gene is expressed in all tissues, the extent depending on the developmental state and regulated by external stresses.

Physiological relevance of the ANT. The ANT plays an important role in oxidative phosphorylation via the import of ADP and the export of ATP. Besides this major function, the ANT seems to be involved in different physiological events such as cytoplasmic male sterility, uncoupling of mitochondria and, in the case of animals, apoptosis.

ANTs have been described as having lower activity in cytoplasmic male sterile petunia [71] and *Sorghum* plants [72] than in fertile lines. In both cases, the export of ATP from mitochondria of normal plants is more efficient than in cytoplasmic male sterility (CMS) plants, and it appears that the affinity of the translocator for ATP in normal plants is higher than in CMS lines. As the translocator is associated with other constituents of the inner membrane, Liu et al. [71] postulated that the low affinity in CMS lines may be caused either by an alteration of the translocator structure or by a different interaction between this molecule and other components of the inner membrane. The smaller ANT activity in CMS lines may contribute to the male sterility via an inadequate supply of ATP in the cytosol leading to a low ATP/ADP ratio. However, Bush and Ninnemann [73] have suggested that these results must be interpreted with caution, having demonstrated that ATP can be formed in the intermembrane space at high ADP concentrations by the adenylate kinase (AK), and this process does not need the ADP/ATP translocator. The activity of this enzyme was not considered in the experiments performed by Lui et al. [71] and Arora et al. [72]; thus their results cannot exclude AK activity. Even so, AK can only work as a short-term adenylate buffer since AMP cannot be allowed to build up. Thus, in the long term, the cells might still lack ATP if the ANT is not functional, which may be a component leading to sterility.

Fatty acids are known to be effective uncouplers for mitochondria. In animals, it has been suggested that proteins of the inner mitochondrial membrane can facilitate the cyclic transfer of fatty acids and that inhibitors of ANT could suppress their uncoupling effect [74]. Furthermore, fatty acid-mediated H^+ transport could be demonstrated in proteoliposomes containing isolated and functional bovine ANT [55]. Involvement of the ANT in this process has also been demonstrated in yeast, studying the effect of fatty acids on the membrane potential ($\Delta\Psi$) of mitochondria isolated from wild-type yeast, or mutant yeast with either defective or deleted ANT. The addition of increasing concentrations of oleate and palmitate induced a decrease of $\Delta\Psi$ in wild-type yeast mitochondria, and this decrease was partially inhibited by bongkrekate. In contrast, the effect of fatty acids on $\Delta\Psi$ from yeast mutant mitochondria was quantitatively different; only a small portion of $\Delta\Psi$ was dissipated. Similar effects of fatty acids have

also been described in plants. The addition of a low palmitate concentration to pea or sunflower mitochondria decreases the $\Delta\Psi$, and CATR partially restores it [75], suggesting that also in plants ANT could be involved in uncoupling by fatty acids. Finally, when grown at 14 °C, seedlings of maize lines selected for high germination at 9.5 °C (C4-H) showed a higher activity of reconstituted ANT than seedlings selected for low germination (C4-L). This higher activity was associated with an increase in the 18-carbon unsaturated fatty acid level and a higher activity of cytochrome oxidase and mitochondrial guaiacol peroxidase [76]. This suggests that the ANT activity is also affected by chilling and might be dependent on the peroxidative modification of specific fatty acids which are connected to the active center of the carrier.

In animal mitochondria, fatty acids are also compounds that promote opening of the mitochondrial permeability transition pore (MTP: a nonselective channel that opens in the inner membrane as a result of Ca^{2+} accumulation in the matrix) [77]. It is now generally accepted that the ANT is one component of this pore because CATR and BGK, known to bind to this carrier, regulate its opening and closing [78]. Supporting this idea, it has been shown that isolated ANT reconstituted in liposomes could, in the presence of Ca^{2+}, form a high-conductance channel for small molecules similar to the MTP [79, 80]. More recent publications have pointed out that the long-chain fatty acids participate in the fatty acid-induced uncoupling of mitochondria by a direct action on the pore [81]. Furthermore, evidence has been presented that in animals opening of the MTP constitutes an important step in the apoptotic process [77, 82]. Considering that plant ANT can be involved in uncoupling of mitochondria by fatty acids, that apoptosis has been described in plants [83–86] and that the presence of an MTP in plant mitochondria has been suggested [87], it is tempting to postulate that also in plant, ANT could have an important function in programmed cell death, being a component of the MTP. This raises the question of the role of mitochondria, especially the function of the ANT or other mitochondrial carriers in plant programmed cell death.

Uncoupling proteins

Mitochondrial uncoupling proteins (UCPs) allow protons present in the mitochondrial intermembrane space to return to the mitochondrial matrix, bypassing ATP synthase and thus dissipating the proton electrochemical potential difference. This process results in an increase in mitochondrial respiration, and the energy liberated by the oxidation of different substrates is dissipated as heat [88].

Uncoupling proteins in mammals. The first uncoupling protein, called 'thermogenin' or UCP and now UCP1, was observed for the first time in rat brown adipose tissue by Ricquier and Kader [89]. These authors have shown that the content of a protein with an apparent molecular weight of 32 kDa was found to increase after cold adaptation. UCP1 has been studied in detail for more than 20 years, and numerous reviews describing this protein are available in the literature [90, 91]. Its role in the nonshivering thermogenesis of animals at birth or during hibernation has now been clearly demonstrated. The primary structure of UCP1 has been established by direct amino acid sequencing [92]. The corresponding gene has been cloned from different mammals [93–96]. Its expression is specific for brown adipose tissue and highly regulated (for a review see [97]). Due to this high specificity, the gene was thought to be unique and to have recently evolved with the appearance of mammals. The protein itself is also strongly regulated. Its activity is increased by free fatty acids [74, 98–100] and long-chain fatty acid Coenzyme A (CoA) esters [101, 102], and is decreased by purine nucleotide di- or triphosphates [98, 103]. UCP1 seems to be the simplest H^+ carrier known, and its transport mechanism requires fatty acids. Two principal mechanisms of H^+ transport for UCP1 are currently discussed. The first model proposes that fatty acids bind to UCP1 and that their carboxyl groups serve as H^+ donors and acceptors to resident carboxyl groups provided by Asp or Glu residues [88, 104]. In this model, fatty acids are not translocated through the membrane. Evidence for the existence of resident H^+ donor/acceptor groups has been given by site-directed mutagenesis. Two histidine residues (e.g. His^{145} and His^{147} of hamster) are proposed to be the final H^+ donors, which 'push' the proton into the mitochondrial matrix [105]. The second model proposes that UCP is not a proton transporter but a fatty acid anion carrier. UCP1 would transport deprotonated fatty acid and the protonated form cross the membrane by flip-flop, the result of which is an indirect transport of proton [74, 106, 107]. Since 1997, several groups have cloned other genes encoding uncoupling proteins [97]. *UCP2* has been isolated in mouse by screening a mouse muscle cDNA library with *UCP1* and in human by PCR amplification from human lung and skeletal muscle cDNA [108]. This gene is widely expressed in all mammalian tissues and might have a function in energy balance, diabetes, obesity and thermoregulatory responses to infection, and is probably a regulator of mitochondrial hydrogen peroxide generation. *UCP3* [109, 110] is preferentially expressed in skeletal muscle, which is thought to be a major site of adaptive thermogenesis in adult humans. However, UCP3 seems to contribute also to thermogenesis in brown adipose tissue in rodents, where its expres-

sion is highly regulated in a manner similar to UCP1. *UCP4* has very recently been identified via the analysis of EST databases, and a full-length cDNA was cloned by Polymerase chain reaction (PCR) reaction [111]. *UCP4* is specifically expressed in both foetal and adult human brain, which might indicate that this protein is involved in heat production and metabolism in the brain. Finally, via the screening of a mouse brain cDNA library with *UCP1* and *UCP2* cDNAs, another mitochondrial carrier called *BMCP1* (brain mitochondrial carrier protein-1) has been isolated. The human homologous cDNA has also been cloned. BMCP1 is equally similar to UCP1, UCP2 and UCP3 and the oxoglutarate malate translocator. However, their uncoupling activity after overexpression in yeast suggest that they could belong to the UCP subfamily [112].

Uncoupling proteins exist also in plants. In plants, the first evidence for the existence of a UCP-like protein called PUMP (plant uncoupling mitochondrial protein) was provided by analysis of potato mitochondrial respiration. A fully coupled respiration state could be obtained in potato mitochondria only when a purine nucleotide such as ATP was present, and simultaneously free fatty acids were absent [113, 114]. The addition of ATP to potato mitochondria produced a 50% decrease in the rate of state 4 respiration and an increase of about 15 mV in the membrane potential. This coupling effect could be modulated by the addition of Borin Serum Albumin (BSA) but was unaffected by oligomycin and carboxyatractyloside [115]. Furthermore, a protein of 32 kDa, named PUMP has been isolated from potato tuber. After reconstitution in proteoliposomes, PUMP was responsible for a fatty acid-mediated H^+ transport similar to that described for UCP1. The activity of the protein was downregulated by purine nucleotides such as ATP and GTP [116] (table 1). Other observations have also argued for the interaction of fatty acids with PUMP. Fatty acids induce the H^+-dependent swelling of potato mitochondria in potassium acetate medium in the presence of valinomycin [117]. Linoleic acid, a naturally abundant plant fatty acid, strongly stimulates the state 4 respiration rate and simultaneously induces a membrane potential drop in potato tuber [115, 117] and in green tomato fruit mitochondria [118]. Linoleic acid seems to effect state 4 and state 3 respiration, since the ADP/O ratio of tomato mitochondria decreases in a fatty acid concentration-dependent manner, indicating that PUMP is active during resting and phosphorylating respiration and might be able to divert energy from oxidative phosphorylation.

The existence of a UCP-like protein in plants was confirmed by the identification of *StUCP*, a potato cDNA encoding a peptide with high similarity to mammalian uncoupling proteins [119]. This cDNA was isolated via the screening of a cDNA library with a cDNA fragment isolated by a high-throughput yeast two-hybrid protein interaction trap. However, although PUMP and StUCP have a similar molecular mass, it has still not been demonstrated that *StUCP* encodes PUMP. A homologous cDNA (*AtPUMP*, or *AtUCP*) from *Arabidopsis* was subsequently cloned via the screening of a cDNA library with an *Arabidopsis* EST [120]. Based on genomic Southern blotting, Maia et al. [120] suggest that *AtPUMP* is a single-copy gene, but a second *Arabidopsis* cDNA called *AtUCP2* (AB021706) has recently been cloned. The deduced amino acid sequence of AtPUMP (AtUCP1), AtUCP2 and StUCP are strongly similar. AtUCP1 and StUCP show 81% identity between themselves. AtUCP2, however, shows only 66 and 67% identity to AtUCP1 and StUCP, respectively, indicating that two different uncoupling proteins exist in *Arabidopsis*. The percentage of identity to sequences of mammalian UCPs is between 37 and 45%. The genomic structures of genes for plant uncoupling protein from potato (*StUCP*) and *Arabidopsis* (*AtUCP*) have been more recently determined and appear to be different from the animal ones [M. Laloi et al., unpublished data]. Nine exons and eight introns form *StUCP* and *AtUCP1* genes. *UCP1* [121, 122], *UCP2* [123, 124] and *UCP3* [125, 126] are encoded by six exons, each coding for a segment with one transmembrane region. Each domain of 100 amino acids thus comprises two exons. In the case of plant uncoupling proteins this structure is found only in the first domain. Three exons encode the second domain and four exons the third domain. Thus, we can guess that mammalian genes have evolved from plant genes with the loss of introns.

Plant uncoupling proteins have the typical mitochondrial transporter structure. They are 300-amino acid proteins which have a predicted molecular mass of approximately 32 kD. Their amino acid sequence can be divided into three domains of about 100 residues, each containing a mitochondrial carrier signature (table 2). The predicted transmembrane topology for plant uncoupling proteins is strongly similar to that of mammalian uncoupling proteins and reveals the presence of six putative transmembrane regions as in all other mitochondrial transporters. Like their mammalian counterparts, they do not have obvious N-terminal mitochondrial-targeting sequences. The amino acid sequence of plant UCPs contains three conserved arginine residues (Arg^{83}, Arg^{182}, Arg^{276}) described in rat UCP1 to be essential for nucleotide binding and inhibition [127, 128]. Furthermore, the histidine pair (H^{145}, H^{147}) thought to be the final H^+ donor in UCP1 [105] is absent in all other known UCPs.

In contrast to *UCP1*, *UCP3* and *UCP4*, which are preferentially expressed in brown adipose tissue, skeletal

muscle and brain, respectively, *StUCP* and *AtPUMP* are, like *UCP2*, ubiquitously expressed. Furthermore, a very strong similarity can be observed when comparing the expression of *StUCP* and *AtUCP1*. They show similar trends, high expression in flowers, fruits and roots, but weak in leaves, and their expression is cold-induced to a similar extent. The amount of transcript increases significantly after 2–3 days' exposure to cold [119, 120]. Furthermore, we have also observed that the amount of *StUCP* transcripts decreases in the tubers as they grow. Using *StUCP* as a probe for Northern blotting, we could also show that the expression of uncoupling protein in tomato decreases with fruit ripening. The gene is strongly expressed in green fruit, but transcript is undetectable in red fruit [M. Laloi et al., unpublished data]. These data agree with the observation that, in tomato, PUMP levels detected by immunoblot analysis decrease during ripening [129]. As with PiC and ANT, uncoupling proteins are expressed in developing organs where cell division occurs intensively. However, with the discovery of AtUCP2, the investigations at hand [119, 120] with Northern analysis must be taken with caution. The expression of this second gene is still not described, and a cross-hybridisation with this gene cannot be excluded in the above results.

What is the role of UCPs in plants? Through heterologous expression in yeast and flow cytometry measurements, we have observed that plant UCPs decrease the growth rate of yeast cells via a partial decrease of the mitochondrial proton electrochemical gradient [119] to the same extent as animal uncoupling proteins UCP1, UCP2, UCP3 [108, 130]. This uncoupling activity added together with the cold induction of plant UCP expression might indicate that these proteins could be involved in heat production. Thermogenesis in plants has been previously described in different plant developmental processes such as fruit ripening and flowering, which are often correlated with a burst of respiration. Heat production has also been observed after exposure to chilling temperature, which might contribute to cold acclimation or resistance to chill [131].

Some observations agree with the idea that uncoupling protein is involved in fruit ripening. Measuring respiratory activity and transmembrane electrical potential of isolated mitochondria, Saviani et al. [132] suggested that PUMP and F_0 from β-detached ATP synthase both provide pathways for energy dissipation in ripened avocado fruit. The use of antibodies raised against potato PUMP leads to the detection of PUMP in many climacteric and nonclimacteric fruits [133] such as tomato, banana, mango, apples, pears, strawberries, papaya, muskmelon, peach, pinapple and orange [115]. Potato fruit and *Arabidopsis* silliques also show strong expression of UCPs. In tomato fruit, the UCP transcript level as well as the amount of protein decreases when the fruit turns red [129] [M. Laloi, unpublished data]. However, the activity of the protein seems to be induced in red fruit by fatty acids [115]. Finally, based on very preliminary experiments performed with an ethylene-insensitive mutant of *Arabidopsis* (*etr1*), Maia et al. [129] proposed that ethylene might be a negative regulator of UCP expression. More experiments must be performed to confirm these data and clarify the role of plant UCPs in fruit ripening.

Heat production in flowers is well documented for plants from the Araceae family. Apart from this family, thermogenesis is found in other monocotyledons such as Cyclanthaceae (Panama hat palms) and Palmae (palms), in dicotyledons such as the Annonaceae, Aristolochaiceae, Nymphaceae and Nelumbonaceae families, and in male reproductive structures of gymnospermic cycads [134, 135]. In Araceae inflorescence there is, before pollination, an increase in respiration and large heat production, which leads to the evaporation of odoriferous compounds in order to attract pollinating insects [136, 137]. Because the respiratory rate, via the alternative oxidase pathway, increases at the peak of heat production, the alternative pathway has been thought to be involved in this process. Even so, Wagner and Krab [138] estimate that the respiratory rate and amount of alternative oxidase are not large enough to produce significant levels of heat. Alternative oxidase is also believed to be involved in chilling protection [131]. However, Breidenbach et al. [139], considering calorimetric, respirometric and heat dissipation data, have determined that the reactions of the alternative oxidase pathway do not have larger exothermic enthalpy changes than those of the cytochrome oxidase pathway. Heat dissipation after cold exposure due to the alternative oxidase is not sufficient to raise tissue or organelle temperature. Thus, they disagree with the idea that the alternative pathway is thermoregulatory, protecting plants from exposure to cold, and seek other ways to explain the relationship between partitioning of the electron flow and physiological conditions such as low temperature. One alternative could be that plant UCPs, the expression of which is also cold-induced, may contribute to heat generation in thermogenic plants or nonthermogenic plants. Furthermore, the inner mitochondrial membrane contains a third overflow mechanism, the internal rotenone-insensitive NADH dehydrogenase, which transfers electrons from NADH to ubiquinone, without coupling to proton transport. Although this pathway seems to proceed only when the NADH/NAD ratio is high, it would be interesting to consider its role in heat production in plants. It is actually not possible to distinguish the precise function of these three proteins in thermogenesis in plants. Further analysis must be performed to determine whether they can act together for heat evolution.

It should be mentioned that the uncoupling proteins and the alternative oxidase have been found in nonthermogenic plants and are also expressed in many organs without cold stress [119, 129, 138]. Thus, heat production might not be the primary function of these plant proteins but rather a secondary effect of a more general function. Based on the fact that the inhibition of PUMP activity by the addition of ATP and BSA increases mitochondrial H_2O_2 generation, it has been suggested that PUMP activity decreases the production of mitochondrial reactive oxygen species [140]. A similar regulatory function has been postulated for the alternative oxidase [141, 142] and for rat UCP2, which could decrease the formation of reactive oxygen species under stress conditions by preventing overreduction of the respiratory chain [143]. Finally, we can postulate that plant UCPs could maintain the energy balance of the cell via the downregulation of ATP production and elimination of energy excess.

In conclusion, it appears that plant mitochondria have two energy-dissipating systems: the alternative oxidase, which may prevent the buildup of the mitochondrial transmembrane potential, and the uncoupling protein, which decreases it. Although it has been recently proposed that these two systems could work sequentially during the life of the plant cell [144], further experiments must be performed to determine the real function of each protein and their roles in plant thermogenesis and regulation of metabolism.

Carriers involved in the transport of TCA cycle intermediates

In plants, the TCA cycle plays an important role not only in the breakdown of respiratory substrates but also in many biosynthetic pathways via the supply of diverse intermediates which are precursors for different biosynthetic reactions in the cell. Both functions of the TCA cycle involve the activity of mitochondrial carriers either for the import of respiratory substrates such as pyruvate, malate and oxaloacetate, or for the constant export of intermediates. Monocarboxylate, dicarboxylate carriers, citrate transporter, oxaloacetate transport system and the oxoglutarate/malate translocator are examples of carriers involved in such exchanges (fig. 3).

Pyruvate transport protein (PTP) or monocarboxylate transporter

Pyruvate uptake in mammalian mitochondria occurs in exchange for OH^- (or in conjunction with H^+), is electroneutral, inhibited by thiol reagents, by α-cyano-4-hydroxycinnamate (CHCA) and α-cyano-β-(1-phenylindol-3-yl)acrylate (UK5099). Pea leaf mitochondria transport pyruvate via a specific carrier which mediates the electroneutral uptake of pyruvate, driven by ΔpH [145]. In isolated corn mitochondria, pyruvate oxidation and swelling in pyruvate solutions is inhibited by CHCA [146]. The accumulation of pyru-

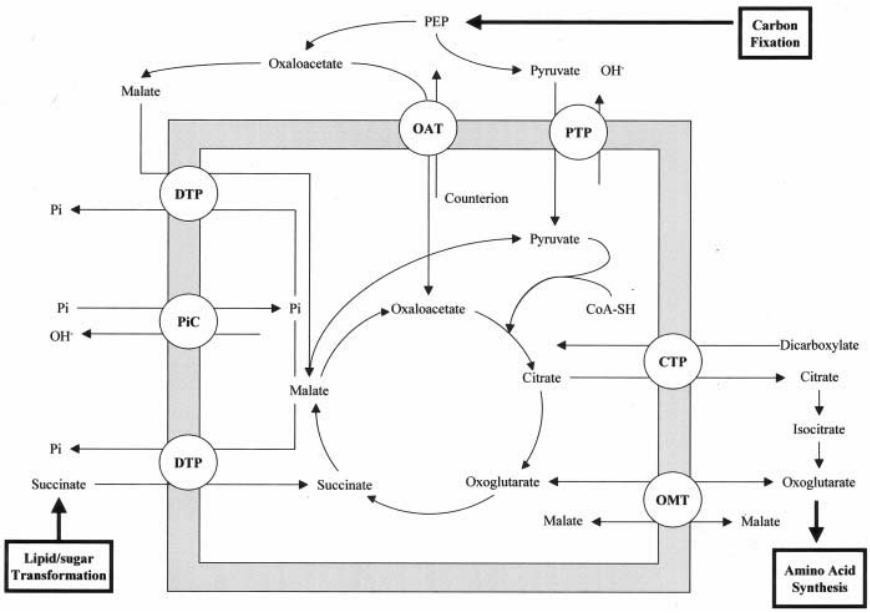

Figure 3. Schematic representation of the plant TCA cycle showing the different carriers involved in the transport of TCA cycle intermediates and the interactions with other plant processes. PiC, phosphate carrier; PTP, pyruvate transport protein; DTP, dicarboxylate transport protein; CTP, citrate transport protein; OMT, oxoglutarate-malate translocator; OAT, oxaloacetate transporter.

vate in mitochondria isolated from the endosperm of *Ricinus communis* is inhibited by UK5099 [147]. Finally, pyruvate uptake has been also described in mung bean [145], and in spadices of *Sauromatum guttatum* and *Arum maculatum* [148]. All these observations demonstrate that plant pyruvate transport has biochemical features similar to mammalian transport and involves a carrier.

Brailsford et al. [149] have solubilized and partially purified the pyruvate carrier from castor bean mitochondria using hydroxylapatite column chromatography (table 1). They were able to isolate six major bands of M_r 74, 66, 34, 32, 30 and 12 kDa. After reconstitution in liposomes the eluate was able to catalyse a pyruvate exchange sensitive to NEM and to analogues of α-cyanocinnamate. It was concluded that one or more proteins in the eluate correspond to the pyruvate carrier. Afterward, a monocarboxylate transporter was identified in pea (*Pisum sativum*) mitochondria by means of a specific monoclonal antibody [150]. A protein fraction was partially purified over a hydroxylapatite column and incorporated into liposomes (table 1). This fraction was responsible for a pyruvate/pyruvate exchange reaction, which was blocked by the antibody and by α-cyano-4-hydroxycinnamate. The antibody was then used to identify the protein involved by Western analysis. The transporter was identified as a 19-kDa protein. This molecular mass seems to be low in comparison with the pyruvate carrier isolated from other organisms. A protein of 34 kDa has been purified from bovine heart mitochondria, rat brain and rat liver [151, 152]. In yeast, the pyruvate carrier was identified in a fraction containing two polypeptides of apparent molecular mass 26 and 50 kDa [153]. Vivekananda and Oliver proposed that the 19-kDa polypeptide might be only one subunit of a multiple subunit protein. If this hypothesis is true, the pyruvate carrier might be different from the other mitochondrial carriers and thus may not belong to the mitochondrial carrier family. However, this seems unlikely, considering the molecular weight of bovine carrier and the presence of three proteins of $\sim$30 kDa in the purified fraction from Castor bean. Thus, we cannot exclude that the molecular weight of 19 kDa is not the real mass of the carrier. Finally, the primary structure of the pyruvate carrier is still unknown, and no genes have been identified in any organisms.

Dicarboxylate transporter

The dicarboxylate transport protein (DTP) catalyses an exchange between phosphate (HPO_4^{2-}) and dicarboxylates^{2-} such as malate, succinate and malonate and also a dicarboxylate^{2-}/dicarboxylate^{2-} exchange. Evidence for the existence of such a transporter in plants was first proposed by indirect techniques such as swelling experiments and oxidation capacities of isolated mitochondria. Plant mitochondria suspended in solutions of ammonium malate, succinate, malonate but not fumarate swell on addition of small amounts of phosphate [12, 20]. Butylmalonate and pentylmalonate inhibited this swelling as well as oxidation of succinate and malate by isolated mitochondria [154–158]. Direct uptake experiments on mitochondria isolated from different plants such as bean [159], potato [160] and castor bean [161] suggest that the transport is electroneutral but dependent on the electrochemical gradient via the phosphate carrier. The electron transport chain activity creates the electrochemical gradient which drives the exchange of hydroxyl and phosphate on the phosphate carrier. The accumulated phosphate can then exchange for external dicarboxylates via the dicarboxylate transporter.

The dicarboxylate transporter from pea leaf mitochondria has been purified using three monoclonal antibodies able to inhibit dicarboxylate transport [162]. A protein fraction was isolated from pea mitochondria by solubilisation and hydroxylapatite chromatography (table 1). This fraction was reconstituted into liposomes, and [^{14}C]-malate uptake was studied. The transport was dependent on preloading with malate and was sensitive to butylmalonate. The protein was able to transport malate, succinate and phosphate and, with a lower efficiency, citrate and 2-oxoglutarate. The authors concluded that the purified fraction also contained tricarboxylate and oxoglutarate transporters. The transporter has been identified as a protein having an apparent molecular mass of 26 kDa using Western blot analysis [162]. Antibodies were used for the screening of a pea cDNA library, but only a partial cDNA (0.7 kb) was isolated [163], and the entire primary sequence of plant DTP has not been described. In contrast, the sequences of dicarboxylate transporters are known in other organisms such as yeast, rat, mouse and *C. elegans*. The primary sequences found in these organisms have the specific tripartite structure of the mitochondrial transporter family and are highly conserved. The yeast protein was identified after overexpression in *E. coli* and reconstitution in liposomes [164, 165]. The recombinant protein was able to transport malate [154] and malonate [165] against malate, phosphate, malonate and succinate. Using the yeast sequence, Fiermonte et al. [166] identified a related protein encoded in the genome of *C. elegans* which was used to identify murine EST sequences, in turn used to isolate a rat cDNA by PCR. After reconstitution in proteoliposomes, the rat and *C. elegans* carriers were able to exchange malate against malate, phosphate, malonate, succinate, sulfate and thiosulfate. The transport activities for both proteins were inhibited by butylmalonate,

benzylmalonate, phenylsuccinate, bathophenanthroline and pyridoxal 5′-phosphate [166]. The rat gene, which seems to be a single-copy gene, is most strongly expressed in liver and kidney, at a lower level in brain and was also detected at a very low level in heart. The high expression level in kidney and liver seems to be related to gluconeogenesis and ureogenesis, which occur mainly in these tissues. However, this carrier might have another unknown function in other tissues.

In plant cells, phosphoenolpyruvate, which is a product of glycolysis, can be converted into pyruvate by pyruvate kinase or into oxaloacetate by the action of PEP carboxylase. Oxaloacetate can then be converted into malate by cytosolic malate dehydrogenase. Pyruvate and malate constitute substrates which enter the mitochondria via the pyruvate carrier and the dicarboxylate carrier, respectively (fig. 3). Few data have pointed out the physiological importance of malate import into the mitochondria via the dicarboxylate carrier. In thermogenic spadices of *Arum maculatum*, butylmalonate, the specific inhibitor for the dicarboxylate carrier, decreases the respiration rate [167]. Furthermore, based on the observation that transgenic tobacco plants without cytosolic pyruvate kinase were indistinguishable from wild-type plants, it has been proposed that malate import could substitute pyruvate uptake into the mitochondria [168] and that in presence of the NAD^+-malic enzyme the TCA cycle can be functional with malate as sole substrate. This gives evidence of the importance of the dicarboxylate carrier in the supply of the major substrate for the TCA cycle.

The dicarboxylate carrier also seems to be important during lipid mobilisation. Ammonium salt-swelling experiments performed with mitochondria isolated from cucumber cotyledons show that pyruvate oxidation is limited by pyruvate uptake into mitochondria isolated from dark-grown cotyledons and light-grown cotyledons less than 6 days old. However, in mitochondria from 6- and 7-day-old light-grown cotyledons the pyruvate uptake increases, indicating that the mitochondrial capacity for pyruvate uptake increases during photosynthesis [169]. These data suggest that during early seedling development, when lipid mobilisation occurs, the activity of the pyruvate carrier is low and perhaps insufficient. Therefore, the role of the dicarboxylate carrier [170] might be even more important during lipid mobilisation when carbon enters the mitochondria as succinate or malate, substrates for the gluconeogenesis.

Citrate transport protein (CTP) or tricarboxylate transporter

Three mechanisms have been postulated for citrate transport into plant mitochondria. A proton/citrate symport with maximum activity at pH 4.5 [171, 172], a direct citrate/phosphate antiport [12, 173] and a tricarboxylate transporter similar to that found in animal mitochondria [20, 159, 160]. It is now accepted that the latter transport system is that one which occurs in vivo. The CTP catalyses the exchange of a tricarboxylate anion ($citrate^{3-}$, isocitrate, cis-aconitate) with a dicarboxylate anion ($malate^{2-}$). The main evidence for this carrier in plants has been obtained from passive swelling experiments [20] and exchange studies [159]. Potato mitochondria swell in ammonium citrate only in presence of both phosphate and a dicarboxylate. Malate, malonate and phosphoenolpyruvate, in contrast to phosphate, sulphate and oxoglutarate, stimulate direct exchange of internal citrate with external anions in isolated mitochondria [159]. 1,2,3-Benzenetricarboxylate inhibits these exchanges. From these experiments it was concluded that three transporters operate in sequence. The phosphate carrier, which requires a pH gradient across the membrane, imports the Pi, the dicarboxylate carrier exchanges this phosphate for dicarboxylate and the tricarboxylate carrier exchanges dicarboxylate for citrate.

A tricarboxylate transporter from pea mitochondria has been partially purified from solubilised mitochondrial membrane by hydroxylapatite chromatography [174]. After reconstitution into artificial membranes (table 1), the transporter exchanges citrate against citrate, succinate and malate. Pyruvate, 2-oxoglutarate, phosphate and ATP had low or no effect on the rate of citrate/citrate exchange. The citrate/citrate exchange was sensitive to *n*-butylmalonate, the sulfhydryl reagent *p*-chloromercuribenzoate, 1,2,3-benzenetricarboxylate and the arginine reagent *p*-hydroxyglyoxal. The exchange reaction exhibited an apparent $K_{0.5}$ of 0.66 mM, which is within an order of magnitude of the $K_{0.5}$ of 0.13 mM and 0.12 mM reported for the reconstituted bovine [175] and rat liver [176, 177] transporters, respectively. All these data demonstrate that pea mitochondria contain a tricarboxylate carrier, which is different from the dicarboxylate carrier, in that it does not respond to phosphate and has high similarity with animal [178] and yeast citrate carriers [179]. However, pea mitochondrial transporter was neither inhibited by nor able to transport isocitrate and phosphoenolpyruvate, which are exchanged by the animal and yeast tricarboxylate carriers.

Until now no gene or cDNA encoding the tricarboxylate carrier has been isolated from plants. In rat, using the PCR reaction with primers derived from amino acid sequence information obtained by direct sequencing of a purified rat liver mitochondrial citrate transporter, a partial cDNA was isolated. This fragment was used to screen a cDNA library in order to isolate a full-length cDNA [180]. This cDNA encodes a mature 298-amino acid protein (32 kDa) which presents all the features of the mitochondrial carrier family and has a presequence

of 13 residues. Homologous cDNAs have now been described in human [181], cow [182] and yeast [179]. The rat and human genomes are believed to contain, respectively, two to three and three to five genes related to the citrate carrier; in contrast the yeast genome seems to contain only one. The yeast citrate carrier has been expressed in *E. coli*, purified and reconstituted in liposomes. The reconstituted protein catalysed a 1,2,3-benzetricarboxylate-sensitive citrate/citrate exchange and displays substrate specificity similar to mammalian carriers [179].

Plant mitochondria produce and export carbon skeletons for the synthesis of primary amino acids Glu and Gln. It was originally postulated that mitochondria synthesise and export 2-oxoglutarate [183]. An alternative pathway has been proposed by Chen and Gadal [184] that consider the high activities of aconitase and Nicotinamide adenine dinucleotide phosphate (NADP)-isocitrate dehydrogenase present in the cytosol [185]. The authors proposed that mitochondria need only to synthesise citrate, which is transferred to the cytosol where it is converted to 2-oxoglutarate. This hypothesis has been reinforced by the analysis of metabolite export from mitochondria isolated from illuminated pea and spinach leaves. During the oxidation of malate or oxaloacetate, citrate is mainly produced, and a much lower amount of 2-oxoglutarate is released [5]. These data provide evidence for the role of citrate carrier in the amino acid metabolism in illuminated plants.

Oxoglutarate/malate translocator

The existence of an oxoglutarate/malate translocator (OMT) has been postulated in bean mitochondria by measuring the direct exchange between intramitochondrial, labelled oxoglutarate and external anions and by testing the inhibitor sensitivity of this process [159]. Oxoglutarate is exchanged for succinate, malate, malonate and oxaloacetate but not for phosphate, citrate or phosphoenolpyruvate. This transporter appears thus to be different from the dicarboxylate and tricarboxylate carriers. Butylmalonate and phenylsuccinate but not mersalyl inhibited the transport of oxoglutarate. In other plant tissues such as thermogenic spadices, green leaf or etiolated plant tissues, 2-oxoglutarate uptake was found to be inhibited by phthalonic acid [186], a potent inhibitor of the 2-oxoglutarate transporter of rat liver.

The transporter has been partially purified from corn shoot mitochondria after solubilisation and hydroxylapatite/celite chromatography in the presence of cardiolipin (table 1). The eluted fraction contained proteins with molecular masses of 12 to 70 kDa [187]. After reconstitution into liposomes, the purified protein fraction exchanges 2-oxoglutarate against internal 2-oxoglutarate, L-malate, malonate, oxaloacetate and succinate. In contrast, a very low exchange was observed against aspartate, glutamate, fumarate, phosphate, sulfate and citrate. Furthermore, the 2-oxoglutarate/2-oxoglutarate exchange was inhibited by phthalonate, pyridoxal-5′-phosphate, mercurials but not NEM, as was reported for intact mitochondria in plant [159] and animals.

Three cDNA clones that show high similarity with bovine mitochondrial 2-oxoglutarate/malate translocator have been isolated from the leaves of *Panicum miliaceum* [188]. The three nucleotide sequences differ only by the presence of introns, indicating that the three cDNAs correspond to the same gene. The encoded protein is made up of 302 amino acids, has a molecular mass of 32 kDa and might contain six predicted transmembrane regions. This structure indicates that this protein might belong to the mitochondrial carrier family, although no mitochondrial signatures were detected in the peptide sequence. The cDNA was overexpressed in *E. coli* in order to purify the encoded protein. After reconstitution into liposomes, the isolated protein was able to transport malate and 2-oxoglutarate but also citrate with a high efficiency. Phosphate, glutamate and fumarate were not transported (table 1). The transport properties of the recombinant protein partly disagree with those of the 2-oxoglutarate/malate translocators purified from animals or maize. It is thus not clear whether this translocator is functionally distinct from other translocators in vivo. Messenger RNA for the *Panicum OMT* gene was detected by Northern analysis. A higher level was found in green leaves than in nonphotosynthetic tissues such as etiolated leaves or roots [189]. When the full-size cDNA fragment was used as a Northern probe, transcripts were detected in mesophyll cells and in bundle-sheath cells; however, if the 3′-untranslated region was used, transcripts were detected only in the bundle-sheath cells, this expression was also confirmed with Western analysis. These results indicate the presence of a general mitochondrial *OMT* gene expressed in both types of cells (bundle-sheath and mesophyll cells) and also a bundle-sheath cell-specific *OMT* gene. The transcript level for the bundle-sheath cell *OMT* rapidly increases with periods of illumination shorter then 24 h. It is also found to increase with cell development as demonstrated by a higher amount of transcript in leaf sections of the tip than of the base of leaf. Taniguchi and Sugiyama [189] suggest that the OMT is likely to participate closely in the C4 photosynthetic pathway. Furthermore, Northern analysis has shown that unspliced mRNAs were present in leaf tissues, which indicates that this gene might also be post-transcriptionally regulated. Finally, using an *Arabidopsis* EST, which showed similarity with the animal 2-oxoglutarate/malate translocators, we screened a

potato leaf cDNA library and have isolated a cDNA which encodes a protein showing high sequence similarity to animal OMT and the *Panicum* malate carrier. The protein has the specific mitochondrial carrier structure and contains one signature (table 2). This gene, which might encode a mitochondrial carrier, is expressed in all potato organs [unpublished data].

Few data are available concerning the physiological function of the oxoglutarate/malate carrier in plants. As mentioned before, this carrier could be involved in the export of 2-oxoglutarate for the biosynthesis of primary amino acids. However, citrate seems to be the most important intermediate produced. In animal mitochondria, the oxoglutarate/malate translocator has been shown to play a central role in the transfer of reducing equivalents from the cytosol to the mitochondria. Together with the aspartate/glutamate carrier, it constitutes the malate/aspartate shuttle, a cyclic pathway allowing the indirect transfer of reducing equivalents through the inner mitochondrial membrane. This shuttle is involved in gluconeogenesis and urea synthesis. Although some data suggest the presence of an aspartate/glutamate carrier in plant mitochondria [190–192], the malate/aspartate shuttle is thought not to be very important in this process since most plant mitochondria oxidise cytoplasmic NAD(P)H via external NADH and NADPH dehydrogenases.

Oxaloacetate transporter (OAT)

Animal mitochondria under normal physiological conditions do not transport oxaloacetate. However, some reports have shown that it can be slowly transported via the dicarboxylate [193] and/or the oxoglutarate/malate carrier [194].

In contrast, plant mitochondria transport oxaloacetate at rapid rates through their inner membrane. This transport was first suggested by the fact that O_2 consumption during glycine or malate respiration in isolated mitochondria is instantaneously inhibited by externally added oxaloacetate. This phenomenon was explained by a lack of NADH for the oxidative phosphorylation, as the NADH produced in the matrix by glycine decarboxylation or malate oxidation is reoxidized via malate dehydrogenase converting oxaloacetate, which enters the mitochondria, to malate [195]. It was later demonstrated that oxaloacetate transport in plant mitochondria is carrier-mediated and inhibited by phthalonate [196]. Furthermore, butylmalonate, an inhibitor of the mitochondrial dicarboxylate carrier has no effect on the oxaloacetate inhibition of respiration [195], indicating that the dicarboxylate transporter is not involved. It has been suggested that the 2-oxoglutarate carrier could be involved, as phthalonic acid, an inhibitor of the 2-oxoglutarate carrier, could reverse the inhibitory effect of oxaloacetate, mentioned above [186, 196, 197]. However, an excess of malate, malonate, succinate, 2-oxoglutarate or phosphate has little effect on the rate of oxaloacetate transport in isolated pea mitochondria, indicating that the transport of oxaloacetate occurs via a carrier different from the dicarboxylate and the 2-oxoglutarate carriers [197]. The possibility of an exchange with hydroxyl ions has also been excluded, since oxaloacetate uptake decreases with decreasing pH [197, 198]. In contrast, other studies of oxaloacetate uptake into isolated mitochondria have proposed a link between oxaloacetate and malate transport such that the uptake of oxaloacetate by mitochondria can be increased in presence of malate [198]. The high activity and low sensitivity toward inhibition by other metabolites led to the idea of a specific malate-oxaloacetate shuttle. Furthermore, the swelling of pea leaf mitochondria resuspended in isotonic solutions of ammonium malate plus phosphate occured until valinomycin was added. This swelling was then strongly inhibited by oxaloacetate. These results suggested that oxaloacetate and malate were transported by electrogenic uniport [199].

More recently, Hanning et al. [200] have reinvestigated the properties of the malate oxaloacetate shuttle using proteoliposomes, into which potato mitochondrial proteins have been reconstituted. They showed that oxaloacetate is taken up into the proteoliposomes only when dicarboxylates such as malate, oxaloacetate, succinate and oxoglutarate, but also citrate and aspartate, were present inside the vesicles. Phthalonate strongly inhibited all these exchanges. This suggests that the same carrier transports all these compounds. Furthermore, exchange of oxaloacetate in the absence of internal malate could not occur even in the presence of valinomycin, which makes the membrane more permeable to K^+. These results indicate that oxaloacetate uptake occurs by an antiport with dicarboxylates or tricarboxylates and not by electrogenic uniport as suggested by Zoglowek et al. [199]. The exchange of external oxaloacetate against internal malate is inhibited by *n*-butylmalonate, an inhibitor of malate transport, only when it is added inside the vesicles, suggesting that the malate-oxaloacetate shuttle might have different binding sites for malate and oxaloacetate. Finally, dithiocyanostilbene disulfonate (DIDS), pyridoxal, mersalyl and *p*-chloromercuribenzene sulfonate (pCMS) inhibited oxaloacetate uptake, indicating that the carrier might contain lysine residues and SH groups. Interestingly, the specificity of the reconstituted carrier in this system agrees with the specificity described for the OMT isolated from *Panicum milaceum*. Both carrier systems are able to transport malate, 2-OG and citrate, which could suggest that both are similar. However, the transport activity for the *Panicum* carrier was not tested

for oxaloacetate, succinate and aspartate as well as the inhibitory effect of phthalonate. Further experiments need to be performed in order to clarify the features of these carriers.

The importance of the malate-oxaloacetate shuttle in the transfer of reducing equivalents has been demonstrated [201–205]. During photorespiration, the reduction of hydroxypyruvate in the peroxisomes requires reducing equivalents, which are produced either during the photosynthetic electron transport in the chloroplast or during substrate respiration in the mitochondria. Three shuttles can perform this kind of transfer: the choroplastic malate-oxaloacetate shuttle, the malate/aspartate shuttle and the malate-oxaloacetate shuttle, both from mitochondria. Using isolated spinach or pea mitochondria, it has been estimated that 25 and 50%, respectively, of the reducing equivalents formed during oxidation of glycine and malate were exported for the reduction of hydroxypyruvate in the peroxisome via the mitochondrial and chloroplastic malate-oxaloacetate shuttles. No major activity of the mitochondrial malate/aspartate shuttle could be detected [5, 201], indicating that this shuttle might have a very small function, if any, in this process.

Other transporters

Amino acid carriers

In plant cells, at least two major pathways involve mitochondrial metabolism of amino acids. In the light many plants produce glycolate, which is oxidised in the peroxisome and leads to the production of glycine. Two molecules of glycine are taken up into the mitochondria where one is oxidised by the glycine decarboxylase to form CO_2, NH_3 and a one-carbon compound, which will join with another glycine to form a serine. Serine subsequently leaves the mitochondria to be converted to sugar. The other metabolic event is proline accumulation under environmental stresses. It is now known that the enzymes of proline catabolism are localized in the mitochondria, whereas the anabolic enzymes are present in the cytosol. Thus, the transport of amino acids through the mitochondrial membrane seems to be important. The mechanism by which amino acids are transported through the mitochondrial membrane is not well understood. According to some authors, glycine transport seems to be diffusional, because saturation kinetics were not observed [202]. In contrast, mitochondria isolated from mung bean hypocotyl swelled when added to an isosmotic solution of proline, serine, methionine, threonine, alanine and glycine. The swelling was inhibited by PCMB and mersalyl [203], indicating the existence of protein-mediated transport of amino acids. Similarly, the transport of glycine into mitochondria isolated from pea leaves has been described to be sensitive to sulfhydryl reagents (mersalyl and PCMB) and to be dependent on the transmembrane pH gradient, as seen from inhibition by uncouplers [204]. This supplies strong evidence for the existence of a glycine transporter. Furthermore, a study of the effect of pH on glycine oxidation has demonstrated glycine hydroxyl ion exchange [205]. The glycine transporter, which could exchange glycine against both hydroxyl ions and serine, would have a high binding affinity for glycine at high pH values and a lower affinity under low pH conditions. It seems more and more evident that glycine uptake into mitochondria involves a carrier which need to be characterised in more detail. It would also be important to clarify whether serine is transported by the same carrier.

Little evidence for the transport of glutamate and aspartate in plant mitochondria is available in the literature. Plant mitochondria seem to be capable of an electroneutral and reversible glutamate/aspartate exchange [190, 206]. Using monoclonal antibodies raised against crude mitochondrial membranes, Vivekananda and Oliver [192] have identified an antibody able to inhibit glutamate and aspartate-dependent oxaloacetate metabolism. This antibody was used to identify and purified a 21-kDa protein. After reconstitution into liposomes, this protein catalysed glutamate/glutamate and glutamate/aspartate exchange. However, glutamate uptake could not be driven by a pH gradient, providing no evidence for an electrogenic exchange. These data differ from those obtained for mammalian mitochondria, where glutamate/aspartate antiport occurs via an electrogenic transport system due to a proton cotransported with glutamate but not with aspartate [207]. Although we cannot exclude that the protein isolated from pea leaf is a glutamate/dicarboxylate exchanger (this possibility has not been tested), it seems that plant mitochondria differ from animal mitochondria by their lack of an electrogenic glutamate/aspartate carrier. In animal mitochondria, glutamate/aspartate exchange which is part of the malate aspartate shuttle, plays an important role in the transfer of reducing equivalents through the inner mitochondrial membrane. In plants, the transfer of reducing equivalents can be solved by two other systems, the oxaloacetate shuttle and an NADH dehydrogenase on the outer side of the inner mitochondrial membrane. Thus, the presence and function of the glutamate/aspartate transporter in plant mitochondria still needs to be clarified.

Two homologous genes encoding amino acids carriers have been isolated in *S. cerevisiae* (*AGR11*) [208] and *N. crassa* (*ARG13*) [209]. These genes encode proteins with the characteristic features of the mitochondrial carrier family. The yeast ARG11 protein has been produced in *E. coli* and, after reconstitution in liposomes,

was responsible for the transport of ornithine in exchange for protons [210]. Thus, we can guess that plant mitochondria contain members of the mitochondrial carrier family which are involved in amino acid transport. These carriers still need to be identified.

Cofactors

Some data indicate that plant mitochondria are also able to transport enzyme cofactors such as NAD^+, coenzymeA (CoA), thiamine pyrophosphate (TPP) and tetrahydrofolate.

Transport of NAD^+, CoA and TPP seems to be carrier-mediated because the accumulation of these cofactors follows saturation kinetics [11, 211–213]. In the presence of uncouplers, NAD^+ uptake is not only inhibited, but a net efflux is observed. Both uptake and efflux of NAD^+ are inhibited by an NAD analogue (*N*-4-azido-2-nitrophenyl-4-aminobutylryl-3′-NAD^+, $NAP_4 - NAD^+$), which indicates that the same carrier is involved in both directions. Thus, it is possible that the NAD^+ carrier regulates the mitochondrial NAD^+ pool and has a regulatory function modulating the activity of all NAD-enzymes. Accumulation of CoA in plant mitochondria is also sensitive to uncouplers but not to NAP_4–NAD^+, indicating that CoA and NAD^+ are transported by two different carriers. The physiological function of the mitochondrial CoA transport system might be to move the CoASH molecule from the cytosol, where it is synthesised, to the mitochondrial matrix, where it is used for the entry of different substrates in the TCA cycle. Thiamine pyrophosphate is a cofactor essential for the oxidation of pyruvate and 2-oxoglutarate catalysed by the pyruvate dehydrogenase and the 2-oxoglutarate dehydrogenase complexes, respectively. Isolated mitochondria appear to be depleted of TPP but can rapidly accumulate it from the external medium in a concentration-dependent manner, indicating that a protein is involved in this process.

Tetrahydrofolate is a coenzyme involved in reactions requiring one-carbon transfer. It is necessary for the glycine decarboxylase complex and serine hydroxymethyltransferase in the mitochondria, but also for thymidylate and methionine synthesis. Plants, in contrast to animals, are able to synthesise this cofactor via a biochemical pathway involving five enzymes. The activity of these enzymes has been detected only in mitochondria [214, 215] indicating that tetrahydrofolate synthesis appears mainly restricted to mitochondria. This implies that this cofactor might be exported toward the cytosol and the chloroplasts where it will be

Table 3. Putative members of the *Arabidopsis thaliana* mitochondrial carrier family with accession numbers in GenBank, chromosome localization, number of amino acid residues, calculated molecular weight, calculated pI, mitochondrial signature and highest identity with mitochondrial carriers from other organisms.

Accession no.	Chr	AA	kDa	pI	Mitochondrial signature sequence (position)	Highest sequence identity
AC002505	II	303	32.9	9.26	PCEVLKQRLQ (137–146) PFDVIKTRMM (228–237)	Yeast Pet8 (32%)
AC004165	II	331	35.9	8.82	PVDTVKTHMQ (58–67) PMDMVKQRLM (152–161)	*Ribes nigrum* (66%) MRS4-MRS3 (36%)
AC005397	II	358	38.8	9.37	PLDVVKTRLQ (41–50) PVELARTRMQ (178–187)	AL035524 (48%) YGR257c (29%)
AL035524	IV	378	41.5	9.6	PLDVVKTRLQ (46–55) PIDLARTRMQ (172–181)	AC005397 (48%) YGR257c (27%)
AF049236	III	381	41.8	9.75	PLDRIKLLMQ (107–116) PLDVLRLRLA (207–216)	Ca-dep solute carrier (32%)
AL021749	IV	379	40.7	9.78	PIERVKLLIQ (99–108) PIDTVRRRMM (304–313)	ANT (70%)
AL035356	IV	313	32.9	9.62	PLDLIKVRLQ (22–31) PVDVIKTRVM (246–255)	OMT/DTP/UCP (32–37%)
AC002329	IV	453	49.7	9.56	PFEAIKVRVQ (292–301)	PiC (50–53%)
AC002535	II	447	49.7	10.12	PHEVVRARLQ (368–377)	YIL006w (38%) YEL006w (37%)
AF007269	IV	352	38.3	9.56	PLDVIRRRMQ (264–273)	YPR011c (40%)
AF077407	IV	336	36.4	9.95	PVDTIKTRLQ (262–271)	KIAA0446 (33%)

Sequences have been identified in the Non-Redundant *Arabidopsis* Protein Database (http://genome-www.stanford.edu) by PatMatch analysis using the following pattern: P-x-[DE]-x-[LIVAT]-[RK]-x-[LRH]-[LIVMFY]-[QMAIGV]. Molecular weights and calculated pI are determined using ExPASy (http://www.expasy.ch), the identity with BLAST at the NCBI (http://www.ncbi.nlm.nih.gov/BLAST).

utilised. However, the mechanism of this transport has not been characterised.

Finally, in *S. cerevisiae* the gene *Flx1p*, which encodes a member of the mitochondrial carrier family, has been shown to be responsible for FAD import into the mitochondria [216]. Thus, it is highly possible that the cofactor carriers described above also belong to this family.

Unknown carriers

The analysis of the complete genome of *S. cerevisiae* has indicated the presence of 35 sequences encoding members of the mitochondrial carrier family [217, 218]. This function, however, is known only for a few of them. Three ANTs, a phosphate carrier, a citrate carrier, a dicarboxylate carrier, a succinate/fumarate carrier and a FAD carrier have been identified. In order to find other mitochondrial carriers in plants, I performed a PatMatch (http://genome-www.stanford.edu/Arabidopsis) with the mitochondrial energy signature (prosite PS00215) in the Non-Redundant *Arabidopsis thaliana* Protein database (http://genome-www.stanford.edu). This allowed me to identify sequences having 3, 2 or 1 mitochondrial signatures. These sequences were further selected by having the other features of mitochondrial carriers: (i) an amino acid sequence of around 300 residues, (ii) a molecular weight of 30 kDa, (iii) a high pI value, (iv) similarity with members of the mitochondrial carrier family. Eleven new sequences having all these characteristics have been thus identified (table 3).

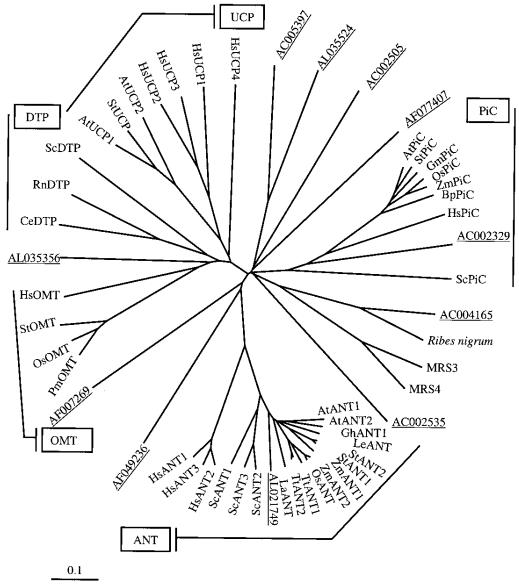

Figure 4. Phylogenetic tree of plant members of the mitochondrial carrier family. Some animal and yeast sequences have been added for comparison. The tree was performed using the ClustalW WWW Service at the European Bioinformatics Institute (http://www2.ebi.ac.uk/clustalw) and the TreeView program [226]. Accession numbers for plant sequences are as indicated in tables 2 and 3. HsUCP1 (U28480), HsUCP2 (U76367), HsUCP3 (U84763), HsUCP4 (AF110532), ScDTP (U79459), RnDTP (AJ223355), CeDTP (U23525), HsOMT (X66114), HsANT1 (J04982), HsANT2 (J05624), HsANT3 (P12236), ScANT1 (Z49703), ScANT2 (Z35791), ScANT3 (Z35954), MRS3 (S55179), MRS4 (S13533), ScPIC (X57478), HsPIC (X60036). At, *Arabidopsis thaliana* (Arabidopsis); Gh, *Gossypium hirsutum* (Cotton); La, *Lupinus albus* (Lupin); St, *Solanum tuberosum* (Potato); Le, *Lycopersicon esculentum* (Tomato); Os, *Oryza sativa* (Rice); Pm, *Panicum miliaceum* (Proso millet); Ribes, *Ribes nigrum* (Blackcurrant); Tt, *Triticum turgidum* (Wheat); Zm, *Zea mays* (Mays); Sc, *Saccharomyces cerevisiae;* Hs, *Homo sapiens*; Rn, *Rattus norvegicus;* Ce, *Caenorhabditis elegans*. For other abbreviations see table 1. Scale bar: '0.1' means 0.1 nucleotide substitutitons per site.

The ORF AL021749 has 70% identity with plant ANTs. It contains two mitochondrial signatures completely identical to the mitochondrial signatures found in all plant ANTs (PIERVKLLIQ, PIDTVRRRMM) and seems to have a presequence. This protein seems to be a member of the ANTs subgroup, as indicated by the phylogenetic tree (fig. 4) and the principal component analysis (fig. 5). Thus it appears that *Arabidopsis* has at least three different ANTs.

The ORF AC002329 has 50 to 53% identity with the plant PiCs. It has one mitochondrial signature identical to the one found in all plant phosphate carriers (PFEAIKVRVQ) and seems to contain a very long presequence. Even if the identity with plant PiC is not very high, this protein seems to fall into the PiC subgroup (figs 4 and 5). Takabate et al. [37], using a phylogenetic tree have pointed out that plant PiCs form distinct subgroups: woody plants, dicots and monocots. Using Southern analysis, they have postulated that plants contain multiple copies of mitochondrial phosphate carriers. Although the function of the protein (AC002329) is not determined, it appears that *Arabidopsis* has at least two different genes encoding putative mitochondrial phosphate carriers.

The ORF AL035356, described as a UCP homologue, has high similarity to OMT and dicarboxylate carriers from animal and plants. This protein has 35–37% identity with dicarboxylate carriers from animals, 35–36% identity with animal OMTs, 32–33% identity with PmOMT and StOMT and 35–36% identity with mammalian and plant UCPs. Based on the percentage identity, the principal component analysis (fig. 5) and on the phylogenetic study (fig. 4), this polypeptide does not seem to encode a UCP. However, it is more difficult to determine whether it encodes an OMT or a DTP. PmOMT, StOMT and OsOMT have more than 80% identity between them and between 45 and 47% identity with animal OMTs. It is thus tempting to postulate that AL035356, which is more distant (32–36% identity with OMTs), could be a plant dicarboxylate carrier. In conclusion, it appears that the UCP, OMT, and DTP groups are closely related and that in plants two different subgroups of carriers having high similarity with OMTs as well as DTPs exist. The substrate specificity of these carriers still remains to be clearly determined.

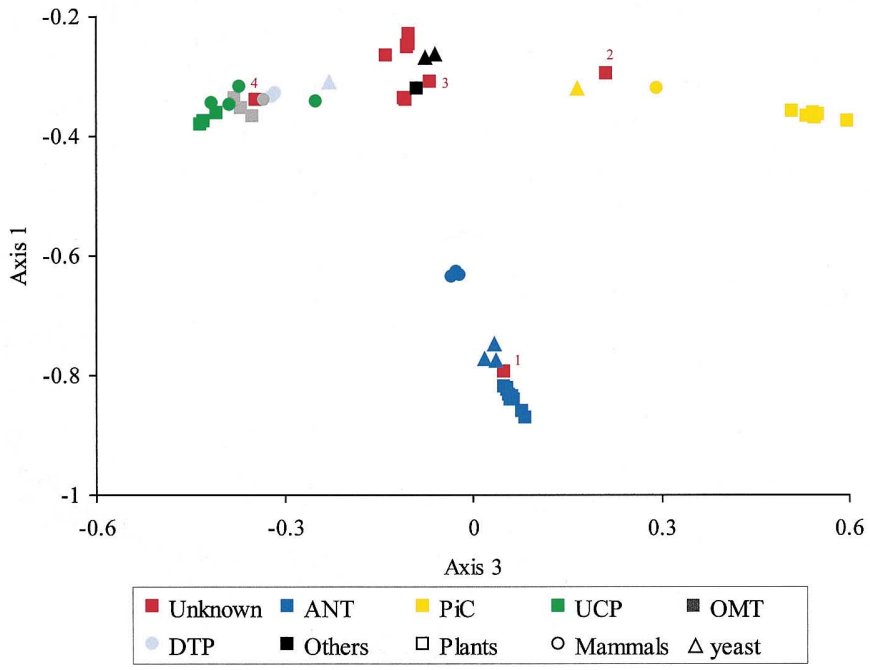

Figure 5. Principal component analysis based on percent identity between the different mitochondrial carriers presented in the phylogenetic tree. This analysis has been performed using the statistical analysis software package NCSS (Number Cruncher Statistical System). 1, AL021749; 2, AC002329; 3, AC004165; 4, AL035356.

The ORF AC004165 has 66% identity with a putative plant mitochondrial carrier cloned in *Ribes nigrum* (fig. 4). The function of this carrier is still not determined, but the gene seems to be differentially expressed during blackcurrant fruit ripening and expressed only at a very low level in leaves [219]. These two proteins show similarity to MRS3 (35%) and MRS4 (36%) yeast genes shown to be members of the mitochondrial carrier family and involved in mitochondrial RNA splicing. When expressed from high copy number plasmids, these two yeast proteins act as suppressors of a mitochondrial mutation that causes RNA splicing defects [220]. The substrates transported by such carriers are still unknown. It is more and more evident that plant mitochondria also contain proteins related to MRS3 and MRS4, even through the phylogenetic analysis indicates that the yeast and plant genes diverged quite early, which might indicate different functions for these genes. Five others ORFs have high similarity to yeast putative mitochondrial carriers [217] with unknown function (table 3). AC002505 has 32% identity with the yeast Pet8 gene. AC005397 and AL035524 have 48% identity between themselves, and 29 and 28% identity with the yeast ORF YGR257c, respectively. AC002535 may be homologous to yeast Yil006w and Yel006w, and AF007269 has 40% identity with Ypr011c. The *Arabidopsis* ORF AF077407 also falls in the mitochondrial family and has the highest similarity to a human carrier (KIAA0446), the function of which is not known.
Finally, the ORF AF049236 shows 32% identity with a rabbit calcium-dependent solute carrier identified as a member of the mitochondrial transporter superfamily. However, immunoelectron microscopy localised the transporter in the peroxisome, though a minor fraction was found in the mitochondria [221]. In plants, the protein brittle 1 has also been shown to have the mitochondrial carrier structure but is located in the amyloplast membrane [222–224]. These few examples raise the question of the presence of mitochondrial carrier family members in other membranes than the inner mitochondrial membrane.

Conclusion

Biochemical and molecular studies performed during the last 20 years have demonstrated the presence of solute carriers in the plant inner mitochondrial membrane. The features and the structures of these proteins are relatively conserved between animals and plants, and molecular information clearly shows that plant carriers belong to the mitochondrial transporter family.

However, some members of this family described in animals or yeast are still not identified in plants, although they have been described at the biochemical level, for example dicarboxylate, citrate and amino acid carriers. Furthermore, advances in the sequencing of the *Arabidopsis* genome have revealed the presence of new plant members of this family, the function and the specificity of which still remain to be determined. It is thus clear that the study of plant mitochondrial transporters is only at its beginning and that more analyses need to be performed in order to deepen the understanding of metabolic interactions between organelles, which are of capital importance for plant metabolism. An interesting area of research will be the analysis of transport systemswhich are only described in plants, such as oxaloacetate transport and glycine/serine exchange [203, 204], and the transport of cofactors [11] such as NAD [211, 225], CoA [213], thiamine pyrophosphate [11] and tetrahydrofolate [214].

Finally, we cannot exclude that exchanges performed at the level of the inner mitochondrial membrane are carried out only by the mitochondrial carrier family described here. It is highly probable that other proteins are involved in these exchanges, as suggested by the discovery of ABC transporters in yeast mitochondria [225].

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