Identification of a Major Soluble Protein in Mitochondria from Nonphotosynthetic Tissues as NAD-Dependent Formate Dehydrogenase

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In many plant species, one of the most abundant soluble proteins (as judged by two-dimensional polyacrylamide gel electrophoresis) in mitochondria from nongreen tissues is a 40-kD polypeptide that is relatively scarce in mitochondria from photosynthetic tissues. cDNA sequences encoding this polypeptide were isolated from a strong homology to an NAD-dependent formate dehydrogenase of 1137 nucleotides whose predicted amino acid sequence shows homology to a NAD-dependent formate dehydrogenase (EC 1.2.1.2) from Pseudomonas sp. 101. Comparison of the cDNA sequence with the N-terminal amino acid sequence of the mature 40-kD polypeptide suggests that the polypeptide is made as a precursor with a 23-amino acid presequence that shows characteristics typical of mitochondrial targeting signals. The identity of the polypeptide was confirmed by assaying the formate dehydrogenase activity in plant mitochondria from various tissues and by activity staining of mitochondrial proteins run on native gels combined with antibody recognition. The abundance and distribution of this protein suggest that higher plant mitochondria from various nonphotosynthetic plant tissues (tubers, storage roots, seeds, dark-grown shoots, caulifower heads, and tissues grown in vitro) might contain a formate-producing fermentation pathway similar to those described in bacteria and algae.

Mitochondria are one of the major sites of energy conversion in eukaryotic cells, providing ATP and various substrates for biosynthetic reactions that occur in the cytoplasm. It is thus understandable that many basic features of structure and function have been conserved between animal and plant mitochondria (e.g., general structure, cyanide-sensitive electron pathway, ATP synthase complex), despite the fact that they diverged a billion years ago. Nevertheless, plant mitochondria display particular characteristics due to the autotrophic metabolism of plant cells. These include the cyanide-insensitive electron pathway (Moore and Siedow, 1991; Rhoads and McIntosh, 1991), respiration-linked oxidation of external NAD(P)H, and rotenone-insensitive oxidation of internal NADH (Douce and Neuburger, 1989). With regard to matrix enzymes, although the tricarboxylic acid cycle functions similarly in all eukaryotes, plant mitochondria differ from their animal counterparts in malate oxidation (NAD-malate enzyme) and levels of Gly oxidation (Gly decarboxylase) (Douce and Neuburger, 1989). Gly is oxidized in the matrix space by the combined action of Gly decarboxylase and Ser hydroxymethyltransferase. This is an important step in the process of photorespiration. The Gly cleavage system purified from plant mitochondria (Neuburger et al., 1986; Walker and Oliver, 1986) is composed of four subunits, P (94 kD), L (60 kD), T (41 kD), and H (15 kD). This complex is very abundant in green tissues but scarce in nonphotosynthetic tissues, and it increases dramatically when exposed to the light. The L subunit (lipoamide dehydrogenase) has been found in root and other etiolated tissues, where it is thought to be part of two other enzyme complexes, the pyruvate and α-ketoglutarate dehydrogenases (Turner et al., 1992).

It is thus clear that the physiological role and enzyme complement of mitochondria differ considerably among different tissues, as in fact does mitochondrial morphology (Bendich and Gauriloff, 1984). To study these differences in more detail, the variation in the protein composition of mitochondria from different plant tissues has been examined in our laboratory. In previous papers, we described the analysis of two-dimensional protein patterns of mitochondria isolated from different pea (Rémy et al., 1987; Humphrey-Smith et al., 1992) and potato (Solanum tuberosum L.) (Colas des Francs-Small et al., 1992) organs. In addition to the above-mentioned abundance of the four subunits of the Gly decarboxylase complex in green tissues, we described a 40-kD polypeptide that is very abundant in mitochondria from tubers, dark-grown shoots, and calli or cell suspensions but scarce in leaf mitochondria (Colas des Francs-Small et al., 1992). This differential distribution between green and nongreen tissues was observed in all plant species tested but shows noticeable quantitative variations between species. It

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Abbreviations: FDH, formate dehydrogenase; IPTG, isopropyl-β-D-thiogalactopyranoside; pl, isoelectric point.

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was found to be extremely abundant in potato tubers and dark-grown shoots, accounting for up to 9% of the total mitochondrial protein revealed by Coomassie blue staining of two-dimensional gels. The abundance of this protein suggests that it plays an important role in plant metabolism, and its distribution (the converse to that observed for Gly dehydrogenase) suggests its participation in a metabolic pathway largely confined to nonphotosynthetic tissues. It is encoded by a nuclear gene because it is not synthesized by isolated mitochondria.

To determine the identity and role of this 40-kD polypeptide, we raised a polyclonal antibody against it, which was used to screen a cDNA expression library. Here we describe the isolation and characterization of the cDNA encoding this polypeptide and its identification as an NAD-dependent formate dehydrogenase (FDH). Although FDH has already been reported from green leaf mitochondria (Halliwell, 1974; Olivier, 1981), its abundance in mitochondria from nonphotosynthetic tissues has not been noted; and we suggest that the importance of this enzyme has been previously underestimated. The implications of the presence of this enzyme in such large amounts in certain tissues are discussed.

**MATERIALS AND METHODS**

**Plant Material**

Potato (*Solanum tuberosum* L.) tubers cv BF15 were either kept in the dark at 16°C or planted in a greenhouse and produced either dark-grown shoots or green leaves. Calli and cell suspensions were obtained as described previously (Colas des Francs-Small et al., 1992).

**agt11 cDNA Library Screening**

A *agt11* cDNA library prepared from tuber poly(A)*+* RNA (cv Désirée) was screened using a rabbit polyclonal antibody against the denatured 40-kD polypeptide (Colas des Francs-Small et al., 1992). Before use, the antisera was incubated with an *Escherichia coli* protein extract, to avoid any cross-reaction. About 2 × 10⁸ phages were plated and overlayed with nitrocellulose filters saturated with IPTG. The antigen was detected using the Protoblot immunoscreening system from Promega. All positive plaques were replated and rescreened four times, until all plaques yielded only positive signals. The sizes of the cDNA inserts were determined by digestion of purified λ-DNA with EcoRI and subsequent agarose gel electrophoresis. Initially, a partial cDNA clone named cDNA 707 (707 bp), discounting the poly(A) tail) and, subsequently, a second larger clone named cDNA 1440 (1440 bp, discounting the poly[A] tail) were isolated and used for further experiments.

**DNA Sequencing and Sequence Analysis**

The cDNAs of interest were subcloned into the plasmid Bluescript (Stratagene). Single-stranded plasmid DNA was prepared according to the method of Ausubel et al. (1987). The DNA was sequenced according to the dyeoxy-nucleotide chain termination method (Sanger et al., 1977) using an Applied Biosystems apparatus as recommended by the manufacturer.

Computer analysis of the sequence was carried out using the University of Wisconsin GCG package of programs (Devereux et al., 1984). Comparison of the sequence with the GenBank, EMBL, PIR, and SwissProt sequence data bases was performed using the BLAST (Altschul et al., 1990) network service at the National Center of Biotechnology Information (Bethesda, MD).

**RNA Isolation and Northern Analysis**

Total RNA was isolated from tubers, dark-grown shoots, calli, cell suspensions, and green leaves according to the procedure of Logemann et al. (1987). mRNAs were purified from the total RNAs using mAP paper from Amersham according to the manufacturer’s instructions. The nucleic acids (about 10 µg per lane) were separated on 3% formaldehyde, 1.5% agarose gels, transferred by blotting onto nitrocellulose, and probed with the 5′ half of the cDNA (a 870-bp fragment generated by a *XhoI* digest of the plasmid *pBluescript* containing the cDNA 1440) labeled by random priming in the presence of [α-³²P]dCTP.

**In Vitro Translation**

Total or poly(A)*+* potato RNAs were translated in a rabbit reticulocyte lysate system from Promega. The polypeptide of interest was immunoprecipitated from the in vitro translation mixture by the addition of the antisera raised against the denatured 40-kD polypeptide and protein A-Sepharose as described by Ausubel et al. (1987). Polypeptides synthesized in the presence of [³⁵S]Met were fractionated by electrophoresis through 12% polyacrylamide gels in the presence of SDS (Laemmli, 1970) and autoradiographed.

The same experiment was done using poly(A)*+* RNA that had previously been selected by hybridization with the cDNA 707 (hybrid selection).

**Expression of Potato FDH in *E. coli***

The cDNAs to be expressed in *E. coli* were generated by polymerase chain reactions using either of two 27-mer oligonucleotides carrying an *NcoI* site (underlined) and the precursor (GTACCCATGGCTAGTCGTGTAGCTTCT), together with a second oligonucleotide complementary to flanking vector sequences. The reaction mixture (100 µL) contained 10 ng of template DNA, 1 µM each oligonucleotide, 0.2 mM each deoxyribonucleotide triphosphate, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 100 µg mL⁻¹ of gelatin, and 2.5 units of Taq polymerase (Perkin-Elmer Cetus). Forty cycles were performed on a Perkin-Elmer apparatus as follows: 1 min at 94°C for denaturation, 2 min at 60°C for annealing, and 1 min (plus 10 s for each cycle) at 72°C for primer extension. The polymerase chain reaction products were digested with *NcoI* and *XhoI* and subsequently ligated into the strictly IPTG-inducible expression vector pTrc99A (Amman et al., 1988). The resulting plasmids thus expressed polypeptides that either contained or did not contain the presence.
Synthesis of potato FDH was carried out using the E. coli strain χ 53a (Tobey and Grant, 1986) transformed with the respective plasmids. After induction with IPTG according to the method of Ausubel et al. (1987), the proteins were separated by SDS-PAGE. The western transfer and subsequent antibody-staining reaction were as described by Colas des Francs-Small et al. (1992).

**Enzyme Assays**

FDH activity was measured as the increase in A340 according to the method of Quayle (1966), using 0.025% Triton X-100-treated tuber or leaf mitochondria or only the soluble mitochondrial proteins that were extracted as previously described (Colas des Francs-Small et al., 1992).

**Electrophoresis**

Nondenaturing electrophoresis was performed on 7% acrylamide gels in the discontinuous buffer system of Laemmli (1970) without SDS. Soluble mitochondrial proteins (20 μg) were loaded on each track, and the gels were run at 100 V for 2 h at 4°C in a Mini Protean I1 Bio-Rad apparatus. Subsequent FDH activity staining in the presence of nitroblue tetrazolium, phenazine methosulfate, formate, and NAD was performed according to the method of Towbin et al. (1979). For the experiment described in Figure 5, after nondenaturing electrophoresis and Coomassie blue staining, a denaturing electrophoresis and Coomassie blue staining, a

**RESULTS**

Isolation and Sequencing of cDNA Clones

**Reacting with the Antibody**

Immunoscreening of the Agt11 cDNA library initially gave one positive clone, which was cloned into the plasmid Bluescript (to give cDNA 707) and fully sequenced. Subsequently, a longer clone was isolated, subcloned (to give cDNA 1440), and almost fully sequenced, to reconstitute as far as possible the complete cDNA sequence. No differences were observed in the approximately 300 bp sequenced from both clones; therefore, both cDNAs probably originate from the same gene. However, the two clones differ in their site of addition of the poly(A) tail: cDNA 1440 contains an extra 200 bp at the 3' end. Based on the size of the mRNA on northern blots (about 1.4 kb), we suspect that the poly(A) site in cDNA 707 is the major site used and that, in fact, although cDNA 1440 is at least as long as the major mRNA, it lacks 100 to 150 nucleotides at the 5' end.

The combined cDNA sequence and the deduced amino acid sequence of the single, long, open reading frame are illustrated in Figure 1. The predicted amino acid sequence includes the 29 N-terminal amino acids previously identified by protein sequencing (Colas des Francs-Small et al., 1992), confirming the correspondence between the cDNA sequence and the 40-kD polypeptide. The size of the protein expected from the 1137-nucleotide open reading frame is 379 amino acids at the 5' end.
and B are from one gel, and lanes C and D are from another gel, the 43-kD product corresponds to rabbit immunoglobulin C band. The translation product of interest was immunoprecipitated using the antibody directed against the 40-kD polypeptide. Lane A, Coomasie blue-stained tuber mitochondrial proteins; lane B, corresponding immunoblot showing the 40-kD polypeptide; lane C, fluorograph of an SDS-gel of the immunoprecipitate showing the 43-kD labeled polypeptide (the extra band that can be seen above the 43-kD product corresponds to rabbit immunoglobulin G band slightly labeled by the unremoved 43-kD product); lane D, fluorograph of the supernatant left after immunoprecipitation. Lanes A and B are from one gel, and lanes C and D are from another gel, which was autoradiographed. Molecular mass markers (Pharmacia kit) are indicated on the left; 40 kD is an estimate.

Figure 2. SDS-PAGE analysis of translation products. Total potato RNAs were translated in a rabbit reticulocyte lysate system, and the translation product of interest was immunoprecipitated using the antibody directed against the 40-kD polypeptide. Lane A, Coomasie blue-stained tuber mitochondrial proteins; lane B, corresponding immunoblot showing the 40-kD polypeptide; lane C, fluorograph of an SDS-gel of the immunoprecipitate showing the 43-kD labeled polypeptide (the extra band that can be seen above the 43-kD product); lane D, fluorograph of the supernatant left after immunoprecipitation. Lanes A and B are from one gel, and lanes C and D are from another gel, which was autoradiographed. Molecular mass markers (Pharmacia kit) are indicated on the left; 40 kD is an estimate.

Figure 3. Alignment of potato FDH and Pseudomonas FDH amino acid sequences. The sequence alignments strongly suggested that the 40-kD polypeptide was an NAD-dependent FDH, and therefore, a number of enzyme activity assays were run on soluble mitochondrial proteins from potato tubers or green leaves. Initially, samples were run on nondenaturing gels (Fig. 4), and then one-half of each track was stained for FDH activity, and the other half was run on a second-dimension SDS gel (Fig. 5). Figure 4 shows the FDH activity revealed on non-denaturing gels for green leaf- and tuber-soluble mitochondrial proteins. The intensity of band revealed on the tuber mitochondria gel is about 5-fold greater than that observed for leaf mitochondrial proteins (Fig. 4). This result was confirmed by spectrophotometric enzyme assays performed on tuber and leaf mitochondria. The FDH activities found were (an average of three separate experiments) 0.27 μmol min⁻¹.

Amino Acid Sequence Comparisons

The amino acid sequence derived from the open reading frame was compared with sequences in the GenBank/EMBL, PIR, and SwissProt data bases. The comparisons revealed similarity between the derived amino acid sequence from cDNA 1440 and several NAD-specific, d-isomer 2-hydroxy acid dehydrogenases, but the most significant homology is with Pseudomonas sp. 101 FDH (Popov et al., 1990) (51% identity in a 325-amino acid overlap, i.e. from amino acids 51–376). The alignment of the two sequences can be seen in Figure 3, where the sequences corresponding to the NAD-binding domain are boxed.

Confirmation of the 40-kD Polypeptide as FDH

The sequence alignments strongly suggested that the 40-kD polypeptide was an NAD-dependent FDH, and therefore, a number of enzyme activity assays were run on soluble mitochondrial proteins from potato tubers or green leaves. Initially, samples were run on nondenaturing gels (Fig. 4), and then one-half of each track was stained for FDH activity, and the other half was run on a second-dimension SDS gel (Fig. 5). Figure 4 shows the FDH activity revealed on non-denaturing gels for green leaf- and tuber-soluble mitochondrial proteins. The intensity of band revealed on the tuber mitochondria gel is about 5-fold greater than that observed for leaf mitochondrial proteins (Fig. 4). This result was confirmed by spectrophotometric enzyme assays performed on tuber and leaf mitochondria. The FDH activities found were (an average of three separate experiments) 0.27 μmol min⁻¹.

Figure 3. Alignment of potato FDH and Pseudomonas FDH amino acid sequences. Gaps inserted for optimal alignment are represented by dots in the sequence. Identical amino acid residue matches between the two sequences are connected by solid vertical lines. Conservative and semiconservative changes are represented by double vertical dots and single dots, respectively. The sequences corresponding to the NAD-binding domain are boxed.
Mitochondrial Formate Dehydrogenase

Figure 4. Nondenaturing acrylamide gel electrophoresis of potato soluble mitochondrial proteins. Coomassie blue-stained gels of green leaf proteins (lane A) and tuber proteins (lane B). Lanes C and D show similar gels stained for FDH activity. Each lane was loaded with 20 μg of soluble mitochondrial proteins.

Figure 6. Immunoblot of the polypeptide expressed in E. coli. Part a of the gel shows the kinetics of the IPTG induction of the expression of the 43-kD precursor protein (plasmid containing the putative full-length cDNA), and part b shows that of the 40-kD mature protein (plasmid containing the cDNA encoding the mature protein). The samples were taken after 0 min (lane 0), 10 min (1), 20 min (2), and 60 min (3) of induction for both experiments. About 5 μg protein were loaded on part a and 50 μg on part b of the gel such that the induced mature protein could be detected. Lane M shows an immunoblot of tuber mitochondrial proteins. These experiments were done using the polyclonal antibody raised against the 40-kD polypeptide. An unidentified 70-kD E. coli polypeptide slightly cross-reacts with the antibody but is clearly not IPTG induced. The sizes indicated are estimated from molecular mass markers.

Figure 5. Identification of the major 40-kD polypeptide of soluble mitochondrial proteins from potato tubers as FDH. (FDH activity co-migrates on nondenaturing gels with the 40-kD polypeptide recognized by the antibody.) A nondenaturing gel of soluble mitochondrial proteins (A) similar to that presented in Figure 4B was subjected to a second-dimensional separation by SDS-PAGE (C). On the left side is a one-dimensional gel of the same denatured sample (B). The position of the major 40-kD polypeptide is indicated by an arrow. On the right side a bracket shows the part of the gel represented in D as an immunoblot. The major polypeptide corresponding to the FDH activity shown in Figure 4 clearly is specifically recognized by the 40-kD polypeptide polyclonal antibody. The sizes of molecular mass markers are indicated on the right.

Figure 7. Northern blot of total potato RNA hybridized with a labeled 870-bp XhoI fragment of the cDNA 1440. Lanes L0 to L20 show the accumulation of the transcript in the leaf after 0 h (L0), 8 h (L8), 16 h (L16), and 20 h (L20) of darkness. The other lanes show hybridization to callus RNA (C), dark-grown shoot RNA (S), and tuber RNA (T). About 10 μg of total RNA (as estimated by absorbance measurements and ethidium bromide staining of the gel) were loaded per lane.

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mg⁻¹ of soluble protein for tuber mitochondria and 0.035 μmol min⁻¹ mg⁻¹ of soluble protein for leaf mitochondria. No FDH activity was found outside mitochondria. Figure 5 shows that the single band that had FDH activity ran as a 40-kD polypeptide on an SDS gel and was recognized by our antibody after western blotting. This experiment was performed because this antibody (raised against the denatured protein) does not recognize the native protein very efficiently.

To further confirm the identity of the 40-kD polypeptide, cDNAs corresponding to the precursor protein or to the mature protein were expressed in E. coli using an IPTG-inducible expression vector. Both cDNAs gave rise to polypeptides of the expected sizes (43 or 40 kD, respectively, on the SDS-gels that were specifically recognized by our anti-
body, but the precursor form was much more highly expressed than the mature form, which was barely detectable (Fig. 6).

**Regulation of Expression of FDH in Plant Tissues**

Figure 7 shows a northern blot hybridized with a 870-bp XhoI fragment of the cDNA 1440. The size of the transcript was estimated to be approximately 1.4 kb using brome mosaic virus RNA and rRNAs as markers. The difference in transcript abundance in various organs is striking; it is abundant in dark-grown shoots and calli (and cell suspensions), but scarce or undetectable in tubers. In leaves, the transcript level is low and does not seem to vary during the day but starts increasing by 8 h of darkness and has increased dramatically after 16 h in the dark.

**DISCUSSION**

The sequence comparisons, activity tests, and antibody recognition data presented in this paper strongly suggest that the cDNA we have sequenced encodes a precursor for a mitochondrial NAD-dependent FDH corresponding to the abundant 40-kD polypeptide previously observed on two-dimensional gels. FDH is a dimeric enzyme that catalyzes the oxidation of formate to CO$_2$ in the presence of NAD$^+$. Although the reverse reaction is theoretically possible, the thermodynamic equilibrium is strongly in favor of CO$_2$ production, and attempts to demonstrate the reverse reaction have been unsuccessful (Mathews and Vennesland, 1950; Davison, 1951). The enzyme has been found previously in plants (Davison, 1951), animals (Mathews and Vennesland, 1950), and microorganisms (Quayle, 1966; Kato et al., 1974). As opposed to the FDH found in *E. coli* and several other bacteria (Ferry, 1990), which is Cyt b$_6$-linked and membrane bound, the plant, yeast, and *Pseudomonas* enzymes are soluble. FDH has been purified from the yeast species *Kloeckera* (Kato et al., 1974) and *Candida boidinii* (Schütte et al., 1976) and from pea (Nason and Little, 1955; Uotila and Koivusalo, 1979). The molecular mass and pI for the potato enzyme are close to those found for the pea (42.4 kD for the monomer and a pI of 6.2), *C. boidinii* (36 kD) (Schütte et al., 1976), and *Candida methanolica* (43 kD) (Izumi et al., 1989) enzymes.

In methanol-using microorganisms, FDH is cytosolic and catalyzes the last step of methanol oxidation, the true substrate being the S-formylglutathione produced by formaldehyde dehydrogenase (Van Dijken et al., 1976). Plants do contain a cytosolic formaldehyde dehydrogenase, which has been purified from pea (Uotila and Koivusalo, 1979), but the lower K_m of the plant FDH for formate (about 2 mM, as opposed to 13 mM [Schütte et al., 1976] or 22 mM [Kato et al., 1974] for yeast FDHs) suggests that the plant enzyme may use formate directly. In higher plants, in which FDH has previously been shown to be mitochondrial (Halliwell, 1974; Oliver, 1981), its function is not clear. Formate can be oxidized to CO$_2$ by FDH or by the peroxidative action of catalase but can also be reduced to hydroxymethyl tetrahydrofolate and participate in Ser synthesis by the Ser hydroxymethyl transferase reaction. What remains obscure is the source of this formate (Cossins, 1980). In leaves, it is thought to arise from the glyoxylate produced by photorespiration, and FDH has been shown to be active in spinach and tobacco leaves (Oliver, 1981). However, our experiments show that the protein is much more abundant in nongreen tissues (i.e. potato tubers, dark-grown shoots, cell suspensions, cauliflower heads, and storage roots such as sweet potato and carrot [Colas des Francs-Small et al., 1992]) than in photosynthetic tissues. The activity we found in tuber mitochondria is 5- to 8-fold greater than the activity in leaf mitochondria. High activities have also been observed in mature seeds (Davison, 1949).

At least part of the tissue-specific distribution of FDH is regulated by mRNA levels. Northern blot experiments show that the transcript is abundant in dark-grown shoots, calli, and cell suspensions, which are actively dividing tissues. In dormant tubers, the protein is abundant but the transcript is scarce, suggesting that protein turnover is low inside the mitochondrion. In leaves, the transcript level seems to be low and constant during the day but accumulates after 16 h in the dark. The slow response suggests a control by changing metabolite levels rather than directly by light levels. This type of regulation is interesting, and the study of the FDH gene promoter is about to start in our laboratory.

In conclusion, the abundance and tissue distribution of FDH suggest that a major uncharacterized metabolic pathway exists in mitochondria from various nonphotosynthetic plant tissues that produces large quantities of formate. In these tissues, the origin of the formate is likely to be different from that in leaves, and it is apparently produced in much higher amounts. In general, high FDH activity seems to be found in nonphotosynthetic tissues that have low surface area to volume ratios (tubers, storage roots, etc.) or that are growing in confined conditions, e.g. in vitro cultures, developing seeds. This suggests to us a correlation with anaerobic metabolism. Formate has long been known to be produced by potato tubers and by roots under anaerobic stress (Davison, 1951), although in recent years much more emphasis has been placed on ethanol production. Formate is also produced in large quantities during anaerobic fermentation in bacteria (Ferry, 1990) and uncellular algae (Kreuzberg, 1984). In these organisms, formate is produced by thioclastic cleavage of pyruvate by the enzyme pyruvate-formate lyase, which is activated under strictly anaerobic conditions by a specific "activase." The enzymes of the formate fermentation pathway found in *Chlamydomonas* are mostly mitochondrial (Kreuzberg et al., 1987). The possible occurrence of such a pathway in higher plants should stimulate interest in the distinct physiological roles of mitochondria in different tissues.

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LITERATURE CITED


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