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Identification of a mitochondrial glycerol-3-phosphate dehydrogenase from *Arabidopsis thaliana*: evidence for a mitochondrial glycerol-3-phosphate shuttle in plants

Wenyun Shen[a], Yangdou Wei[b], Melanie Dauk[a], Zhifu Zheng[a], Jitao Zou[a,*]

[a] *Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, SK, Canada S7N OW9*
[b] *Department of Biology, University of Saskatchewan, Saskatoon, SK Canada, S7N 5E2*

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Abstract **We report molecular characterization of an *Arabidopsis* gene encoding a mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase (FAD-GPDH) that oxidizes glycerol-3-phosphate (G-3-P) to dihydroxyacetone phosphate. We demonstrate through in vitro targeting assays that the encoded gene product can be imported into mitochondrial membrane systems. Enzyme activity of the protein was confirmed through heterologous expression in *Escherichia coli*. The *Arabidopsis* gene is expressed throughout plant development, but at the highest level during seed germination. We also show that expression of the *Arabidopsis FAD-GPDH* gene is coupled to oxygen consumption and affected by ABA and stress conditions. Together with an NAD^+-dependent GPDH, this enzyme could form a G-3-P shuttle, as previously established in other eukaryotic organisms, and links cytosolic G-3-P metabolism to carbon source utilization and energy metabolism in plants.**

Key words: FAD-glycerol-3-phosphate dehydrogenase; Glycerol metabolism; Glycerol-3-phosphate shuttle; *Arabidopsis*

1. Introduction

Mitochondrial metabolism not only provides energy, but also serves as a major link to other biosynthetic and catabolic processes within the cell. The central role of mitochondrial metabolism is in part reflected by the array of mitochondrial transporters and shuttles that transfer metabolites and reducing equivalents between mitochondria and the cytosol [1–5]. The direction of metabolite exchange is generally dependent on the concentration of the participating metabolites in the two compartments, and is often modulated by the redox status of the cytosol. On the outer surface of the inner membrane of plant and yeast mitochondria, multiple NAD(P)H dehydrogenases are present and potentially function in the regulation of cytosolic redox status [6].

In yeast and mammalian mitochondria, there also exists a glycerol-3-phosphate shuttle (G-3-P shuttle), which transfers cytosolic reducing equivalents to mitochondria and is required for glycerol metabolism [7]. The G-3-P shuttle consists essentially of two components: (i) an NAD^+-glycerol-3-phosphate dehydrogenase (NAD^+-GPDH, EC 1.1.1.8) in the cytosol; and (ii) a membrane-bound FAD-GPDH (EC 1.1.99.5) located on the outer surface of the inner mitochondrial membrane. The cytosolic NAD^+-GPDH reduces dihydroxyacetone phosphate (DHAP) to G-3-P using NADH as a reducing equivalent [8]. In conjunction, the FAD-GPDH catalyzes the conversion of G-3-P to DHAP and forms $FADH_2$ from FAD [9]. The coordinated action of the two enzymes, without the involvement of a membrane carrier, results in the transfer of two reducing equivalents from G-3-P to ubiquinone [10,11]. In plant cells, multiple isoforms of NAD^+-GPDH that reduce DHAP to G-3-P have been identified [12,13]. In contrast, there is only preliminary biochemical information suggesting the existence of a mitochondrial GPDH that oxidizes G-3-P to DHAP, a metabolic step known to be essential for glycerol metabolism [14,15].

In this paper, we report molecular characterization of an *Arabidopsis* gene encoding a mitochondrial FAD-GPDH, which, in combination with the cytosolic NAD^+-GPDH, is necessary and sufficient to constitute a mitochondrial G-3-P shuttle. The *Arabidopsis FAD-GPDH* gene is expressed at a high level during germination when metabolism of glycerol derived from storage lipid is expected to occur. Moreover, expression of the gene appears to be dependent on oxygen availability. Additionally, ABA, and stress conditions of dehydration and salinity were found to influence its expression. Thus, this study provides evidence for a role of FAD-GPDH in glycerol metabolism, and suggests that a G-3-P shuttle system is present in higher plants.

2. Materials and methods

2.1. Plant materials

Arabidopsis thaliana (Columbia ecotype) was grown in controlled environment chambers at 20–22°C under long day conditions (16 h of cool-white fluorescent light). Leaf, shoot, flower and developing siliques of *Arabidopsis* used for RNA extractions were sampled from plants grown in Terra-Lite (W.R. Grace Co., Ajax, ON, Canada). Root materials were collected from seedlings growing on the surface of vertically positioned half-strength MS [16] medium plates.

2.2. cDNA cloning and construction of Escherichia coli expression vector

The primers used for RT-PCR to amplify the full length FAD-GPDH cDNA are: (CTCTCGCTTCCATTCGCC) and (GCTTTAGTTGTGCTTGCC). The PCR product was fully sequenced. Amino acid sequence alignments were performed according to the MagaAlign program of the software DNASTAR™.

*Corresponding author. Fax: (1)-306-975 4839.
E-mail address: jitao.zou@nrc.ca (J. Zou).

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2.3. Enzyme activity characterization

To construct an *E. coli* expression vector pFAD-GPDH, primers (GGAGAACCATGGGTGATCCCACCGCTTCTG) and (GAAGAAGGATCCGCTTTAGTTGTGCTTGCC) were used to amplify the *FAD-GPDH* by PCR. The PCR product was inserted into the *Bam*HI/*Nco*I sites of vector pQE60 (Qiagen) to generate *pFAD-GPDH*. *E. coli* strain DH5α containing plasmid *pFAD-GPDH* was cultured in LB medium supplemented with 50 mg/l ampicillin at 37°C. Induction of *FAD-GPDH* expression was achieved by adding IPTG. Cells were harvested and broken down through sonication. The DH5α strain harboring the pQE60 vector was cultured and treated in parallel as a control.

FAD-GPDH activity was determined by the method of Gardner [17]. Briefly, bacterial lysates containing 100 μg protein were incubated in 0.4 ml reaction mixtures containing 4 mM *p*-iodonitrotetrazolium violet (INT), 1 mM KCN, and 50 mM bicine buffer (pH 8.0) in the presence or absence of 40 mM L-glycerol-3-phosphate (D-L isomers), and in combination with and without 0.1 mM FAD, at 37°C for 30 min. The reaction was terminated by adding 1 ml ethyl acetate, and subsequently spun for 1 min at 3000 rpm. The absorbancy of the reduced INT in the ethyl acetate extract was measured at 490 nm.

2.4. Mitochondrial import assay

The FAD-GPDH cDNA and a cDNA corresponding to At4g01950 were cloned in-frame into the PCR 2.1-TOPO vector bearing the T7 promoter. In vitro synthesis of the polypeptide was carried out using TNT Quick Coupled Transcription/Translation systems (Promega), and labeled with [^{35}S]methionine.

Mitochondria were isolated from 12-day-old green pea seedlings, and purified twice with discontinuous Percoll gradient as described by Fang et al. [18]. The import of the in vitro synthesized polypeptide into isolated mitochondria was carried out according to Cleary et al. [19]. Samples were analyzed using 4–20% SDS–polyacrylamide gels (Bio-Rad) and BenchMark pre-stained protein ladder (Gibco) as a molecular weight marker. After electrophoresis, the gels were fixed in a solution of 50% methanol, 10% acetic acid for 30 min, and then soaked in 7% acetic acid, 7% methanol and 1% glycerol for 10 min before gel drying. The labeled products in the dried gel were visualized by autoradiography.

2.5. Nucleic acid isolation and Northern analysis

Total RNA extraction was performed as described [20]. Approximately 20 μg of total RNA from various tissues was separated on a 1.2% agarose-formaldehyde gel and stained with ethidium bromide to confirm equal loading of the lanes based on ribosomal RNA bands. Gels were blotted on nylon membranes (Zetaprobe, Bio-Rad) and baked at 80°C for 2 h under vacuum. Pre-hybridization for 30 min and hybridization overnight were performed at 65°C in Church buffer [21]. The membranes were washed at 65°C, twice with 20 mM sodium phosphate buffer and 5% SDS, and twice with 20 mM sodium phosphate buffer and 1% SDS.

2.6. Stress and ABA treatment of plant seedlings

Two-week-old seedlings germinated on filter paper were sprayed directly with 50 μM ABA. Salinity treatment was performed by placing the seedlings on filter paper in Petri dishes containing 50 mM NaCl. Dehydration was imposed by exposing the seedlings in an open Petri dish to the air-flow of a flow hood for various durations. Anoxia was induced by placing seedlings in an Atmosbag (Aldrich) continuously flushed with an N_2 stream.

3. Results

3.1. Identification of an Arabidopsis cDNA encoding FAD-GPDH

The FAD-dependent GPDH in *Saccharomyces cerevisiae* is encoded by the *GUT2* gene [22,23]. A blast search [24] of the *Arabidopsis* genome database using the amino acid sequence of Gut2p (P32191) yielded results with an ORF (At3g10370) on chromosome III which predicts a 629 aa polypeptide that exhibits 50% sequence similarity over its entire sequence to Gut2p. Further blast searches with the nucleotide sequence

Fig. 1. Alignment of the predicted protein sequences for *Arabidopsis* (FAD-GPDH; At), fruit fly (FAD-GPDH; Dm) and yeast (FAD-GPDH; Sc) mitochondrial G-3-P dehydrogenases. Identical amino acids are denoted by an asterisk (*), and conserved amino acid substitutions are marked beneath with colon (:) and dot (.). Boxed regions indicate putative FAD-binding domains. Sequences potentially involved in G-3-P binding are highlighted in gray.

of this ORF revealed several *Arabidopsis* ESTs, indicating that the gene, which we designated as *FAD-GPDH*, is indeed expressed.

The *FAD-GPDH* cDNA was amplified by RT-PCR with primers synthesized according to the sequence of the identified ORF. As expected, the deduced amino acid sequence of *FAD-GPDH* shares no sequence homology with the cytosolic NAD^+-GPDH (AJ347019). However, it can be aligned with other known FAD-dependent GPDHs (Fig. 1), strongly suggesting that it is an ortholog from *Arabidopsis*. A putative FAD-binding site as well as sequences involved in G-3-P binding could be easily identified. However, similar to the yeast

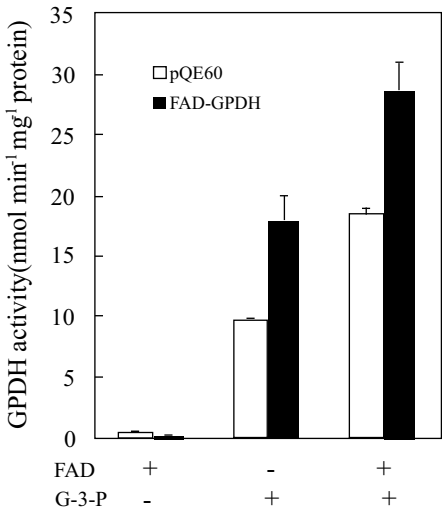

Fig. 2. G-3-P dehydrogenase enzyme activity from *E. coli* harboring the empty vector (pQE60) or expressing the *Arabidopsis* FAD-GPDH. The reaction was performed in the presence (+) and absence (–) of G-3-P and FAD, respectively.

FAD-GPDH and unlike the animal FAD-GPDH, the *Arabidopsis* protein lacks the EF-hand calcium-binding domain, suggesting that its activity is not affected by cytosolic Ca^{2+} [25].

3.2. Enzyme activity confirmation of FAD-GPDH

To confirm its enzyme activity, the *Arabidopsis FAD-GPDH* cDNA was cloned into a bacterial expression vector pQE60 and introduced into *E. coli*. Since *E. coli* possesses endogenous FAD-GPDH activity, the enzyme activity of the *FAD-GPDH*-expressing lysate was evaluated in reference to the same strain harboring a control vector (pQE60). As shown in Fig. 2, in the absence of G-3-P, the *E. coli* lysate exhibited a very low level of enzyme activity regardless whether *FAD-GPDH* was expressed or not. However, in the presence of G-3-P, there was more than a two-fold increase in GPDH activity due to the expression of the *Arabidopsis FAD-GPDH* cDNA. Furthermore, addition of FAD greatly stimulated the enzyme activity. Thus, the increased enzyme activity detected in *E. coli* expressing *FAD-GPDH* was G-3-P as well as FAD dependent, and therefore indicated that the *Arabidopsis* cDNA encodes a functional FAD-GPDH.

3.3. The FAD-GPDH protein can be targeted to isolated mitochondria

According to the plant organellar targeting sequence prediction program Predotar, the FAD-GPDH protein has in its N-terminal an apparent mitochondrial targeting sequence (see http://aramemnon.botanik.uni-koeln.de). Program MitoPro_v2 also predicted the protein as likely to be targeted to mitochondria, albeit with a low prediction score. On the other hand, prediction from program TargetP_v1 yielded negative results with respect to mitochondrial targeting. In order to validate these predictions, we performed import assays using isolated pea mitochondria. The in vitro translated FAD-GPDH had a molecular weight similar to the predicted size of 68 kDa (Fig. 3A, lane 1), indicating that a full-length protein was synthesized. When the protein was incubated with isolated mitochondria and subjected to centrifugation through a 20% sucrose cushion, the protein was not only found to be associated with the mitochondria (Fig. 3A, lane 2), but was also protected from proteinase K digestion (Fig. 3A, lane 3). Proteinase K treatment of the mitochondria in the presence of Triton X-100, which destroys the mitochondrial membrane systems, resulted in a complete digestion of the protein (Fig. 3A, lane 4). To further test if the polypeptide was inserted into the mitochondrial membrane system, mitochondria with the imported protein were lysed in a low osmolarity buffer (20 mM Tes-NaOH), and the membranes were subsequently recovered by ultra-centrifugation. As shown in Fig. 3A, lane 5, the protein was tightly associated with mitochondria membrane systems. It appeared that the protein was imported into mitochondria as an intact protein without cleavage of an N-terminal sequence as there was no reduction in protein size. Our mitochondrial targeting experiment also included an *Arabidopsis* protein encoded by At4g01950, whose function is unknown at the present time. As shown in Fig. 3B, although the protein was properly synthesized, no evidence of association with mitochondria was found, and thereby served as a negative control for our assay.

3.4. Expression profile of FAD-GPDH transcript

In a Northern blot with total RNA prepared from roots, leaves, flowers, developing siliques, germinating seeds and young seedlings, a single hybridization signal could be detected in all tissues examined (Fig. 4). However, the transcript level was significantly elevated in germinating seedlings. Such an expression pattern is consistent with a previous biochemical study in which a great amount of mitochondrial G-3-P activity was found in germinating seeds (fatty seedlings) [14].

Since the function of FAD-GPDH is presumably coupled to respiration, the effect of oxygen availability on its expression was also investigated. Northern blot analysis with RNA prepared from *Arabidopsis* seedlings exposed under an N_2 stream showed that expression of *FAD-GPDH* was severely reduced under prolonged anoxia treatment (Fig. 4B). The question of whether other stress conditions would affect the expression of *FAD-GPDH* was also investigated. Northern analysis results with RNA isolated at different time points after ABA treatment are shown in Fig. 4C. In the first 30 min, ABA negatively regulated the level of *FAD-GPDH* transcript. However, within 3 h a noticeable increase of *FAD-*

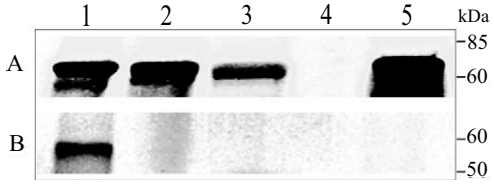

Fig. 3. In vitro import of the FAD-GPDH protein into isolated mitochondria from pea. A: Lane 1, polypeptide generated through transcription-coupled translation of the *FAD-GPDH* cDNA in the presence of ^{35}S-labeled Met; lane 2, polypeptide retained in mitochondria after incubation with the in vitro translation mixture; lane 3, polypeptide protected from proteinase K digestion after import into mitochondria; lane 4, treatment of mitochondria with proteinase K in the presence of 0.5% Triton X-100 led to the disappearance of the polypeptide; lane 5, polypeptide associated with membrane systems after mitochondria were broken with low osmolarity buffer. B: Import assay under identical conditions with a polypeptide generated from a cDNA corresponding to At4g01950.

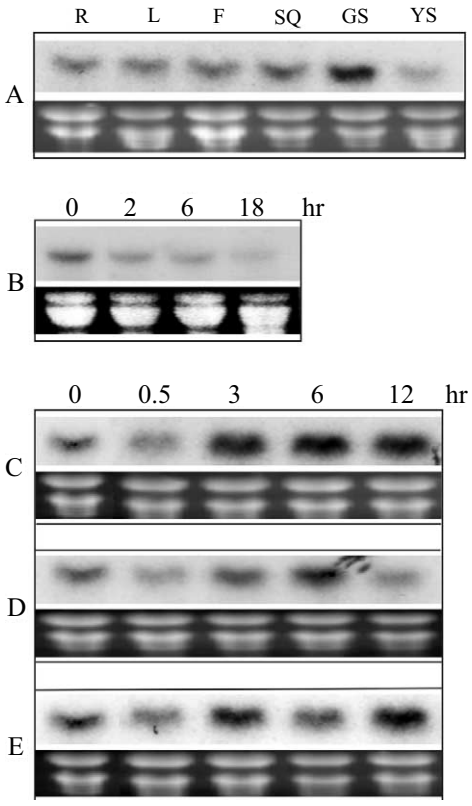

Fig. 4. Expression profile analyses of the *FAD-GPDH* gene in *Arabidopsis*. Upper sections of the panels, Northern hybridization signals; lower portions of the panels, ethidium bromide staining of the RNA gels. A: Total RNA was extracted from roots (R), leaf (L), flowers (F), developing siliques (SQ), germinating seeds (GS) and 2-week-old seedlings (YS). B: RNA was prepared from young seedlings subjected to anoxia under an N_2 stream for various periods of time. C: RNA prepared from seedlings treated with 50 μM ABA. D: RNA prepared from seedlings under dehydration. E: RNA prepared from seedlings treated with salt.

GPDH expression took place, generating transcript at a level higher than the control. Salinity and dehydration treatment also affected the expression of *FAD-GPDH* (Fig. 4D,E). In both cases, an initial decline of transcript was observed in the first 30 min. Recovery of the transcript level also occurred in a few hours, but it did not seem to reach beyond that of the control. Moreover, dehydration treatment after 12 h reduced the expression.

4. Discussion

G-3-P is a central metabolite interconnected to several primary biochemical pathways. It is known that G-3-P can be generated through the reduction of DHAP by an NAD^+-GPDH [12,26]. G-3-P can also be produced through phosphorylation of glycerol by glycerolkinase using ATP [27,28]. Besides its role in generating the glycerol backbone for glycerolipids [29], other aspects of G-3-P metabolism in plants have been surprisingly neglected. The oxidation of G-3-P to DHAP is a metabolically significant step particularly relevant to glycerol dissimilation. A substantial amount of glycerol is generated during seed germination in species such as *Arabidopsis*, whose major seed carbon and energy source is storage lipids. Moreover, plant cells of vegetative tissues are known to accumulate glycerol at concentrations ranging from 0.1–0.5 mM [30], and glycerol at low concentrations can sustain a certain degree of growth in plant tissue culture [31–33]. Therefore, plant cells apparently possess a glycerol dissimilation pathway. The first step of glycerol metabolism is the phosphorylation of glycerol resulting in the production of G-3-P. Subsequent glycerol utilization necessitates an enzyme that catalyzes the conversion of G-3-P to DHAP, which could then participate in a recycling of triosphosphate to hexose phosphate [34]. The conversion of G-3-P to DHAP in plants was previously attributed to the G-3-P dehydrogenase activity of the NAD^+-GPDH [35]. However, the G-3-P to DHAP reaction catalyzed by the cytosolic and plastidic NAD^+-GPDH requires a high pH (pH 9.5), and the reaction has an extremely small equilibrium constant of 10^{-12} M [13,35,36]. Results obtained from this study provide convincing evidence that, similar to other eukaryotic systems [37–39], the *Arabidopsis* FAD-GPDH is a mitochondrial enzyme that converts G-3-P to DHAP. Consistent with a potential role in glycerol metabolism, the highest transcript level of the identified gene in *Arabidopsis* was detected in tissues of germinating seeds where metabolism of glycerol derived from storage lipid breakdown is assumed to be very active [14,15].

A previous biochemical study has shown that caster bean mitochondria were capable of oxidizing G-3-P to reduce cytochrome C [14]. With the mitochondrial preparation, the presence of G-3-P enabled uptake of oxygen, and G-3-P oxidation was fully inhibited if the mitochondrial electron chain was blocked [14]. We found that the transcription of the *Arabidopsis FAD-GPDH* was repressed by prolonged anoxia, thereby supporting the notion that its function is coupled to mitochondrial respiration. Similar to the yeast FAD-GPDH, the *Arabidopsis* enzyme lacks the EF-hand calcium-binding domain conserved in animal FAD-GPDHs [25]. It thus suggests that, in contrary to the mitochondrial NAD(P)H dehydrogenases which are regulated by Ca^{2+} and are likely to be inactive in an unstressed plant cell [6], FAD-GPDH activity is not dependent on Ca^{2+} changes. Nonetheless, the expression of the *Arabidopsis FAD-GPDH* is indeed modulated by stress conditions that are known to affect cellular redox status and reactive oxygen species (ROS) production [40]. Interestingly, a common effect of ABA, salinity and dehydration on the expression of *FAD-GPDH* was an initial decline of the transcript level in the first 30 min. At the present time, we can only speculate the significance of such an observation. It is conceivable that the decreased expression reflects a perturbed mitochondrial electron transport activity in plants due to stress. Alternatively, it is also possible that the transient decrease of *FAD-GPDH* expression is a response to a higher demand of NAD(P)H, which serves as an electron donor for ROS production [41]. Thus, this and previous studies [14] provide strong evidence that FAD-GPDH, through oxidizing cytosolic G-3-P, could fulfill a role of transferring reducing equivalents from the cytoplasm to the mitochondrial electron transport chain. However, whether the activity of FAD-GPDH is coupled with the cytosolic NAD^+-GPDH to complete an irreversible shuttle for cellular redox control awaits further study.

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