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Yao Zheng, Cécile Cabassa-Hourton, Séverine Planchais, Sandrine Lebreton,  
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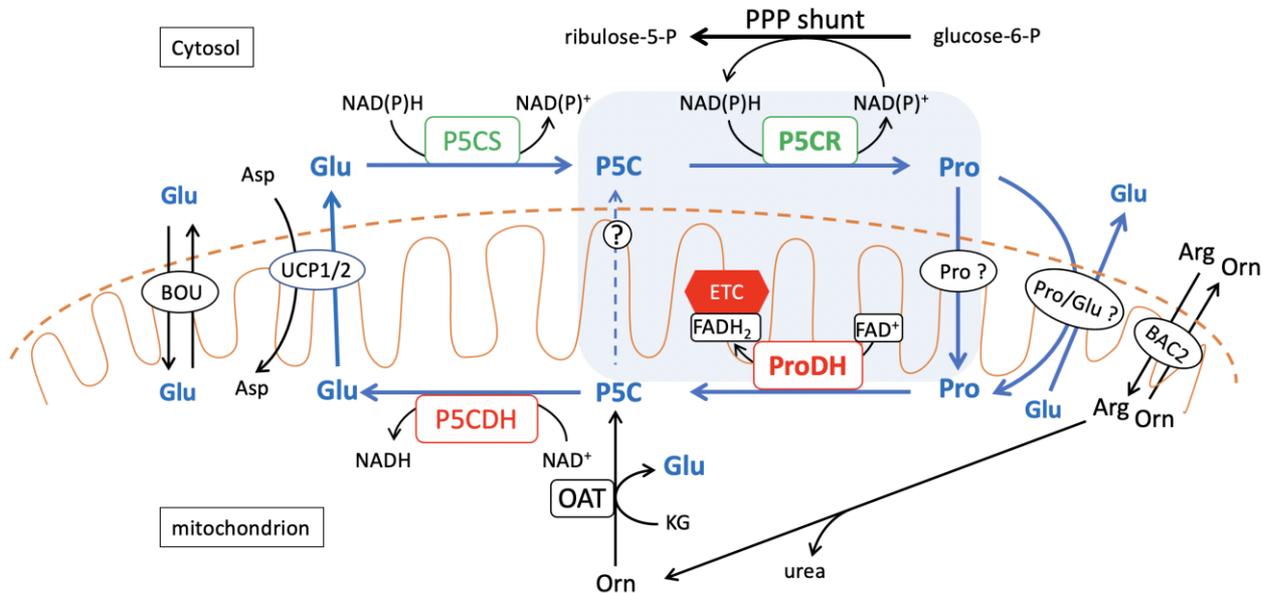


Fig. 1. Proline metabolism in plants and its links to the ornithine and pentose phosphate pathways. The proline cycle is highlighted by blue arrows and the putative proline-P5C cycle by a square in light blue. Asp, aspartate; BAC, basic amino acid carrier; BOU, a bout de souffle; ETC, electron transfer chain; Glu, glutamate; KG,  $\alpha$ -ketoglutarate; OAT, ornithine  $\delta$ - aminotransferase; Orn, ornithine; PPP shunt, pentose phosphate pathway; P5C, pyrroline-5-carboxyate; P5CDH, P5C dehydrogenase; P5CS, P5C synthetase; ProDH, proline dehydrogenase; Pro, proline; UCP, mitochondrial uncoupling protein; ? indicates unknown transporter(s).

## The proline cycle as a eukaryotic redox valve

Yao Zheng, Cécile Cabassa-Hourton, Séverine Planchais, Sandrine Lebreton, Arnould Savoure<sup>1</sup>

Sorbonne Université, UPEC, CNRS, IRD, INRAE  
 Institute of Ecology and Environmental Sciences of Paris (iEES),  
 F-75005 Paris, France

1 Corresponding author  
 arnould.savoure@sorbonne-universite.fr

### Running title

The proline cycle

### Highlight

The proline cycle regulates energy and redox power in a shuttle mechanism with a valve function during plant development and in stress conditions



1 **Abstract**

2 The amino acid proline has been known for many years to be a component of proteins as well as an  
3 osmolyte. Many recent studies have demonstrated that proline has other roles such as regulating redox  
4 balance and energy status. In animals and plants, the well-described proline cycle is concomitantly  
5 responsible for the preferential accumulation of proline and shuttling of redox equivalents from the  
6 cytosol to mitochondria. The impact of the proline cycle goes beyond regulating proline levels. In  
7 this review, we focus on recent evidence of how the proline cycle regulates redox status in relation to  
8 other redox shuttles. We discuss how the interconversion of proline to glutamate shuttles reducing  
9 power between cellular compartments. Spatial aspects of the proline cycle in the entire plant are  
10 considered in terms of proline transport between organs with different metabolic regimes  
11 (photosynthesis versus respiration). Furthermore, we highlight the importance of this shuttle in the  
12 regulation of energy and redox power in plants, through a particularly intricate coordination notably  
13 between mitochondria and cytosol.

14

15

16 **Keywords**

17 Mitochondria; Proline metabolism; Proline cycle; Proline transport; Redox status; Redox shuttle;  
18 Redox valve

19

20

21 **Introduction**

22 Redox homeostasis during development is vital for all branches of the tree of life, particularly in  
23 response to environmental changes (Scheibe *et al.*, 2005). Compartmentalization of metabolism and  
24 other cellular functions in eukaryotic cells implies that pools of metabolites, ions and reducing  
25 equivalents are concentrated locally in a regulated manner (Lunn, 2006). Maintenance of metabolism  
26 as environmental conditions vary, whether regularly, rapidly or to extremes, is a challenge for all  
27 eukaryotic organisms, and particularly plants for several reasons. As sessile organisms, plants cannot  
28 move away to more clement conditions, so they are subject to dynamic environmental alterations. In  
29 eukaryotic photosynthetic organisms, the regulation of energy metabolism is particularly complex  
30 because it involves two organelles, the mitochondrion and the chloroplast, as major sources of ATP  
31 and reducing power in the form of pyridine nucleotides NADH and NADPH (Foyer and Noctor,  
32 2020). Additional complexity of redox and energy metabolism arises from the daily cycles of light  
33 and dark periods, and the presence of green and non-green tissues within the same individual. In  
34 daylight, photon energy is transformed in green tissues by photosystems into ATP and NADPH,  
35 which in turn are used to fix CO<sub>2</sub> by the Calvin–Benson cycle. In mitochondria, oxidative

36 phosphorylation breaks down carbohydrates to generate ATP in all tissues at any time, but remains  
37 the only powerhouse system operative during the night and in non-green tissues. Tight spatiotemporal  
38 coordination of supply and demand to the two powerhouse systems of plant cells is required to meet  
39 the energy needs of anabolic processes such as primary assimilation of carbon and nitrogen, as well  
40 as transport of substrates, intermediates and products.

41 Specific translocators enable the direct or indirect exchange of reducing power between cell  
42 compartments (Palmieri *et al.*, 2009). The cofactor  $\text{NAD}^+$ , the oxidized form of NADH, is synthesized  
43 in the cytosol and must be imported into organelles to ensure timely supply to many enzymatic  
44 reactions. In Arabidopsis, the two  $\text{NAD}^+$  carrier proteins NDT1 and NDT2 have been shown to  
45 respectively localize in plastid and mitochondrial membranes, allowing direct transport of  $\text{NAD}^+$  into  
46 these organelles (Palmieri *et al.*, 2009). Recent data locate NDT1 exclusively in the mitochondrial  
47 membrane (de Souza Chaves *et al.*, 2019). On the contrary, no transporter has been identified as a  
48 carrier of reduced form NADH from one subcellular compartment to another. Membranes are also  
49 impermeable to both NADPH cofactor and its oxidized form  $\text{NADP}^+$ . An  $\text{NADP}^+$  carrier has not been  
50 identified yet, suggesting that this compound is likely to be formed by an  $\text{NAD}^+$  kinase. Indeed  $\text{NAD}^+$   
51 kinases have been found to be localized in the cytosol and chloroplasts (Gakière *et al.*, 2018;  
52 Dell'Aglio *et al.*, 2019). Furthermore,  $\text{NAD}^+$  kinase has been also detected in the mitochondrial  
53 matrix of yeasts and mammals (Outten and Culotta, 2003; Ohashi *et al.*, 2012), but not of plants  
54 (Gakière *et al.*, 2018).

55 As organellar membranes are not permeable to reduced forms of pyridine nucleotides, the  
56 compartmentalized redox couples ( $\text{NAD}^+/\text{NADH}$  and  $\text{NADP}^+/\text{NADPH}$ ) rely on shuttles for their  
57 translocation between subcellular compartments. A shuttle system is a series of biochemical  
58 interconversions that temporarily bind the redox or other molecular entity in a permeable form that  
59 can be ferried across the membrane barrier, releasing or reconstituting the entity on the other side of  
60 the barrier. The number of steps, enzymes, substrates, and transporters involved depends on the type,  
61 site and function of the shuttle. Shuttles are thus dynamic control systems that redirect metabolic flux  
62 so certain compounds can be overproduced. Organellar and cytosolic NADH and NADPH pools have  
63 been shown to be regulated by multiple shuttles or metabolic valves, such as the glycerol-3-phosphate  
64 (G3P) shuttle and the malate-aspartate shuttle for the NADH pools, and the malate-oxaloacetate  
65 (OAA) shuttle for NADPH pools. The concept of a metabolic valve is that build-up of an entity, such  
66 as a reducing equivalent, on one side of a membrane barrier can be released or purged in a regulated  
67 manner, redistributing the entity in the cell while resetting the conditions on either side of the barrier.  
68 In this sense, a valve might not be expected to operate stoichiometrically. Such shuttle and valve  
69 mechanisms enable redox and energy homeostasis in both normal and stressed states. Proline  
70 metabolism involves the interconversion of glutamate and proline in a process linked to cellular

71 compartments and energetics. Therefore, enzymes involved in proline metabolism could participate  
72 in NAD(P)<sup>+</sup>/NAD(P)H homeostasis in a shuttle mechanism with a valve function during plant  
73 development and in stress conditions.

74

## 75 **Proline metabolism**

76 Abiotic stresses perturb cellular redox homeostasis and proline metabolism is thought to act as an  
77 important redox buffer in both plants and animals (Krishman *et al.*, 2008; Sharma *et al.*, 2011; Ben  
78 Rejeb *et al.*, 2014, 2015; Phang, 2019). Proline plays important roles in various developmental stages  
79 of plants as well as in stress tolerance (Kavi Khishor and Sreenivasulu, 2014; Bhaskara *et al.*, 2015;  
80 Trovato *et al.*, 2019). Indeed most plant species have been shown to accumulate proline in response  
81 to abiotic and biotic stresses. In plants, proline biosynthesis occurs in the cytosol starting from  
82 glutamate (Fig. 1). Glutamate is phosphorylated and reduced by the  $\Delta^1$ -pyrroline-5-carboxylate  
83 synthetase (P5CS) using NADPH as a cofactor to form glutamate semialdehyde (GSA) in equilibrium  
84 with pyrroline-5-carboxylate (P5C). P5C is further reduced into proline by P5C reductase (P5CR)  
85 also using NAD(P)H as a cofactor. Székely *et al.* (2008) presented evidence that P5CS1 may be  
86 localized in chloroplasts during salt stress, indicating that proline may also be synthesized in this  
87 organelle. Recent work using spectrally resolved fluorescence imaging showed exclusive cytosolic  
88 localization of P5CS1 and P5CS2 in Arabidopsis protoplasts, suggesting that plastids do not  
89 contribute to P5CS-mediated proline biosynthesis (Funck *et al.*, 2020). The subcellular localization  
90 of P5CS1 therefore remains ambiguous and may depend on growth and/or stress conditions. Further  
91 research is required to unravel these discrepancies.

92 In animals, the conversion of P5C to proline is also mediated by P5CR, commonly named PYCR.  
93 The organization of the proline biosynthesis pathway is more complex at least in humans where  
94 PYCR is encoded by three different genes, *PYCR1*, *2*, and *L*, with both PYCR1 and 2 localized in  
95 mitochondria and PYCRL in the cytosol (De Ingeniis *et al.*, 2012). P5CR was shown to be exclusively  
96 cytosolic in Arabidopsis cells (Funck *et al.*, 2012).

97 When stress is relieved, proline is rapidly transported from the cytosol into mitochondria by either a  
98 mitochondrial proline symporter or a proline/glutamate antiporter (Di Martino *et al.*, 2006); however,  
99 genes encoding these transporters have yet to be identified. Mitochondrial proline is then oxidized by  
100 the sequential action of proline dehydrogenase (ProDH) and P5C dehydrogenase (P5CDH) to release  
101 glutamate. Mitochondrial glutamate transporters “a bout de souffle” (BOU) and mitochondrial  
102 uncoupling proteins UCP1/2 have now been identified and it is conceivable that they could participate  
103 in the shuttling of glutamate during the proline cycle (Monné *et al.*, 2018; Porcelli *et al.*, 2018).  
104 ProDH, also known as proline oxidase (POX), catalyzes the first and rate-limiting step of proline  
105 catabolism using FAD as a cofactor (Servet *et al.*, 2012). In both animals and plants, ProDH is

106 localized on the matrix side of the mitochondrial inner membrane (Elthon and Stewart, 1981;  
107 Cabassa-Hourton *et al.*, 2016) where it transfers electrons released from proline oxidation to the  
108 mitochondrial electron chain. While P5CDH activity was detected in the mitochondrial inner  
109 membrane in *Zea mays* (maize) (Elthon and Stewart, 1981), this enzyme is localized in the  
110 mitochondrial matrix in animals (Brunner and Neupert, 1969) and other plants (Deuschle *et al.*, 2001).  
111 Recently, Ren *et al.* (2018) have shown that the two enzymes may physically interact with the  
112 inhibitory protein DFR1 in Arabidopsis to regulate proline catabolism.

113 In mitochondria, ornithine  $\delta$ -aminotransaminase (OAT) contributes to the formation of GSA and  
114 glutamate by transferring the ornithine  $\delta$ -amino group to  $\alpha$ -ketoglutarate (KG). There have been some  
115 reports of proline biosynthesis from ornithine in plants (Delauney *et al.*, 1993; Roosens *et al.*, 1998)  
116 and in animals (De Ingeniis *et al.*, 2012). An increase in proline content was also shown to be  
117 correlated with higher OAT activity in salt-stressed cashew plants (da Rocha *et al.*, 2012) and in rice  
118 overexpressing OAT (You *et al.*, 2012). In comparison, Funck *et al.* (2008) demonstrated that, at least  
119 in Arabidopsis, P5C is involved in glutamate formation through the action of P5CDH rather than  
120 proline biosynthesis.

121 In bacteria, invertebrates and plants such as halophytes, proline accumulation has been shown to  
122 contribute to osmotic adjustment of cells to counterbalance water loss (Slama *et al.*, 2015; Forlani *et*  
123 *al.*, 2019). Proline can act as a metabolic signaling molecule to modulate mitochondrial functions and  
124 influence cell death (Szabados and Savouré, 2010; Zhang and Becker, 2015; Phang, 2019; Senthil-  
125 Kumar and Mysore, 2012; Rizzi *et al.*, 2016). Proline has also been shown to participate in the redox  
126 balance and energy status of the cell. Proline metabolism may provide stress protection by  
127 maintaining the NADPH/NADP<sup>+</sup> balance and the levels of antioxidants (Ben Rejeb *et al.*, 2014). On  
128 a cellular scale, proline metabolism may bridge compartments to allow redox changes across  
129 membranes. Given this reach and multifunctionality, we now focus on the characteristics of three  
130 redox shuttle systems to provide a framework in which to consider whether proline metabolism could  
131 fulfil a similar purpose in eukaryotes.

132

### 133 **Eukaryotic redox shuttles**

134

#### 135 *The glycerol-3-phosphate (G3P) shuttle*

136 In mitochondria, electrons released from NADH oxidation are transferred to the electron transport  
137 chain, which in turn feeds the energy necessary to pump protons across the inner mitochondrial  
138 membrane. This creates an electrochemical proton gradient that drives ATP synthesis. In addition to  
139 ATP generation, mitochondrial membrane potential drives the transport of metabolites between  
140 mitochondrial and cytosolic compartments. In yeasts, plants and animals, a G3P shuttle involved in

141 redox homeostasis has been described (Ansell *et al.*, 1997; Shen *et al.*, 2003, 2006; Mráček *et al.*,  
142 2013). A key component of the shuttle is a G3P dehydrogenase on the outer surface of the inner  
143 mitochondrial membrane which donates electrons directly to the ubiquinone pool. The G3P shuttle  
144 irreversibly consumes cytosolic NADH generating mitochondrial FADH<sub>2</sub> (Fig. 2A; Shen *et al.*, 2006;  
145 McKenna *et al.*, 2006). Briefly, electrons from NADH are transferred to dihydroxyacetone phosphate  
146 (DHAP) via the cytosolic G3P dehydrogenase (cGPDH) to form G3P. Then, G3P is converted to  
147 DHAP on the outer surface of the inner mitochondrial membrane by mitochondrial G3P  
148 dehydrogenase (mGPDH). Next, electrons are transferred to FAD to form FADH<sub>2</sub> within the  
149 mitochondria, coupled with the reduction of ubiquinone (McKenna *et al.*, 2006). This G3P shuttle is  
150 especially prominent in muscle cells enabling an extremely high rate of oxidative phosphorylation to  
151 be sustained. Some insects that lack lactate dehydrogenase are completely dependent on the G3P  
152 shuttle for the regeneration of cytoplasmic NAD<sup>+</sup> (Mráček *et al.*, 2013). Generally, the G3P shuttle  
153 provides a less important mechanism of redox state regulation in mammalian cells than the malate-  
154 aspartate shuttle (described in detail below) because most tissues have only low levels of mGPDH  
155 (Mráček *et al.*, 2013). In plants, the *FAD-GPDH* gene has been reported to be highly expressed during  
156 seed germination and repressed upon water stress (Shen *et al.*, 2006; Quettier *et al.*, 2008). However,  
157 the role of G3P shuttle remains elusive in plants.

158

### 159 *The malate-aspartate shuttle*

160 Like the G3P shuttle, the more complex malate-aspartate shuttle acts to irreversibly transfer reducing  
161 equivalents from cytosolic NADH to mitochondria (Borst, 2020), but in contrast it transfers NADH  
162 from the cytosol into mitochondrial matrix forming NADH again. This system involves two cytosolic  
163 and two matrix enzymes as well as two inner membrane transporters. In the cytosol, electrons from  
164 NADH are transferred to OAA by cytosolic malate dehydrogenase (cMDH) to generate malate (Fig.  
165 2B). Malate is multifunctional because it is an intermediate in energy metabolism that is also involved  
166 in the transfer of reducing equivalents, and possibly metabolic trafficking between different cell types  
167 in eukaryotes. Malate enters the mitochondrial matrix via the malate–KG carrier (OGC) in exchange  
168 for KG. The mitochondrial malate dehydrogenase (mMDH) converts malate to OAA, transferring the  
169 electrons to NAD<sup>+</sup> to form NADH in the matrix. Subsequently, OAA is transaminated to aspartate  
170 via mitochondrial glutamate OAA aminotransferase (GOT) with the simultaneous conversion of  
171 glutamate to KG. Finally, aspartate is exported from the mitochondria via the aspartate–glutamate  
172 carrier (AGC) in exchange for cytosolic glutamate, which brings one proton into the mitochondria.  
173 The efflux of aspartate from mitochondria is stoichiometric with entry of glutamate plus a proton in  
174 an electrogenic exchange that provides directionality while controlling the rate (LaNoue *et al.*, 1974a,  
175 b). Consequently, the malate-aspartate shuttle is powered by the electrochemical proton gradient to

176 export aspartate and so aids the import of reducing equivalents into mitochondria. It is not clear what  
177 regulates the balance between malate-aspartate shuttle and G3P shuttle.

178 The malate-aspartate shuttle is found in yeast and animals (Cavero *et al.*, 2003; McKenna *et al.*, 2006;  
179 Satrústegui and Bak, 2015). However, it is still unclear whether this shuttle also occurs in plant  
180 mitochondria. There is evidence for this type of shuttle in plant-bacteria interactions in root nodules  
181 of *Alnus glutinosa* (Akkermans *et al.*, 1981) and *Pisum sativum* with an associated role in nitrogen  
182 fixation (Appels and Haaker, 1991). This shuttle has also been described as operating between  
183 glyoxysomes and mitochondria from the endosperm of germinating castor bean (Mettler and Beever,  
184 1980).

185

### 186 *The malate-OAA shuttle*

187 In plant cells, the regulation of energy metabolism is particularly complex because both mitochondria  
188 and chloroplasts are involved. The malate-OAA shuttle, sometimes referred to as the malate valve, is  
189 a powerful system for balancing metabolic fluxes through indirect transport of reducing  
190 equivalents. The valve is composed of cytosolic, mitochondrial, plastid, and peroxisomal malate  
191 dehydrogenases (MDH) in cooperation with malate-OAA translocators (MOT) (Fig. 3). For example,  
192 higher levels of NAD(P)H drive plastid MDH to convert OAA to malate. Then, malate is transferred  
193 to cytosol or mitochondria by MOTs. In the mitochondrial matrix, malate is oxidized by  
194 mitochondrial MDH which reduces  $\text{NAD}^+$  to NADH (Selinski and Scheibe, 2019). Theoretically, the  
195 malate-OAA shuttle should be able to transfer reducing equivalents in either direction depending on  
196 the prevailing redox conditions on either side of the membrane. Oxidation of malate to OAA by  
197 mitochondrial MDH generates NADH, which is then oxidized by Complex I producing ROS.  
198 Generation of ROS from malate oxidation can trigger cell death in both animals and plants suggesting  
199 that such mechanisms are conserved (Zhao *et al.*, 2020). However, in plant cells this shuttle may only  
200 serve to export reducing equivalents from mitochondria and plastids in normal growth conditions  
201 (Selinski and Scheibe, 2019). It should be noted that the exchange of malate and OAA mediated by  
202 MOTs across different cellular compartments is not driven by the electrochemical proton gradient,  
203 so this exchange can only move reducing equivalents from a relatively reduced compartment to a  
204 relatively oxidized compartment. Since the mitochondrial matrix is much more reduced than the  
205 cytosol with respect to the  $\text{NADH}/\text{NAD}^+$  ratio, the OAA transporter is likely to work primarily to  
206 export reducing equivalents from mitochondria to the cytosol (Selinski and Scheibe, 2019).

207

### 208 **The proline cycle as a reducing-equivalent shuttle**

209 Considering that metabolism of proline involves the oxidation of NAD(P)H in the cytosol and the  
210 reduction of  $\text{NAD}^+$  in mitochondria, we postulate that proline metabolism may also play an important

211 role as a redox regulator in eukaryotic cells. Indeed, P5CS and P5CR oxidize NAD(P)H in the cytosol  
212 while in mitochondria ProDH and P5CDH reduce FAD and NAD<sup>+</sup> respectively.

213 In the cytosol, induction of P5CS and/or P5CR expression may help to maintain a low  
214 NADPH/NADP<sup>+</sup> ratio (Liang *et al.*, 2013). Coordination between proline and redox metabolism can  
215 be deduced from the phenotype of the *Arabidopsis p5cs1* mutant because it displays hypersensitivity  
216 to salt stress, accumulation of ROS and strongly enriched expression of genes involved in redox  
217 metabolism of the mitochondria and chloroplasts (Székely *et al.*, 2008; Shinde *et al.*, 2016). Using a  
218 forward genetic screen based on a *ProDH1*-promoter luciferase reporter, Shinde *et al.* (2016) found  
219 that mutants affected in the synthesis of very-long-chain fatty acids (VLCFA) or in cuticle deposition  
220 also accumulate more proline. This implies a strong coordination between proline and lipid  
221 metabolism in relation to redox status. Proline and lipid metabolism both help buffer cellular redox  
222 status under stress.

223 Regulation of P5CR activity in *Arabidopsis* seems to depend on a complex pattern of regulation  
224 including the ratios of reduced to oxidized pyridine nucleotides, the preference for phosphorylated  
225 over non-phosphorylated pyridine nucleotides, and the presence of ions (Giberti *et al.*, 2014;  
226 Ruszkowski *et al.*, 2015). Equimolar concentrations of NADP<sup>+</sup> completely suppress the NADH-  
227 dependent activity of P5CR, whereas the NADPH-dependent reaction is mildly affected (Giberti *et*  
228 *al.*, 2014). Moreover, NADH-dependent but not NADPH-dependent activity of P5CR is inhibited by  
229 an excess of proline. Excess of salt inhibits the NADH-dependent and activates the NADPH-  
230 dependent activity of P5CR. Similarly, in the protozoan *Trypanosoma cruzi*, TcP5CR is an NADPH-  
231 dependent cytosolic enzyme whose activity is fine-tuned by NADPH cytosolic pools (Marchese *et*  
232 *al.*, 2020). Thus, the cytosolic reduced status dependent on proline cycle functioning would be mostly  
233 driven by NADPH homeostasis regulation.

234 In mitochondria, regulation of the reduced state by the proline cycle occurs through the production  
235 of both NADH and FADH<sub>2</sub>, which impact the electron transfer chain functioning as well as ROS  
236 production. Several studies in both animal and plant cells have shown that increasing the reduced  
237 state of mitochondria by metabolic or mutational manipulation leads to proline biosynthesis, while  
238 decreasing mitochondrial reduced state and/or ATP synthesis impairs proline accumulation (Huang  
239 *et al.*, 2013; Schwörer *et al.*, 2020). A decrease in the activity of the mitochondrial electron transfer  
240 chain caused by a defect in one of its super protein complexes leaves cells prone to the damage of  
241 excess reducing power. Enhancing proline biosynthesis and down-regulating its degradation would  
242 be an alternative way to trap reducing power and limit saturation of the electron transfer chain and  
243 formation of ROS. Recently, SSR1, a mitochondrial protein with a tetratricopeptide repeat domain,  
244 was shown to be involved in maintaining the function of the mitochondrial electron transfer chain  
245 (Han *et al.*, 2021). When the *ssr1-1* mutant was treated with proline, the ROS content was higher, the

246 ATP level was lower and AOX function was enhanced. ProDH was hypothesized to directly generate  
247 ROS because this enzyme was shown to reduce oxygen *in vitro* (White *et al.*, 2007) and its  
248 overexpression increased ROS production in cancers and apoptosis (Pandhare *et al.*, 2009; Moxley  
249 *et al.*, 2011). However, Goncalves *et al.* (2014) demonstrated that superoxides are not produced by  
250 ProDH itself but by specific components of the electron transfer chain. ProDH/POX was found to  
251 bind directly to Coenzyme Q suggesting that proline-derived electrons can directly reduce oxygen at  
252 Complex III to generate superoxides (Hancock *et al.*, 2016). On the other hand, *p5cs1-4*, an  
253 Arabidopsis mutant impaired in stress-induced proline accumulation, was shown to be associated  
254 with strongly upregulated expression of a number of genes encoding NAD(P)H dehydrogenases  
255 and/or related to mitochondrial respiration (Shinde *et al.*, 2016). Similarly, Lovell *et al.* (2015)  
256 revealed that proline concentration was strongly affected by cytoplasmic genome variation in  
257 Arabidopsis mapping population in response to drought. Mitochondrial DNA polymorphisms were  
258 linked to two genes coding NADH dehydrogenase subunits indicating that proline accumulation is  
259 tightly regulated by cellular redox status and that proline catabolism is important in drought tolerance.  
260 These results combined support the idea of a tight regulation between proline metabolism and cellular  
261 redox status with mitochondria having a key role, possibly dissipating energy for drought stress  
262 tolerance (Atkin and Macherel, 2009). This would be consistent with the view that the dynamics of  
263 proline metabolism, probably through a proline cycle between biosynthesis and catabolism, is  
264 important for cell homeostasis.

265

266 The importance of the proline cycle in development and stress

267 Various environmental stresses such as drought or salt stress have been shown to trigger proline  
268 accumulation by upregulating proline biosynthesis and downregulating proline catabolism (Peng *et al.*  
269 *et al.*, 1996; Parre *et al.*, 2007; Leprince *et al.*, 2015). If ProDH is absent, the proline cycle may not be  
270 active in such stress conditions, although a low level of ProDH may be sufficient to allow cycling  
271 between proline and glutamate to support the maintenance of a proper NAD(P)<sup>+</sup> to NAD(P)H ratio.  
272 For example, expression of ProDH2 has been shown to be triggered by salt stress whereas ProDH1  
273 expression is repressed (Funck *et al.*, 2010). Other studies have shown the importance of  
274 mitochondrial proline catabolism in the regulation of cell redox status in response to drought stress  
275 (Sharma *et al.*, 2011). In such stress conditions, the presence of ProDH1, including some with putative  
276 post-translational modifications, was observed in western blots of protein extracts (Bhaskara *et al.*,  
277 2015). This discrepancy could be due to the different growth conditions and/or sensitivities of the  
278 antibodies used, and it would be important to determine the activity of ProDH with an appropriate  
279 assay to clarify this issue (Lebreton *et al.*, 2020). In addition, transcriptome analyses have revealed  
280 that *ProDH1* transcript levels oscillate during light-dark cycles, being downregulated during the day

281 and upregulated during the night. Drought downregulates ProDH1 transcript levels but only during  
282 the day. Interestingly, VLCA biosynthesis genes had a similar expression profile to the *ProDH1* gene  
283 expression profile (Dubois *et al.*, 2017), which corroborates the idea that proline metabolism is  
284 coordinated with VLCA biosynthesis when cellular redox status is altered.

285 Transgenic plants overexpressing *P5CS1* under the control of a heat shock promoter accumulated  
286 proline in a heat-dependent manner and, interestingly, were less tolerant to heat shock. Indeed, higher  
287 *ProDH1* expression and ROS contents were measured in these transgenic plants suggesting that the  
288 proline cycle had been stimulated (Lv *et al.*, 2011). High levels of P5C were also detected in the yeast  
289 *put2* mutant (mutated in the yeast gene for P5CDH) (Nomura and Takagi, 2004) and the Arabidopsis  
290 *p5cdh* mutant in addition to high levels of ROS upon proline treatment (Deuschle *et al.*, 2004; Miller  
291 *et al.*, 2009). These works revealed the importance of the proline cycle in maintaining cellular ROS  
292 homeostasis.

293 The proline cycle is also involved in NADP<sup>+</sup>/NADPH homeostasis during plant development through  
294 a spatial and temporal compartmentalization of proline synthesis and proline degradation. Such a  
295 distribution of metabolism between different organs implies that proline as well as glutamate must be  
296 transported somehow. Proline is known to move between tissues through vascular vessels (Girousse  
297 *et al.*, 1996) and from cell to cell by plasma membrane transporters such as the amino acid/auxin  
298 permease (AAP) and proline transporters (ProT) (Schwacke *et al.*, 1999; Hirner *et al.*, 2006; Lehmann  
299 *et al.*, 2011).

300 Interestingly proline is mainly synthesized in photosynthetically active tissues, although its oxidation  
301 is localized in sink non-green tissues (Sharma *et al.*, 2011). Proline is transported from source to sink  
302 tissues such as growing regions of root and shoot where the oxidation of proline will provide energy  
303 to support growth. Source-sink interactions are also key determinants of leaf senescence (Dellero *et al.*,  
304 2020). During plant senescence, specific degradation of Calvin cycle enzymes is triggered,  
305 lowering the NADPH levels in chloroplasts or senescent organs. Expression of *ProDH1* and to a  
306 lesser extent *ProDH2* has been shown to be triggered when leaf senescence is induced by darkness,  
307 leading to higher ProDH activity (Launay *et al.*, 2019). In addition, proline was demonstrated to be  
308 an alternative metabolic substrate whose oxidation fuels the electron transfer chain to generate energy  
309 (Launay *et al.*, 2019). Proline oxidation is therefore important for restoring the NADP<sup>+</sup> to NADPH  
310 ratio of the cell.

311 In another example, transport of proline was shown to be important during the formation of plant  
312 reproductive organs. Proline content was 56 times more concentrated in flowers (sink organs) than in  
313 source leaves (Schwacke *et al.*, 1999). Proline accumulated in pollen grains accounted for 70% of the  
314 total free amino acids and was essential for pollen fertility (Mattioli *et al.*, 2018). The *LeProT1* gene,  
315 which encodes a proline transporter, is expressed in the germinating pollen tube. However, the proline

316 transport through this route is insufficient to fulfill the demand of developing microspore cells  
317 (Mattioli *et al.*, 2018). The proline that accumulates in pollen derives from local synthesis inside  
318 developing microspores and mature pollen grains. In this case, it was also suggested that proline  
319 would have a role in supplying energy during the rapid growth of the pollen tube.

320 Proline accumulation may also be a very important mechanism to balance redox potential oscillating  
321 during the day. Indeed, light is known to affect transcript levels of both proline biosynthesis and  
322 degradation genes in plants (Hanson and Tully, 1979; Sanada *et al.*, 1995). *P5CS* gene expression is  
323 promoted during the light period and repressed during dark periods, whereas *ProDH* transcript levels  
324 show the opposite behavior (Hayashi *et al.*, 2000; Dubois *et al.*, 2017). It is conceivable that light  
325 regulation also occurs at the protein level. By using a proteomic approach, Marchand *et al.* (2010)  
326 have shown that P5CDH could be a target of thioredoxin. Thioredoxins (Trx) are ubiquitous proteins  
327 catalyzing reversible disulfide-bond formation involved in enzyme redox and activity regulation.  
328 Different Trx isoforms are present in mitochondria (Trx o, Trx h) and cytosol (Trx h) and the reducing  
329 power for Trx reduction is provided by NADPH through NADPH-thioredoxin reductase (NTR)  
330 (Geigenberger *et al.*, 2017). Interestingly, less proline and more glutamate and malate accumulated  
331 in *trxol* and *ntra ntrb* double mutants compared to the wild type (Daloso *et al.*, 2015). A 30%  
332 reduction in proline accumulation was also observed in a *trxI* mutant under low water potential  
333 conditions by Verslues *et al.* (2014). All these data reflect a probable regulation of proline metabolic  
334 enzymes by Trx, that are able to transmit the light signal from the chloroplast to other organelles like  
335 mitochondria. Moreover, as the proline cycle may shuttle reducing power from cytosol to  
336 mitochondria, we can hypothesize that it also contributes to the NTR/Trx system control. Thus, the  
337 proline cycle may be important during the night when photosynthesis is not active to generate redox  
338 power and energy.

339

340 The proline-P5C cycle and its functions

341 Interestingly, when it is in equilibrium with GSA in a reversible non-enzymatic reaction, P5C is an  
342 intermediate for both proline biosynthesis from either glutamate or ornithine and proline oxidation.  
343 It is a product of ProDH activity in mitochondria and a substrate of P5CR in the cytosol. If P5C could  
344 move back and forth between the cytosol and the mitochondrial matrix, P5CR and ProDH would then  
345 be said to form a metabolic ring, the proline-P5C cycle. Notionally, the prerequisite for a functional  
346 proline-P5C cycle is for both P5CR and ProDH to be expressed at the same time and for either GSA  
347 or P5C to be transported through mitochondrial membranes. The existence of this cycle in plants is  
348 far from proven, not least because no transporter of P5C has been identified yet.

349

350 The proline-P5C cycle was first proposed by Hagedorn and Phang (1986) when they demonstrated  
351 the catalytic functioning of the shuttle to generate cytosolic  $\text{NADP}^+$  without stoichiometric  
352 consumption of proline. Proline is exported into mitochondria by a transporter whose gene has not  
353 been identified yet. Proline is then oxidized to P5C, which may be transported back into the cytosol,  
354 with the help of a putative unknown transporter, to be further reduced to proline by P5CR. By this  
355 route, mitochondrial P5C would replenish cytosolic proline biosynthesis and avoid at the same time  
356 the consumption of cytosolic ATP and production of mitochondrial NADH by bypassing P5CS and  
357 P5CDH, respectively. This proline-P5C cycle would then allow NADPH to be consumed in the  
358 cytosol, while  $\text{FAD}^+$  would be reduced in mitochondria. Thus, reducing power would be shuttled in  
359 a single direction from the cytosol to the mitochondrial matrix in this cycle (Figure 1). The proline-  
360 P5C cycle has been proposed to maintain the redox balance between mitochondria and the cytosol.  
361 In animals, the proline-P5C cycle has been shown to play important roles in the regulation of cell  
362 growth and cell death. This cycle acts by transferring the reducing power formed by the pentose  
363 phosphate pathway into mitochondria for the production of either ROS or ATP. ROS signaling  
364 mediated by ProDH was shown to trigger apoptosis, suggesting that ProDH is a mitochondrial tumor  
365 suppressor (Liu and Phang, 2012).

366 Miller *et al.* (2009) proposed that a proline-P5C cycle also operates in plants with P5C transport. This  
367 plant biochemical cycle may support the preferential accumulation of proline as well as the shuttling  
368 of redox equivalents from the cytosol to mitochondria. This cycle would maintain a high cellular  
369 proline to P5C ratio and direct electrons to the electron transfer chain.

370 Increases in ProDH expression and activity have been observed when the oxidative burst occurs  
371 during the hypersensitive response (Cecchini *et al.*, 2011; Monteoliva *et al.*, 2014). In this instance,  
372 ProDH was proposed to participate in the hypersensitive response through the proline-P5C cycle by  
373 generating ROS to trigger cell death in order to prevent pathogen spreading in plants. It is possible  
374 that proline toxicity may be due to an overflow of electrons in the mitochondrial electron transfer  
375 chain.

376 P5C may also originate from the arginine pathway. In most organisms, arginine catabolism  
377 contributes to the formation of ornithine and urea through the action of arginase. In general, OAT  
378 catalyzes the transfer of the  $\delta$ -amino group from ornithine to KG yielding GSA/P5C and glutamate,  
379 contributing to P5C input into mitochondria to serve as the substrate for P5CDH (Figure 1). In  
380 intestine cells, OAT also participates in the degradation of ornithine to form citrulline, which results  
381 in a consumption of P5C (Ginguay *et al.*, 2017). In plants, a direct contribution of OAT to stress-  
382 induced proline accumulation is still debated. A study of salt-stressed *Arabidopsis oat* knockout  
383 mutants provided strong evidence that OAT does not contribute to stress-induced proline  
384 accumulation (Funck *et al.*, 2008). Therefore, it is unlikely that the OAT pathway contributes to the

385 proline-P5C cycle, at least in salt stress conditions. It has nevertheless been proposed that high  
386 concentrations of free arginine may increase the affinity of P5CDH for P5C, possibly influencing the  
387 balance between P5C export into the cytosol and its mitochondrial oxidation to glutamate (Forlani *et*  
388 *al.*, 2015).

389

### 390 **Conclusions and perspectives**

391 As mitochondrial membranes are not permeable to NAD(P)<sup>+</sup> or NAD(P)H, eukaryotes have evolved  
392 redox shuttles, like the G3P, malate-aspartate and malate-OAA shuttles, to allow transfer of reducing  
393 power from one compartment to another. It is not clear yet whether a malate-aspartate shuttle operates  
394 in plant cells.

395 The availability of a redox shuttle that allows redox exchange between cytosol and mitochondria is  
396 of particular interest for plant cells. Proline metabolism is a unique pathway because it involves the  
397 interconversion of proline and glutamate, a process linked to consumption of reducing power for  
398 proline biosynthesis in the cytosol. The existence of the proline cycle allows the biosynthesis of  
399 proline and concomitant oxidation of NADPH molecules in the cytosol. Furthermore, glucose  
400 oxidation from the pentose phosphate pathway in the cytosol can be linked to ATP production via the  
401 sequential transfer of the generated NADPH to proline, which would be then oxidized by ProDH in  
402 the mitochondrion.

403 When proline is transported into mitochondria, its oxidation supports oxidative phosphorylation by a  
404 mechanism independent of NADH oxidation because electrons enter the electron transport system at  
405 the level of the flavoenzyme ProDH, which directly feeds the mitochondrial electron transfer chain  
406 and drives the synthesis of ATP. This proline cycle enables electrons from cytosolic NADPH to be  
407 transported into mitochondria against the NADH concentration gradient. It is unlikely that the proline  
408 cycle acts as a continuous major source of energy because firstly ProDH has to be expressed and  
409 active, and secondly this cycle produces only a limited amount of the ATP produced by succinate  
410 entering the tricarboxylic acid cycle. Nevertheless, there may be special conditions when this shuttle  
411 acts as a transient yet significant source of energy for development or survival. The proline cycle may  
412 be required when the tricarboxylic acid cycle cannot operate maximally, as postulated to occur during  
413 the initiation of leaf senescence. The proposed proline cycle appears to play an important role in  
414 regulating both cytosolic and mitochondrial redox states. Regulation at the level of ProDH and P5CR  
415 enzymes and/or transport of proline, P5C and glutamate across mitochondrial membranes requires  
416 more study, but given the influence and reach of the proline shuttle, any new findings may be relevant  
417 in the study and development of resilient crops.

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419

420

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426

427 **Author contribution**

428 AS conceived and planned the review topic. AS wrote the manuscript with support from YZ, CC,  
429 SP and SL. All authors approved the final version of the manuscript.

430

431 **Conflict of interest**

432 The authors declare that this research was conducted in the absence of any commercial or financial  
433 relationships that would cause any potential conflicts of interest.

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## Figure legends

Fig. 1. Proline metabolism in plants and its links to the ornithine and pentose phosphate pathways. The proline cycle is highlighted by blue arrows and the putative proline-P5C cycle by a square in light blue. Asp, aspartate; BAC, basic amino acid carrier; BOU, a bout de souffle; ETC, electron transfer chain; Glu, glutamate; KG,  $\alpha$ -ketoglutarate; OAT, ornithine  $\delta$ - aminotransferase; Orn, ornithine; PPP shunt, pentose phosphate pathway; P5C, pyrroline-5-carboxyate; P5CDH, P5C dehydrogenase; P5CS, P5C synthetase; ProDH, proline dehydrogenase; Pro, proline; UCP, mitochondrial uncoupling protein; ? indicates unknown transporter(s).

Fig. 2. The glycerol-3-phosphate (G3P) (A) and the malate–aspartate (B) shuttles for transporting reducing equivalents from cytosolic NADH into the mitochondrial matrix or mitochondrial oxidative phosphorylation pathway respectively. AGC, aspartate-glutamate carrier; Asp, aspartate; DHAP, dihydroxyacetone phosphate; GPDH, glycerol-3-phosphate dehydrogenase; G3P, glycerol-3-phosphate; Glu, glutamate; GOT, glutamate oxaloacetate aminotransferase; IMS, intermembrane space; KG,  $\alpha$ -ketoglutarate; Mal, malate; MDH, malate dehydrogenase; OAA, oxaloacetate; OGC, KG-malate carrier.

Fig. 3. Malate-oxaloacetate shuttle, or the malate valve, involves the interconversion of malate and oxaloacetate by malate dehydrogenase (MDH). Isoenzymes of MDH are present in each cellular compartment, the cytosol, chloroplasts and mitochondria. MDH, malate dehydrogenase; OAA,

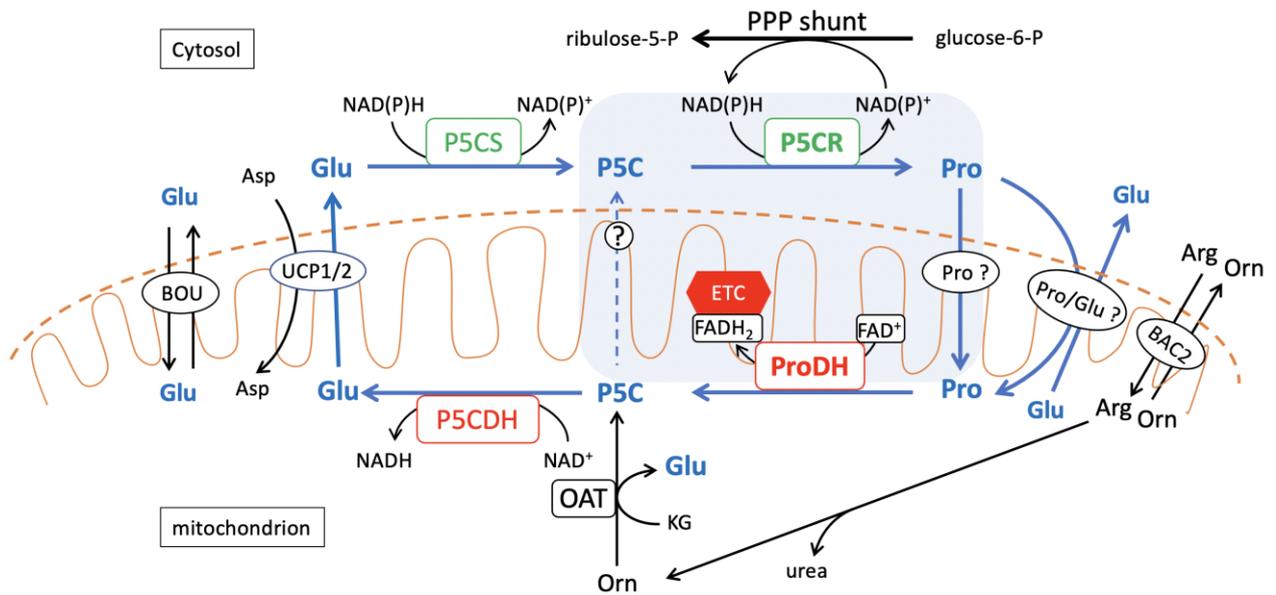


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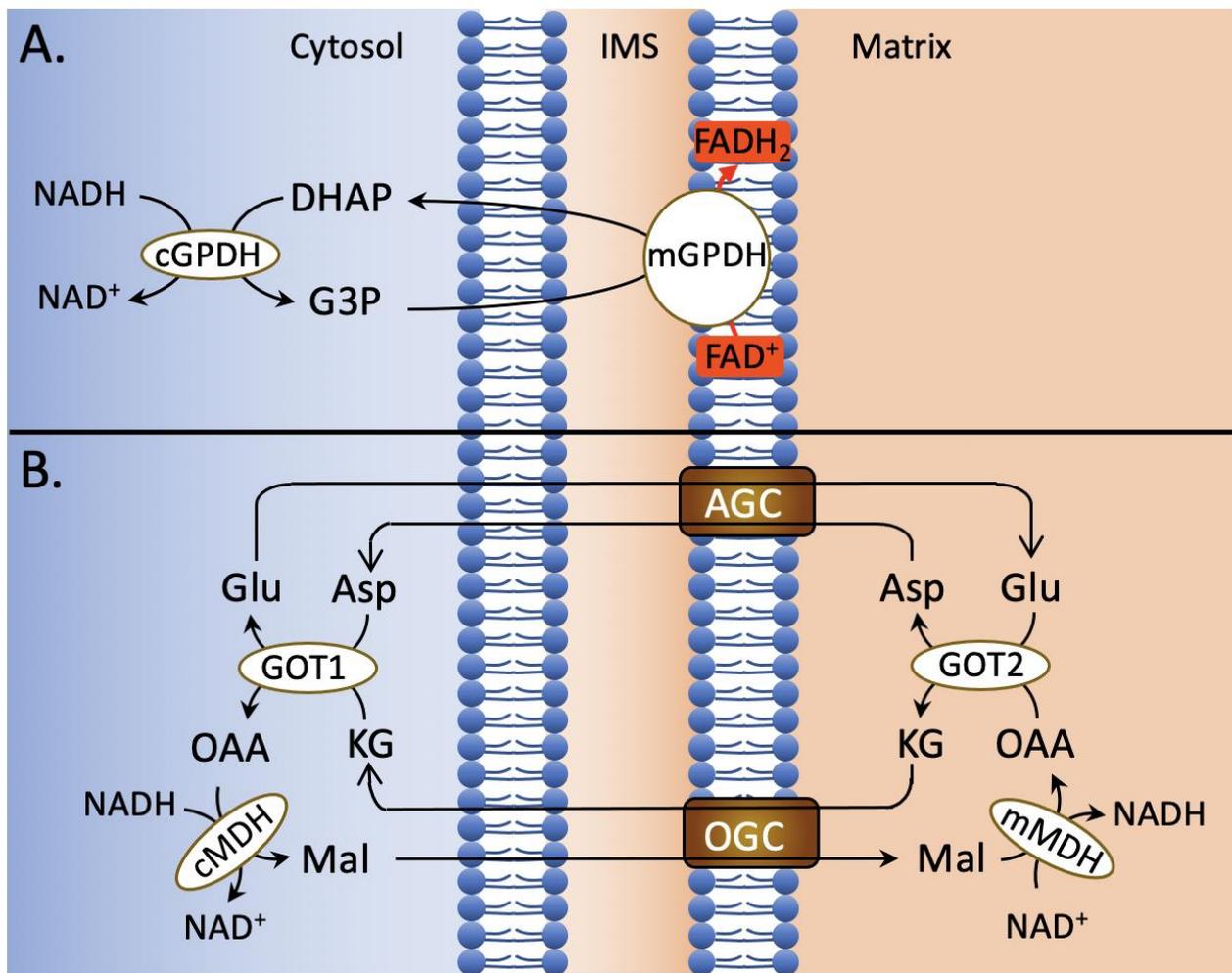


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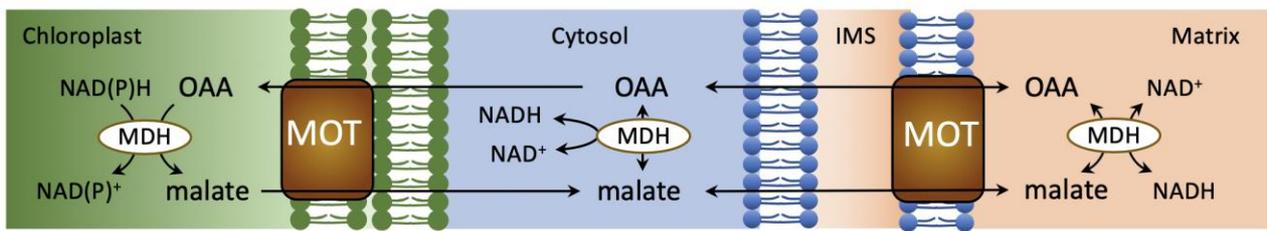


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