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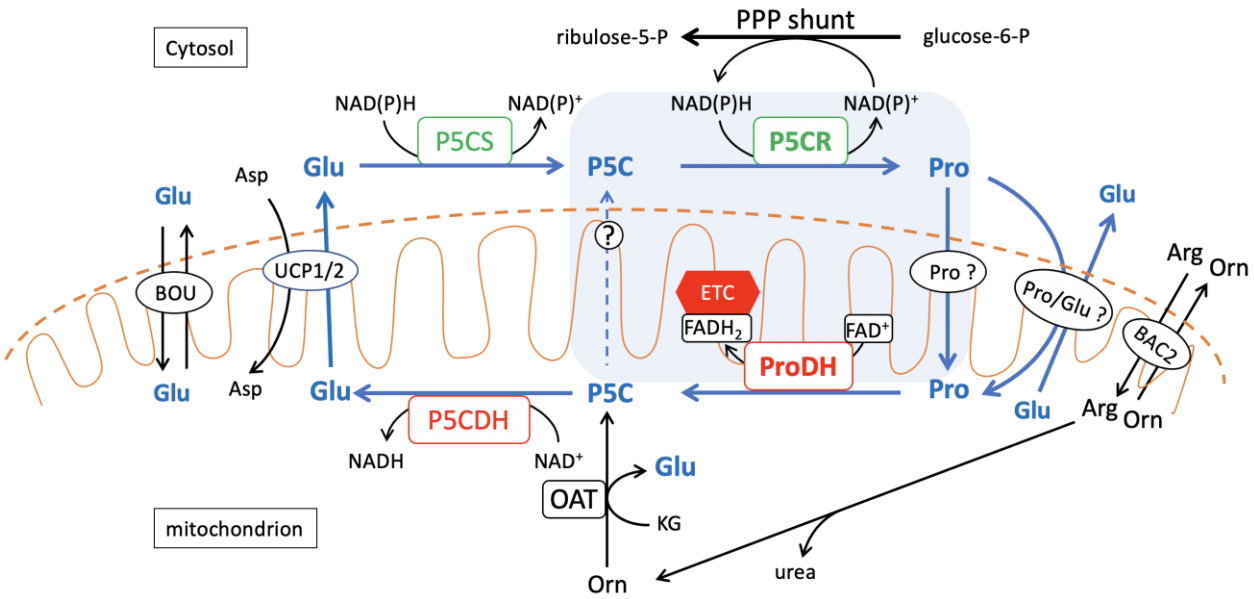

Fig. 1. Proline metabolism in plants and its links to the ornithine and pentose phosphate pathways. The proline cycle is highlighted by blue arrows and the putative proline-P5C cycle by a square in light blue. Asp, aspartate; BAC, basic amino acid carrier; BOU, a bout de souffle; ETC, electron transfer chain; Glu, glutamate; KG, α-ketoglutarate; OAT, ornithine δ- 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).

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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

Abstract

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

Keywords

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

Introduction

Redox homeostasis during development is vital for all branches of the tree of life, particularly in response to environmental changes (Scheibe *et al.*, 2005). Compartmentalization of metabolism and other cellular functions in eukaryotic cells implies that pools of metabolites, ions and reducing equivalents are concentrated locally in a regulated manner (Lunn, 2006). Maintenance of metabolism as environmental conditions vary, whether regularly, rapidly or to extremes, is a challenge for all eukaryotic organisms, and particularly plants for several reasons. As sessile organisms, plants cannot move away to more clement conditions, so they are subject to dynamic environmental alterations. In eukaryotic photosynthetic organisms, the regulation of energy metabolism is particularly complex because it involves two organelles, the mitochondrion and the chloroplast, as major sources of ATP and reducing power in the form of pyridine nucleotides NADH and NADPH (Foyer and Noctor, 2020). Additional complexity of redox and energy metabolism arises from the daily cycles of light and dark periods, and the presence of green and non-green tissues within the same individual. In daylight, photon energy is transformed in green tissues by photosystems into ATP and NADPH, which in turn are used to fix CO_2 by the Calvin–Benson cycle. In mitochondria, oxidative

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

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

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

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

Proline metabolism

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

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

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

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

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

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

Eukaryotic redox shuttles

The glycerol-3-phosphate (G3P) shuttle

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

redox homeostasis has been described (Ansell *et al.*, 1997; Shen *et al.*, 2003, 2006; Mráček *et al.*, 2013). A key component of the shuttle is a G3P dehydrogenase on the outer surface of the inner mitochondrial membrane which donates electrons directly to the ubiquinone pool. The G3P shuttle irreversibly consumes cytosolic NADH generating mitochondrial $FADH_2$ (Fig. 2A; Shen *et al.*, 2006; McKenna *et al.*, 2006). Briefly, electrons from NADH are transferred to dihydroxyacetone phosphate (DHAP) via the cytosolic G3P dehydrogenase (cGPDH) to form G3P. Then, G3P is converted to DHAP on the outer surface of the inner mitochondrial membrane by mitochondrial G3P dehydrogenase (mGPDH). Next, electrons are transferred to FAD to form $FADH_2$ within the mitochondria, coupled with the reduction of ubiquinone (McKenna *et al.*, 2006). This G3P shuttle is especially prominent in muscle cells enabling an extremely high rate of oxidative phosphorylation to be sustained. Some insects that lack lactate dehydrogenase are completely dependent on the G3P shuttle for the regeneration of cytoplasmic NAD^+ (Mráček *et al.*, 2013). Generally, the G3P shuttle provides a less important mechanism of redox state regulation in mammalian cells than the malate-aspartate shuttle (described in detail below) because most tissues have only low levels of mGPDH (Mráček *et al.*, 2013). In plants, the *FAD-GPDH* gene has been reported to be highly expressed during seed germination and repressed upon water stress (Shen *et al.*, 2006; Quettier *et al.*, 2008). However, the role of G3P shuttle remains elusive in plants.

The malate-aspartate shuttle

Like the G3P shuttle, the more complex malate-aspartate shuttle acts to irreversibly transfer reducing equivalents from cytosolic NADH to mitochondria (Borst, 2020), but in contrast it transfers NADH from the cytosol into mitochondrial matrix forming NADH again. This system involves two cytosolic and two matrix enzymes as well as two inner membrane transporters. In the cytosol, electrons from NADH are transferred to OAA by cytosolic malate dehydrogenase (cMDH) to generate malate (Fig. 2B). Malate is multifunctional because it is an intermediate in energy metabolism that is also involved in the transfer of reducing equivalents, and possibly metabolic trafficking between different cell types in eukaryotes. Malate enters the mitochondrial matrix via the malate–KG carrier (OGC) in exchange for KG. The mitochondrial malate dehydrogenase (mMDH) converts malate to OAA, transferring the electrons to NAD^+ to form NADH in the matrix. Subsequently, OAA is transaminated to aspartate via mitochondrial glutamate OAA aminotransferase (GOT) with the simultaneous conversion of glutamate to KG. Finally, aspartate is exported from the mitochondria via the aspartate–glutamate carrier (AGC) in exchange for cytosolic glutamate, which brings one proton into the mitochondria.

The efflux of aspartate from mitochondria is stoichiometric with entry of glutamate plus a proton in an electrogenic exchange that provides directionality while controlling the rate (LaNoue *et al.*, 1974a, *b*). Consequently, the malate-aspartate shuttle is powered by the electrochemical proton gradient to

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

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

The malate-OAA shuttle

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

The proline cycle as a reducing-equivalent shuttle

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

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

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

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

In mitochondria, regulation of the reduced state by the proline cycle occurs through the production of both NADH and $FADH_2$, which impact the electron transfer chain functioning as well as ROS production. Several studies in both animal and plant cells have shown that increasing the reduced state of mitochondria by metabolic or mutational manipulation leads to proline biosynthesis, while decreasing mitochondrial reduced state and/or ATP synthesis impairs proline accumulation (Huang *et al.*, 2013; Schwörer *et al.*, 2020). A decrease in the activity of the mitochondrial electron transfer chain caused by a defect in one of its super protein complexes leaves cells prone to the damage of excess reducing power. Enhancing proline biosynthesis and down-regulating its degradation would be an alternative way to trap reducing power and limit saturation of the electron transfer chain and formation of ROS. Recently, SSR1, a mitochondrial protein with a tetratricopeptide repeat domain, was shown to be involved in maintaining the function of the mitochondrial electron transfer chain (Han *et al.*, 2021). When the *ssr1-1*mutant was treated with proline, the ROS content was higher, the

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

The importance of the proline cycle in development and stress

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

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

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

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

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

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

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

Proline accumulation may also be a very important mechanism to balance redox potential oscillating during the day. Indeed, light is known to affect transcript levels of both proline biosynthesis and degradation genes in plants (Hanson and Tully, 1979; Sanada et al., 1995). *P5CS* gene expression is promoted during the light period and repressed during dark periods, whereas *ProDH* transcript levels show the opposite behavior (Hayashi *et al.*, 2000; Dubois *et al.*, 2017). It is conceivable that light regulation also occurs at the protein level. By using a proteomic approach, Marchand *et al.* (2010) have shown that P5CDH could be a target of thioredoxin. Thioredoxins (Trx) are ubiquitous proteins catalyzing reversible disulfide-bond formation involved in enzyme redox and activity regulation. Different Trx isoforms are present in mitochondria (Trx o, Trx h) and cytosol (Trx h) and the reducing power for Trx reduction is provided by NADPH through NAPDH-thioredoxin reductase (NTR) (Geigenberger *et al.*, 2017). Interestingly, less proline and more glutamate and malate accumulated in *trxo1* and *ntra ntrb* double mutants compared to the wild type (Daloso *et al.*, 2015). A 30% reduction in proline accumulation was also observed in a *trx1* mutant under low water potential conditions by Verslues *et al.* (2014). All these data reflect a probable regulation of proline metabolic enzymes by Trx, that are able to transmit the light signal from the chloroplast to other organelles like mitochondria. Moreover, as the proline cycle may shuttle reducing power from cytosol to mitochondria, we can hypothesize that it also contributes to the NTR/Trx system control. Thus, the proline cycle may be important during the night when photosynthesis is not active to generate redox power and energy.

The proline-P5C cycle and its functions

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

The proline-P5C cycle was first proposed by Hagedorn and Phang (1986) when they demonstrated the catalytic functioning of the shuttle to generate cytosolic $NADP^+$ without stoichiometric consumption of proline. Proline is exported into mitochondria by a transporter whose gene has not been identified yet. Proline is then oxidized to P5C, which may be transported back into the cytosol, with the help of a putative unknown transporter, to be further reduced to proline by P5CR. By this route, mitochondrial P5C would replenish cytosolic proline biosynthesis and avoid at the same time the consumption of cytosolic ATP and production of mitochondrial NADH by bypassing P5CS and P5CDH, respectively. This proline-P5C cycle would then allow NADPH to be consumed in the cytosol, while FAD^+ would be reduced in mitochondria. Thus, reducing power would be shuttled in a single direction from the cytosol to the mitochondrial matrix in this cycle (Figure 1). The proline-P5C cycle has been proposed to maintain the redox balance between mitochondria and the cytosol.

In animals, the proline-P5C cycle has been shown to play important roles in the regulation of cell growth and cell death. This cycle acts by transferring the reducing power formed by the pentose phosphate pathway into mitochondria for the production of either ROS or ATP. ROS signaling mediated by ProDH was shown to trigger apoptosis, suggesting that ProDH is a mitochondrial tumor suppressor (Liu and Phang, 2012).

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

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

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

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

Conclusions and perspectives

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

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

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

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Author contribution

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

Conflict of interest

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

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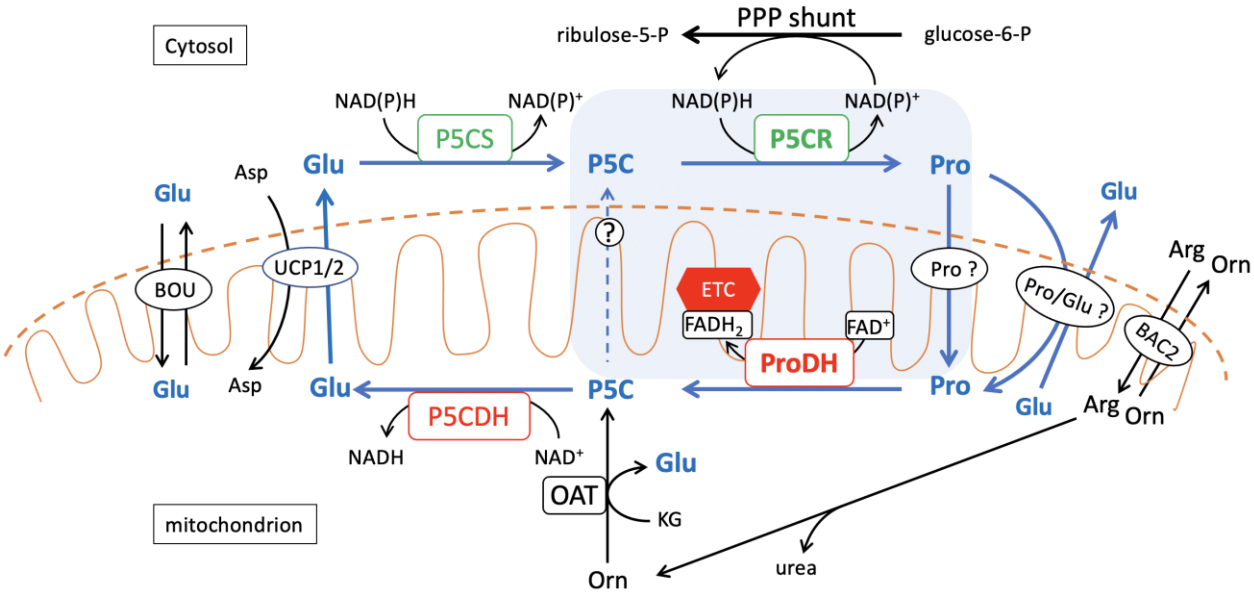
Figure legends

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, α-ketoglutarate; OAT, ornithine δ- 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, α-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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oxaloacetate; MOT, malate-α-ketoglutarate transporter.

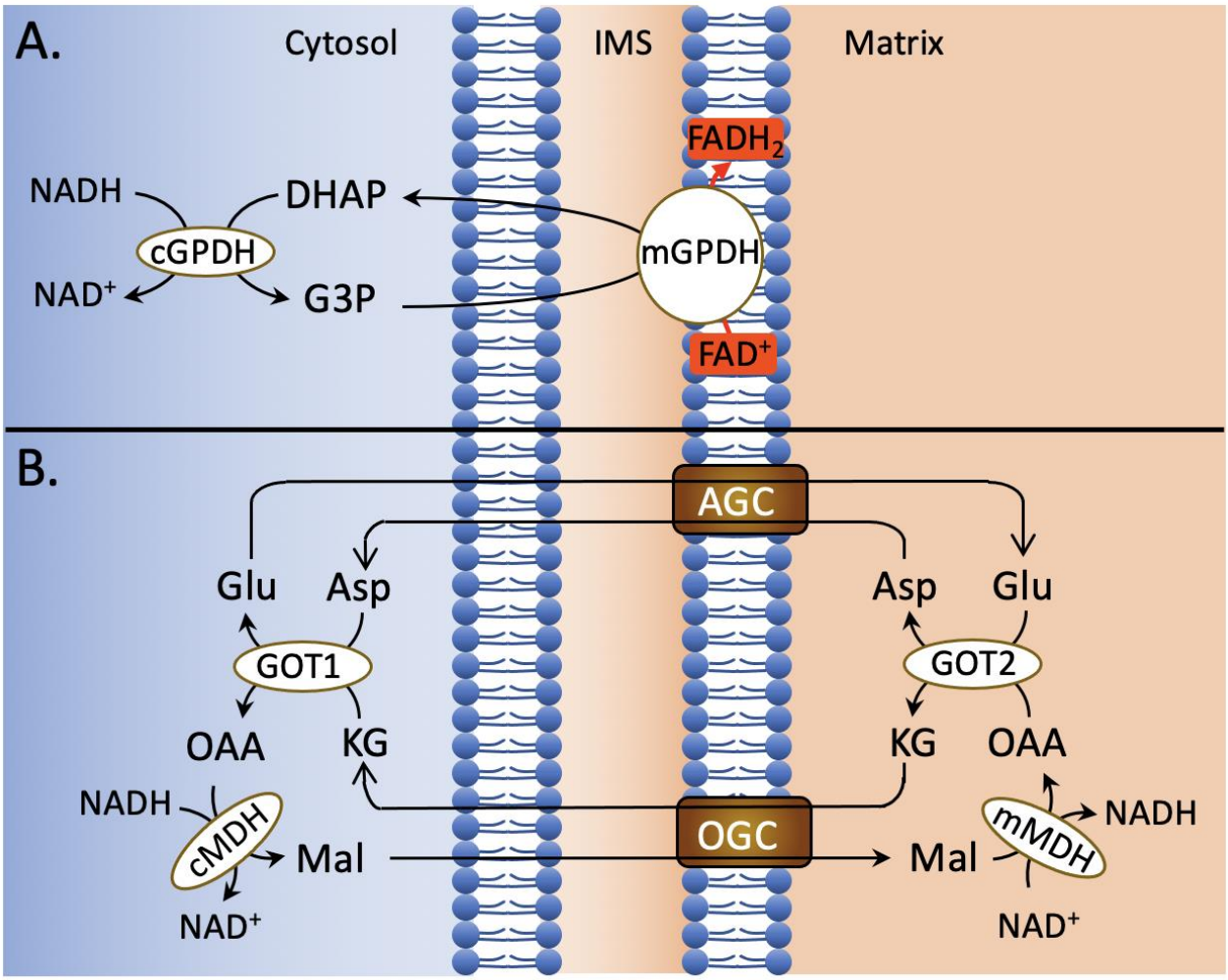

Fig. 2. The glycerol-3-phosphate (G3P) (A) and the malate–aspartate (B) shuttles for transporting reducing equivalents from cytosolic NADH into the mitochondrial matrix or mitochondrial oxidative phosphorylation pathway respectively. AGC, aspartate-glutamate carrier; Asp, aspartate; DHAP, dihydroxyacetone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

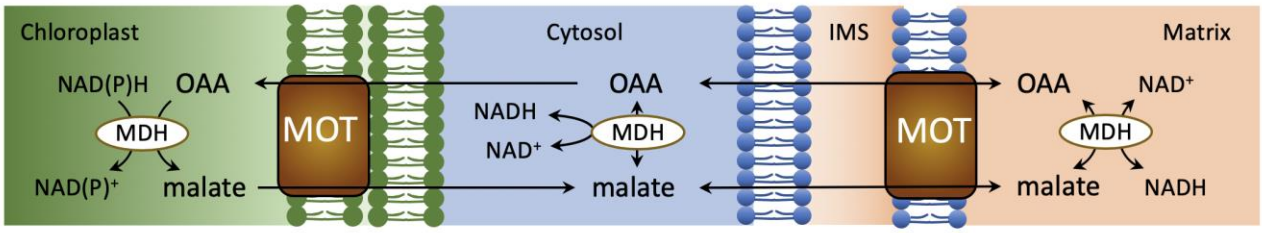

Fig. 3. Malate-oxaloacetate shuttle, or the malate valve, involves the interconversion of malate and oxaloacetate by malate dehydrogenase (MDH). Isoenzymes of MDH are present in each cellular compartment, the cytosol, chloroplasts and mitochondria. MDH, malate dehydrogenase; OAA, oxaloacetate; MOT, malate-α-ketoglutarate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