

Proline: a multifunctional amino acid

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Proline accumulates in many plant species in response to environmental stress. Although much is now known about proline metabolism, some aspects of its biological functions are still unclear. Here, we discuss the compartmentalization of proline biosynthesis, accumulation and degradation in the cytosol, chloroplast and mitochondria. We also describe the role of proline in cellular homeostasis, including redox balance and energy status. Proline can act as a signaling molecule to modulate mitochondrial functions, influence cell proliferation or cell death and trigger specific gene expression, which can be essential for plant recovery from stress. Although the regulation and function of proline accumulation are not yet completely understood, the engineering of proline metabolism could lead to new opportunities to improve plant tolerance of environmental stresses.

Proline accumulation in plants

Proline is a proteinogenic amino acid with an exceptional conformational rigidity, and is essential for primary metabolism. Since the first report on proline accumulation in wilting perennial rye grass (*Lolium perenne*) [1], numerous studies have shown that the proline content in higher plants increases under different environmental stresses. Proline accumulation has been reported during conditions of drought [2] high salinity [3] high light and UV irradiation [4], heavy metals [5], oxidative stress [6] and in response to biotic stresses [7,8]. An osmoprotective function of proline was discovered first in bacteria, where a causal relationship between proline accumulation and salt tolerance has long been demonstrated [9,10]. Such data led to the assumption that proline accumulation in stressed plants has a protective function, which has been emphasized in numerous reviews [11–13]. However, the correlation between proline accumulation and abiotic stress tolerance in plants is not always apparent. For example, high proline levels can be characteristic of salt- and cold-hypersensitive *Arabidopsis* (*Arabidopsis thaliana*) mutants [14,15]. Proline content is also high in drought-tolerant rice varieties [2], but is not correlated with salt tolerance in barley (*Hordeum vulgare*) [16,17]. Nevertheless, several comprehensive studies using transgenic plants or mutants demonstrate that proline metabolism has a complex effect on development and stress responses, and that proline accumulation is important for the tolerance of certain adverse environmental conditions [18–21].

Compartmentalization of proline metabolism in plants

In plants, proline is synthesized mainly from glutamate, which is reduced to glutamate-semialdehyde (GSA) by the pyrroline-5-carboxylate synthetase (P5CS) enzyme, and spontaneously converted to pyrroline-5-carboxylate (P5C) [22,23] (Figure 1). P5C reductase (P5CR) further reduces the P5C intermediate to proline [24,25]. In most plant species, P5CS is encoded by two genes and P5CR is encoded by one [25–27]. Proline catabolism occurs in mitochondria via the sequential action of proline dehydrogenase or proline oxidase (PDH or POX) producing P5C from proline, and P5C dehydrogenase (P5CDH), which converts P5C to glutamate. PDH is encoded by two genes, whereas a single *P5CDH* gene has been identified in *Arabidopsis* and tobacco (*Nicotiana tabacum*) [28–31]. As an alternative pathway, proline can be synthesized from ornithine, which is transaminated first by ornithine-delta-aminotransferase (OAT) producing GSA and P5C, which is then converted to proline [32,33].

Intracellular proline levels are determined by biosynthesis, catabolism and transport between cells and different cellular compartments. Computer predictions suggest a mainly cytosolic localization of the biosynthetic enzymes (P5CS1, P5CS2 and P5CR), whereas a mitochondrial localization is predicted for the enzymes involved in proline catabolism, such as PDH1/ERD5, PDH2, P5CDH and OAT (Table 1). Although signal peptides could not be identified within the primary structure of P5CS1, P5CS2 and P5CR enzymes, the PDH1, P5CDH and OAT proteins have well recognizable mitochondrial targeting signals.

P5CS1-GFP is normally localized in the cytosol of leaf mesophyll cells, but in embryonic cells and roots it is associated with organelles that are similar to fusiform bodies. When cells are exposed to salt or osmotic stress, P5CS1-GFP, but not P5CS2-GFP, accumulates in the chloroplasts. GFP-labeled *Arabidopsis* P5CS2 has been shown to be predominantly localized in the cytosol [20]. The P5CR protein and activity has been detected in the cytosol and plastid fraction of leaf, root and nodule cells of soybean (*Glycine max*) [24,34]. In pea (*Pisum sativum*) mesophyll protoplasts, P5CR activity was localized in chloroplasts, suggesting that P5CR accumulates in plastids under high osmotic conditions [35]. Housekeeping proline biosynthesis probably occurs in the cytosol and, in *Arabidopsis*, it is controlled by the *P5CS2* gene [20]. During osmotic stress, proline biosynthesis is augmented in the chloroplasts, which is controlled by the stress-induced *P5CS1* gene in *Arabidopsis* [20,23,26]. Therefore, proline can be synthesized in different subcellular compartments, depending on the environmental conditions (Figure 1).

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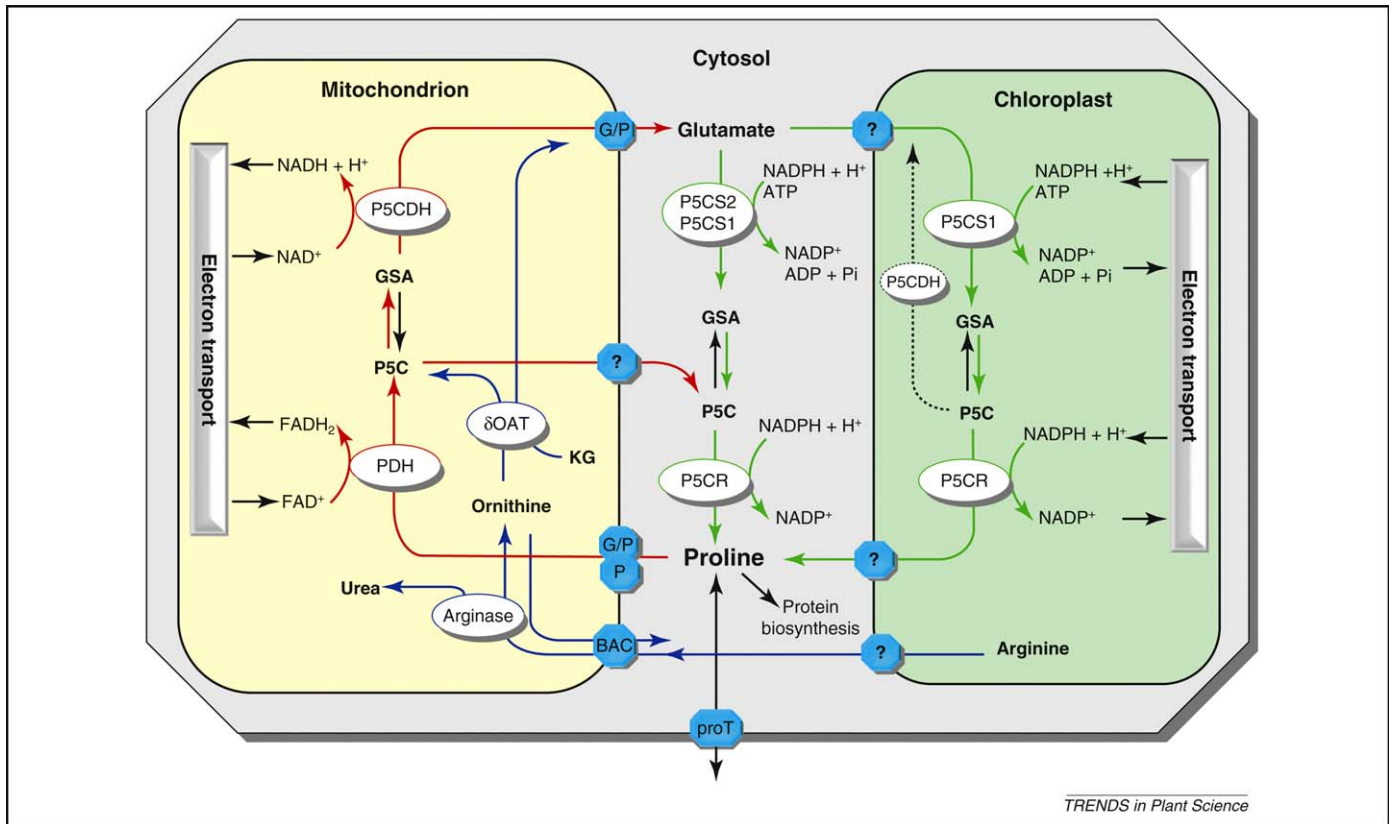


Figure 1. Proposed model for proline metabolism in higher plants. Most data were obtained using *Arabidopsis*, but might also be valid for other species. The biosynthetic pathway is marked with green lines, the catabolic pathway with red lines and the ornithine pathway with blue lines. In meristematic and embryonic cells, housekeeping proline biosynthesis occurs in the cytosol and is mediated by P5CS and P5CR enzymes. Under stress conditions, P5CS1 accumulates in the chloroplasts, leading to enhanced proline biosynthesis in the plastids. Proline degradation occurs in the mitochondria, where proline is oxidized to P5C and glutamate through sequential action of PDH and P5CDH. The ornithine pathway uses arginine and produces P5C and glutamate in mitochondria. Mitochondrial P5C can be recycled to proline in the cytosol by P5CR. Enzymes are depicted as ellipses and transporter proteins as blue octagons. Abbreviations: BAC, basic amino acid transporter involved in arginine and ornithine exchange; Glu, glutamate; G/P, mitochondrial glutamate/proline antiporter; KG, alpha-ketoglutarate; P, mitochondrial proline transporter; Pi, inorganic phosphate; ProT, plasma membrane proline transporter; ?, predicted transporters.

Table 1. Subcellular localization of the enzymes involved in proline metabolism: computer predictions and experimental data^a

Protein	P5CS1	P5CS2	P5CR	PDH1	PDH2	P5CDH	OAT
<i>Arabidopsis</i> gene	AT2G39800	AT3G55610	AT5G14800	AT3G30775	AT5G38710	AT5G62530	AT5G46180
Computer predictions^b							
SUBA	Cyto, Plas	Cyto, ER	Cyto, Mit	Mit	Mit	Mit	Mit, Plas
SherLoc2	Cyto	Cyto	Cyto	Mit	Mit	Cyto, Mit	Mit
Plant-Ploc	Cyto	Cyto	Cyto	Mit	Mit	Mit	Mit
Wolf Psort	ER, Plas	ER, Plas	Cyto	ER, Cyto	Mit, Plas	Mit, Plas	Mit, Plas
ElsPred2	Cyto	Cyto	Cyto	Mit	Plas	Mit	Mit
TargetP	-	-	-	-	Mit	Mit	Mit
MultiLoc2	Cyto	Cyto	Cyto	Cyto, Mit,	Mit	Mit	Mit
LOctree	Cyto	Cyto	Cyto	Mit.	Mit.	Mit.	Mit.
Experimental data							
Plant	<i>Arabidopsis</i>	<i>Arabidopsis</i>	Pea	<i>Arabidopsis</i>		<i>Arabidopsis</i>	<i>Arabidopsis</i>
Location (method) [Ref.]	Plas, Cyto (GFP) [20]	Cyto (GFP) [20]	Plas (biochem.) [35]	Mit (biochem.) [28]		Mit (GFP) [30], Plas (mass spec.) [38]	Mit. (GFP) [40]
Plant			Soybean	Maize, wheat, barley			
Location (method) [Ref.]			Cyto, Plas (biochem.) [24,34]	Mit. (biochem.) [36,37]			
Location (method) [Ref.]				Mit, Cyto (biochem.) [34]			

^aAbbreviations: Biochem, biochemical data; Cyto, cytosol; ER, endoplasmic reticulum; GFP, localization with green fluorescent protein fusion; mass spec, detection by mass spectrometry; mit, mitochondria; plas, plastid.

^bComputer predictions were collected using seven internet services and the SUB-cellular location database for *Arabidopsis* proteins (SUBA). URL sites of the internet services are: SUBA (<http://suba.plantenergy.uwa.edu.au/>); SherLoc2 (<http://www-bs.informatik.uni-tuebingen.de/Services/SherLoc2/>); Plant-Ploc (<http://www.csbio.sjtu.edu.cn/bioinf/plant/>); Wolf Psort (<http://wolfpsort.org/>); ElsPred2 (<http://www.imtech.res.in/raghava/eslpred2/>); TargetP (<http://www.cbs.dtu.dk/services/TargetP/>); MultiLoc2 (<http://www-bs.informatik.uni-tuebingen.de/Services/MultiLoc2/>); and LOctree (<http://cubic.bioc.columbia.edu/services/loctree/>).

PDH and P5CDH are mitochondrial enzymes that use FAD and NAD⁺ as electron acceptors and generate FADH₂ and NADH respectively, delivering electrons for mitochondrial respiration [30,36,37]. In soybean nodules, PDH was detected in the cytosol, suggesting that proline oxidation provides energy for bacteroids during nitrogen fixation [34]. Recently, a P5C–proline cycle has been described showing that P5C, produced from proline in the mitochondria, can be transported into the cytosol and reduced to proline by cytosolic P5CR [21]. When P5CDH activity is limited, the P5C–proline cycle can transfer more electrons to the mitochondrial electron transport chain and generate reactive oxygen species (ROS). P5CDH has been found in chloroplasts by proteome analysis, suggesting that P5C is also converted to glutamate in plastids [38].

The ornithine pathway has been suggested to be important during seedling development and in some plants for stress-induced proline accumulation [27,33,39]. Recently, the significance of the pathway and OAT in proline biosynthesis has been questioned, because proline levels were not affected in *Arabidopsis oat* knockout mutants. Instead, OAT facilitates nitrogen recycling from arginine through P5C, which is converted to glutamate by P5CDH [40].

Compartmentalization of proline metabolism implies that extensive intracellular proline transport must occur between the cytosol, chloroplasts and mitochondria (Figure 1). Physiological data suggest that proline uptake into mitochondria is an active process, hinting at the existence of specific amino acid transporters [41]. Plasma membrane proline transporters, identified in several plants, mediate proline transport between cells and organs, but are not involved in organellar transport [42–44]. Two proline carriers have recently been identified in the mitochondria of durum wheat (*Triticum durum*): a proline uniporter, which facilitates proline transport into the mitochondrial matrix; and a proline/glutamate antiporter, which appears to have an important role in the

Pro/Glu shuttle between the mitochondrial matrix and the cytosol [45]. Basic amino acid transporters (BAC) can deliver arginine and ornithine through the mitochondrial membrane [46]. In the halophyte species *Limonium latifolium*, proline was sequestered to vacuoles in non-stressed plants, whereas in salt-stressed plants, a high proline content was detected in the cytosol, suggesting the importance of *de novo* proline biosynthesis as well as transport for proline accumulation [47].

Regulation of proline metabolism

Although proline metabolism has been studied for >40 years in plants, little is known about the signaling pathways involved in its regulation. Proline biosynthesis is activated and its catabolism repressed during dehydration, whereas rehydration triggers the opposite regulation [3,26,28–30,39] (Figure 2). Proline biosynthesis is controlled by the activity of two *P5CS* genes in plants, encoding one housekeeping and one stress-specific *P5CS* isoform. Although the duplicated *P5CS* genes share a high level of sequence homology in coding regions, their transcriptional regulation is different [26,27,39]. In *Arabidopsis*, *P5CS2* is the housekeeping gene, which is active in dividing, meristematic tissues, such as the shoot and root tips, inflorescences and cell cultures [20,26,48]. Both *P5CS* genes are active in floral shoot apical meristems, and supply proline for flower development [49]. It is intriguing that, in *Arabidopsis*, the *P5CS2* gene was identified as one of the targets of CONSTANS (CO), a transcriptional activator that promotes flowering in response to long day length [50]. *P5CS2* can be activated also by avirulent bacteria, salicylic acid (SA) and ROS signals, which trigger a hypersensitive response (HR) [7]. *Arabidopsis P5CS1* is induced by osmotic and salt stresses and is activated by an abscisic acid (ABA)-dependent and ABA insensitive 1 (ABI1)-controlled regulatory pathway and H₂O₂-derived signals [3,26,51,52]. Moreover, *P5CS1* activation and proline accumulation is

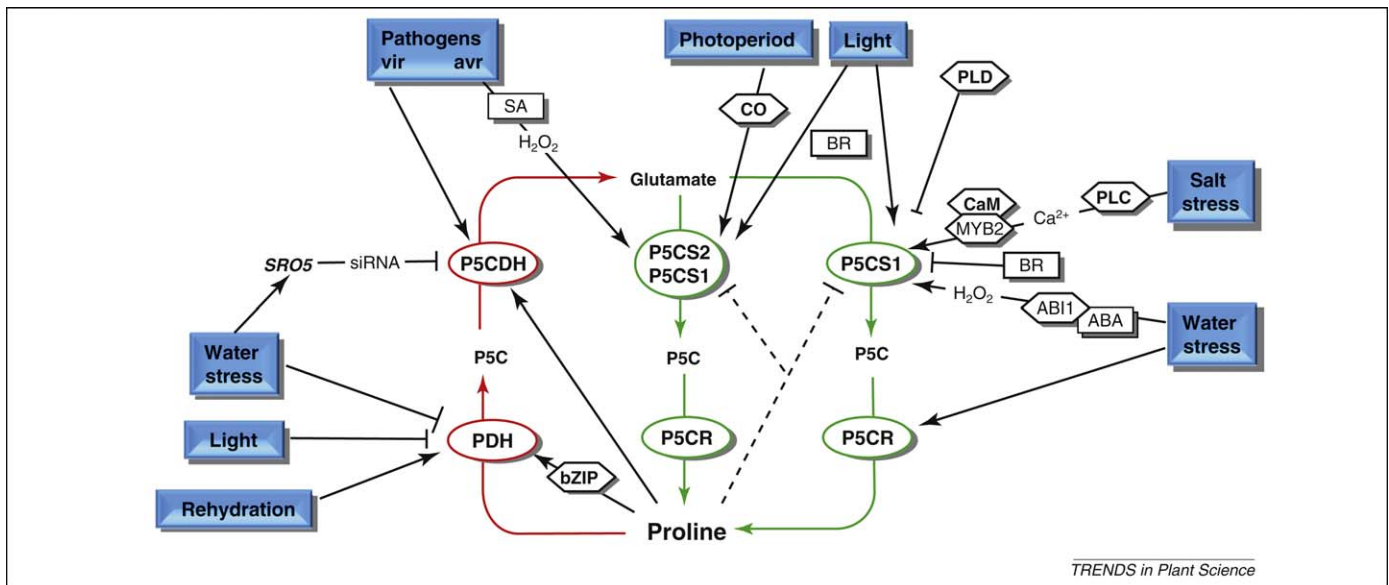


Figure 2. Regulation of proline metabolism in plants. The biosynthetic pathway is indicated with green lines and the catabolic pathway with red lines. Environmental effects are highlighted. Hormones: ABA, BR (brassinolides) and SA. Metabolites and other factors: Ca²⁺ (free calcium ion); H₂O₂ (hydrogen peroxide); P5C and siRNA. Enzymes: P5CDH; P5CS; P5CR and PDH. Regulatory genes and proteins: ABI1; bZIP; CaM (calmodulin); CO; MYB2; PLC; PLD; and SRO5, which has a 3' overlapping region with P5CDH. Pathogens: avr, avirulent; vir, virulent.

promoted by light and repressed by brassinosteroids [48,53]. Under non-stressed conditions, phospholipase D (PLD) functions as a negative regulator of proline accumulation [54], whereas calcium signaling and phospholipase C (PLC) trigger *P5CS* transcription and proline accumulation during salt stress [55,56]. In the halophyte *Thellungiella halophila*, PLD functions as positive regulator, whereas PLC exerts a negative control on proline accumulation [57]. Calcium signals can be transmitted by a specific CaM4 calmodulin, which interacts with the MYB2 transcription factor and upregulates *P5CS1* transcription [58].

As well as transcriptional regulation, P5CS activity is under metabolic control, as seen by feedback inhibition of P5CS by proline [22,59]. Similar allosteric inhibition of bacterial proB has also been described [9]. Loss of feedback inhibition of P5CS leads to elevated proline accumulation [18]. In mammalian cells, alternative splicing has been shown to generate different P5CS isoforms, which differ in their tissue specificity, organ localization, hormonal regulation and feedback inhibition [60]. In *Arabidopsis*, P5CS splice variants have been annotated but not characterized (<http://www.arabidopsis.org>). Conversion of P5C to proline is not a rate-limiting step in proline biosynthesis, yet the control of P5CR activity implies a complex regulation of transcription, which was shown to be under developmental and osmotic regulation [25]. Promoter analysis of *Arabidopsis* P5CR identified a 69-bp promoter region that is responsible for tissue-specific expression [61]. However, *trans*-acting factors that can bind to this promoter region have not yet been identified.

Whereas proline biosynthesis is upregulated by light and osmotic stresses, proline catabolism is activated in the dark and during stress relief, and is controlled by PDH and P5CDH (Figure 2). *PDH* transcription is activated by rehydration and proline but repressed by dehydration, thus preventing proline degradation during abiotic stress [28,29]. *PDH1* transcription is repressed during daylight

and induced in darkness; therefore illumination has opposite effects on *P5CS1* and *PDH1* transcription [48,53]. Promoter analysis of *PDH1* identified the proline and hypo-osmolarity-responsive element (PRE) motif ACT-CAT, which is necessary for the activation of the *PDH* gene [62]. Basic leucine zipper protein (bZIP) transcription factors (AtbZIP-2, -11, -44, -53) have been identified as candidates for binding to this motif [63]. Chromatin immunoprecipitation and genetic analysis revealed combinatorial control of *PDH* expression by a network of group-S bZIP transcription factors [64]. The *P5CDH* gene is expressed at a low basal level in all *Arabidopsis* tissues, and can be upregulated by proline [30]. A short sequence similar to the PRE motif has been identified on the promoters of *P5CDH* genes in *Arabidopsis* and cereals [65]. The *FIS1* gene encodes P5CDH in flax (*Linum usitatissimum*) and is activated during virulent pathogen attack and wounding [66,67]. Natural antisense overlapping of the 3' UTR regions of *P5CDH* and the salt-induced *SIMILAR TO RCD ONE 5 (SRO5)* gene generate endogenous small interfering RNA (24-nt and 21-nt siRNA) in *Arabidopsis*, which cleaves the *P5CDH* RNA and reduces transcript levels during stress. Transcriptional gene silencing is therefore important in the control of *P5CDH* gene activity [68].

Proline accumulation and stress tolerance

For a long time, proline was considered as an inert compatible osmolyte that protects subcellular structures and macromolecules under osmotic stress [10–12]. However, proline accumulation can influence stress tolerance in multiple ways (Figure 3). Proline has been shown to function as a molecular chaperone able to protect protein integrity and enhance the activities of different enzymes. Examples of such roles include the prevention of protein aggregation and stabilization of M4 lactate dehydrogenase during extreme temperatures [69], protection of nitrate

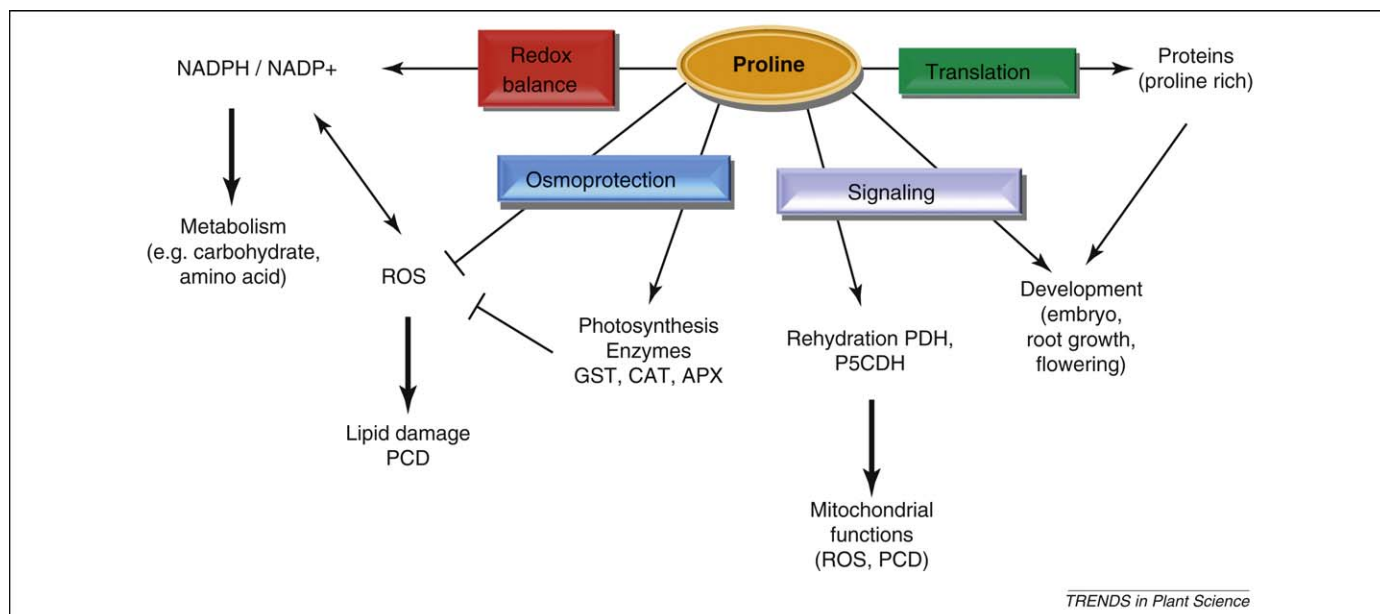


Figure 3. Multiple functions of proline in plants. Proline is used for protein synthesis, has protective functions as an osmolyte, contributes to the maintenance of the redox balance, can regulate development and is a component of metabolic signaling networks controlling mitochondrial functions, stress relief and development. Abbreviations: APX, ascorbate peroxidase; CAT, catalase; PCD, programmed cell death.

reductase during heavy metal and osmotic stress [70], and stabilization of ribonucleases and proteases upon arsenate exposure [71].

Several studies have attributed an antioxidant feature to proline, suggesting ROS scavenging activity and proline acting as a singlet oxygen quencher [72,73]. Proline treatment can diminish ROS levels in fungi and yeast, thus preventing programmed cell death [74], can protect human cells against carcinogenic oxidative stress [75], and can reduce lipid peroxidation in alga cells exposed to heavy metals [76]. Proline pretreatment also alleviated Hg²⁺ toxicity in rice (*Oryza sativa*) through scavenging ROS, such as H₂O₂ [77]. The damaging effects of singlet oxygen and hydroxyl radicals on Photosystem II (PSII) can be reduced by proline in isolated thylakoid membranes (PSII) [78]. Free radical levels were reduced in transgenic algae and tobacco plants engineered for hyperaccumulation of proline by P5CS overexpression and acceleration of the proline biosynthetic pathway [18,79].

By contrast, compromised proline accumulation in *p5cs1* insertion mutants led to accumulation of ROS and enhanced oxidative damage [20]. A similar effect was observed in yeast, where low proline levels in PUT1 (proline dehydrogenase)-overexpressing lines led to enhanced ROS, whereas higher proline content in *put1* mutants correlated with increased protection from oxidative damage [80]. As an alternative to direct ROS scavenging feature, proline can protect and stabilize ROS scavenging enzymes and activate alternative detoxification pathways. In salt-stressed tobacco cells, proline increased the activities of methylglyoxal detoxification enzymes, enhanced peroxidase, glutathion-S-transferase, superoxide dismutase and catalase activities, and increased the glutathion redox state [81,82]. In the desert plant *Pancreatium maritimum*, catalase and peroxidase were found to be stabilized by proline during salt stress [83]. The salt-hypersensitive *p5cs1 Arabidopsis* mutant shows reduced activities of key antioxidant enzymes of the glutathione-ascorbate cycle, leading to hyperaccumulation of H₂O₂, enhanced lipid peroxidation and chlorophyll damage [20].

As well as having protective or scavenging features, it is feasible that proline metabolism can stabilize cellular homeostasis during stress conditions in way that is still poorly understood. Accumulation of P5CS1 and P5CR in chloroplasts during salt stress suggests that, under adverse conditions, glutamate-derived proline biosynthesis increases in plastids, where photosynthesis occurs [20,35]. During stress conditions, the rate of the Calvin cycle is diminished, which prevents oxidation of NADPH and restoration of NADP⁺. When combined with high light, electron flow in the electron transport chain is suppressed by the insufficient electron acceptor NADP⁺ pool, leading to singlet oxygen production in the PSI reaction center and accumulation of ROS [84]. Proline biosynthesis is a reductive pathway, and requires NADPH for the reduction of glutamate to P5C and P5C to proline, and generates NADP⁺ that can be used further as electron acceptor. The phosphorylation of glutamate consumes ATP and produces ADP, which is a substrate for ATP biosynthesis during photosynthesis. An enhanced rate of proline biosynthesis in chloroplasts during stress can maintain the

low NADPH:NADP⁺ ratio, contribute to sustaining the electron flow between photosynthetic excitation centers, stabilize the redox balance, and reduce photoinhibition and damage of the photosynthetic apparatus [11]. In transgenic soybean plants, the inhibition of proline biosynthesis and NADPH–NADP⁺ conversion by antisense *P5CR* led to drought hypersensitivity, whereas overexpression of *P5CR* resulted in moderate drought tolerance, confirming that proline biosynthesis is important for maintaining NADP⁺ pools during stress [85]. Such a connection between photosynthesis and proline metabolism is supported by light-dependent proline accumulation, which is regulated by the light-controlled reciprocal *P5CS* and *PDH* gene activation [48,53].

In mitochondria, proline has distinct protective functions. After stress, proline pools supply a reducing potential for mitochondria through the oxidation of proline by PDH and P5CDH, provide electrons for the respiratory chain and therefore contribute to energy supply for resumed growth [11,12]. Proline was shown to protect Complex II of the mitochondrial electron transport chain during salt stress and therefore stabilized mitochondrial respiration [86]. The recently discovered P5C–proline cycle can deliver electrons to mitochondrial electron transport without producing glutamate and, under certain conditions, can generate more ROS in the mitochondria [21]. Proline catabolism is, therefore, an important regulator of cellular ROS balance and can influence numerous additional regulatory pathways.

Although species-specific differences in proline accumulation exist, it is an open question whether differences in proline accumulation have an adaptive value. Halophyte relatives of *Arabidopsis*, such as *Thellungiella halophila* and *Lepidium crassifolium*, have elevated proline levels under unstressed conditions and accumulate proline to higher levels than does *Arabidopsis* when exposed to high salinity [87,88]. In *Thellungiella*, high proline accumulation results from enhanced *P5CS* and reduced *PDH* expression levels [88,89]. High proline levels can improve the salt tolerance of the halophyte plant *Pancreatium maritimum*, by stabilizing detoxifying enzymes and protein turnover machinery and stimulating the accumulation of stress protective proteins [83]. Other halophytes, such as *Camphorosma annua* or *Limonium* spp, do not have high proline content; instead, they accumulate carbohydrate or betain-derived osmolytes [47,87]. In *Silene vulgaris*, constitutive proline content was higher in metal-tolerant ecotypes, whereas metal-induced proline accumulation was higher in a non-tolerant ecotype [5]. The capacity for proline hyperaccumulation therefore accompanies the extremophile character of certain plant species and is likely that it contributes to their stress tolerance; however, it is not an absolute requirement for adaptation to extreme environmental conditions.

Regulatory functions of proline

Increasing amounts of data suggest that proline has certain regulatory functions, controls plant development and acts as a signal molecule. Antisense expression of *P5CS1* in transgenic *Arabidopsis* led to proline depletion and resulted in abnormal leaf morphology and defective inflor-

escences [90]. A *p5cs2*-knockout mutant displays embryo lethality and proline-rescued mutant plants show aberrant growth, implying that an adequate proline supply is essential for embryo and plant development [20]. *P5CS2* and proline were suggested to regulate cell division and embryogenesis [49]. Overexpression of *P5CS1* and enhanced proline content led to the early flowering of transgenic *Arabidopsis* plants, whereas the *p5cs1* mutant with reduced proline content showed late flowering [19,49]. Promotion of flower transition by *CONSTANS* can be partially mediated by activation of *P5CS2* transcription and local rise of proline content [50].

Although proline is usually considered to be a metabolite with protective functions, several reports show that, under certain conditions, exogenous proline can be deleterious to plants and can inhibit growth and cell division [91]. Proline treatment inhibited *Arabidopsis* seed germination [92], restricted growth of *Petunia* plants [93] and arrested root growth of *Thellungiella halophila*, a plant with low *PDH* activity [89]. Externally added proline was more toxic to transgenic plants expressing antisense *PDH* as well as to *pdh Arabidopsis* mutants than to wild-type plants [94,95]. In such plants, feedback inhibition of *P5CS* enzyme by proline might block proline biosynthesis and affect the $\text{NADP}^+/\text{NADPH}$ ratio and redox balance in plastids, leading to accelerated chlorophyll damage. Transcript profiling in *Arabidopsis* has revealed that one third of rehydration-inducible plant genes can also be induced by proline [96]. Up to 21 rehydration- and proline-inducible genes have been identified in *Arabidopsis*, most of which have the conserved *PRE cis*-acting element in their promoter regions, which is a target of specific bZIP-type transcriptional activators [63,64,96]. In yeast, proline can interact with the Put3p transcription factor and convert it from a transcriptionally inactive to an active form. Active Put3p promotes the expression of specific genes, including those that control proline catabolism [97]. Some of the proline-activated genes can be important for stress adaptation [91]. Uncoupled induction of *PDH* and *P5CDH* in *p5cdh* mutants leads to P5C and ROS accumulation, which can function as stress signal and cause programmed cell death [21,30,98].

In human cells, proline catabolism is implicated in mitochondria-dependent signaling, which controls programmed cell death and apoptosis. Enhanced proline oxidation in human carcinoma cells generates ROS, which in turn functions as an apoptotic signal and triggers programmed cell death [99,100]. Human *PDH/POX* is strongly induced by p53, one of the most important tumor suppressor genes [101]. A p53 binding site was localized in the promoter of human *PDH/POX*, confirming that p53 is a direct activator of that gene [102]. Proline-dependent apoptosis is mediated by the TRAIL death receptor pathway and activates caspase-8 [100]. *PDH/POX* activation controls various signaling pathways: reduces Mitogen-activated protein kinase kinase (MEK) and Extracellular signal-Regulated Kinase (ERK) phosphorylation, downregulates the cyclooxygenase-2 (COX-2) pathway, suppresses the epidermal growth factor receptor (EGFR) pathway and the Wnt/beta-catenin pathway, which in turn reduces phosphorylation of glycogen synthase kinase (GSK-3) [100]. *PDH/POX*

can therefore function as a mitochondrial tumor suppressor and proline can control cell proliferation and promote apoptosis through *PDH/POX*-mediated mitochondrial ROS signals in human cancer cells [103,104].

Proline metabolism can also influence programmed cell death in plants. In *Arabidopsis*, incompatible plant-pathogen interactions trigger a HR via ROS signals, which is accompanied by local activation of *P5CS2* and proline accumulation [7]. Proline can enhance *PDH* expression in such cells and lead to P5C accumulation, which in combination with ROS can function as an apoptotic signal and trigger HR during infection with avirulent pathogens [7,98]. In flax, virulent pathogen infection was shown to activate the rust-inducible gene from flax (*FIS1*) gene, which encodes P5CDH [105]. Although the exact role of *FIS1* activation in virulent infection remains unclear, removal of P5C by *FIS1/P5CDH* during pathogen attack can reduce P5C-derived signals, diminish ROS levels and, therefore, suppress defenses, such as HR [66]. Proline was recently proposed to modulate the plant defense response to *Agrobacterium tumefaciens*. Proline accumulates in plant tumors, and functions as a competitive antagonist of gamma-aminobutyric (GABA)-dependent plant defense, interfering with the GABA-induced degradation of quorum-sensing signal [8]. Proline can therefore promote *Agrobacterium* infection and horizontal transfer of the Ti plasmid. The above-listed examples suggest that proline functions as versatile cellular signal in both animal and plant cells (Figure 3).

Concluding remarks and perspectives

Although proline has long been considered as a compatible osmolyte, recent results highlight its multiple functions in stress adaptation, recovery and signaling. Compartmentalization of proline biosynthesis and degradation in the cytosol, chloroplast and mitochondria add to the complexity of functional diversification of proline metabolism. Stabilization of proteins and protein complexes in the chloroplast and cytosol, protection of the photosynthetic apparatus and enzymes involved in detoxification of ROS are an important, but not the only function of proline accumulation during stress. The enhanced rate of proline biosynthesis in the chloroplasts can contribute to the stabilization of redox balance and maintenance of cellular homeostasis by dissipating the excess of reducing potential when electron transport is saturated during adverse conditions. Proline catabolism in the mitochondria is connected to oxidative respiration and administers energy to resumed growth after stress. Moreover, proline oxidation can regulate mitochondrial ROS levels and influence programmed cell death. Analogies with animal and yeast models also suggest an intimate interaction between proline turnover, cell-cycle control, differentiation and programmed cell death in plants. As well as modulating responses to abiotic and biotic stresses, proline appears to function as a metabolic signal that regulates metabolite pools and redox balance, controls the expression of numerous genes and influences plant growth and development. The application of system biology approaches might help to understand regulation of proline-dependent and proline-mediated signaling in plants.

The complexity of the regulation of proline metabolism and multiple functions of proline illustrate the difficulties of improving plants of agronomic interest by the modified expression of genes involved in this metabolism. One option is the enhancement of proline biosynthesis via increased expression of rate-limiting genes in transgenic plants. Salt tolerance could be improved by the constitutive overexpression of the *Vigna* P5CS in tobacco [106], and further improvements have been achieved when the feedback-insensitive form of *Vigna* P5CS has been used [18]. The feasibility of this approach has been confirmed in other engineered plants [93,107] and algae [79]. Using a similar strategy, feedback-insensitive bacterial proBA genes were overexpressed in transgenic *Arabidopsis*, leading to proline hyperaccumulation and enhanced osmotolerance [108]. Inhibition of proline degradation can also lead to proline accumulation. Contrasting results however have led to the feasibility of this strategy to be questioned. Whereas anti-sense *PDH* was reported to improve salt and cold tolerance in one study [109], other studies reported an unchanged stress tolerance, accompanied by abnormal seed and plant development and increased proline hypersensitivity of the transgenic lines and mutants [31,94]. These results suggest that it might not be the actual proline content, but the enhanced rate of proline biosynthesis that is an important factor for stress adaptation. Engineering strategies could therefore target the proline biosynthetic pathway, and aim to accelerate proline biosynthesis in the chloroplasts.

Improvement of drought or salt tolerance of crop plants via engineering proline metabolism is an existing possibility and should be explored more extensively. The fact that proline can act as a signaling molecule and influence defense pathways, regulate complex metabolic and developmental processes, offers additional opportunities for plant improvement. Further studies are required to study the feasibility to engineer flowering time or to improve defenses against certain pathogens via the targeted engineering of proline metabolism.

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