Regulation of Proline Accumulation and Its Molecular and Physiological Functions in Stress Defence

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1 Introduction

Among the building blocks needed for protein synthesis, proline is the only cyclic amino acid, i.e. a secondary amine in which two carbons are bonded to the amine nitrogen forming a five-membered heterocyclic ring. Because of this, proline plays a unique role in determining protein structure by influencing backbone folding and stability (Ge and Pan 2009). Proline is synthesized in a two-step reduction and cyclization of glutamate (Fichman et al. 2015), although other possible routes have been hypothesized to occur in some plant species (da Rocha et al. 2012). Preliminary evidence supporting an at least partial localization of the anabolic pathway in the chloroplast (Szekely et al. 2008) has not been further supported; thus proline production is regarded as a cytosolic process. As a consequence, free proline is present in the cytoplasm, where homeostatic levels depend on the balance between the rates of its production and utilization for protein synthesis, but also on its translocation to the mitochondrion (Di Martino et al. 2006), where proline is oxidized back to glutamate via an equally short catabolic pathway. The biosynthetic route is controlled mainly through feedback regulation of the enzyme catalysing the first step, namely,

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δ[1]-pyrroline-5-carboxylate (P5C) synthetase (P5CS) (Hu et al. 1992; Hong et al. 2000). The turnover of amino acids into proteins and vice versa seems rapid, since similar proportions of ^{14}C were found in unstressed cells following ^{14}C labelling (Dong et al. 2018).

Besides the involvement in protein synthesis, proline is believed to play a main role in the plant response to several (a)biotic stresses. Starting from the 1960s of the last century, hundreds of papers have reported rapid accumulation of high free proline levels in cells subjected to a variety of stress conditions (Delauney and Verma 1993; Hayat et al. 2012; Verbruggen and Hermans 2008) (Table 1). In response to stress, protein synthesis is reduced, favouring amino acid accumulation (Dong et al. 2018). Notwithstanding this, in most cases the rise of free proline content largely exceeds those of the other proteinogenic amino acids. Accordingly, genetically modified plants with increased intracellular proline content showed higher stress tolerance (Per et al. 2017). The specific increase in proline content may derive from either a reduction in its mitochondrial oxidation (Nanjo et al. 1999), an increase of its biosynthesis (Zhang et al. 1995) or import from other tissues (Lehmann et al. 2010; Wang et al. 2017) via phloem sap. The latter seems to contribute to developmentally related variations in proline homeostasis, as in reproductive organs (Biancucci et al. 2015), whereas increased biosynthesis appears the main mechanism occurring after the exposure to abiotic stress, such as drought, salinity or freezing (Yoshiba et al. 1995, 1997). Because P5CS is feedback inhibited by millimolar concentrations of proline, and homeostatic proline level results from the combination of synthesis, catabolism and transport rates, the accumulation of increased levels of proline in the cytosol requires fine regulation of its metabolism under stress. Consistently, an impressive number of putative *cis*-regulatory elements recognized by different classes of transcription factors were found in the promoter region of the involved genes (Fichman et al. 2015; Zarattini and Forlani 2017).

2 Regulation of Proline Accumulation Under Stress Conditions

A variety of diverse abiotic and biotic stress conditions were found to induce the accumulation of proline in plants (Szabados and Savouré 2010). Proline accumulation was suggested to be due to an increase of the de novo biosynthesis rather than lower catabolism or greater protein degradation (Chiang and Dandekar 1995; Szabados and Savouré 2010; Hildebrandt 2018). Under stress, proline is mainly biosynthesized from glutamate by two enzymatic steps consuming NADPH preferably and catalysed by P5CS and P5C reductase (P5CR) (Forlani et al. 2015c; Giberti et al. 2014). Most plants have two P5CS isoforms, one housekeeping and the other inducible, the latter being mainly responsible for proline accumulation under stress (Signorelli and Monza 2017). Proline accumulation is more significant in photosynthetic tissues (e.g. leaves) but also takes place in roots (Sharma et al. 2011; Signorelli et al. 2013a; Verslues and Sharp 1999). At the cellular level, proline accumulation is

Table 1 Stress-induced accumulation of intracellular proline levels in response to stress conditions in some selected plant species

Species	Stressor	Tissue	Pro content (fold increase)	Reference
Nicotiana tabacum	NaCl (171 mM)	Cultured cells	48	Binzel et al. (1987)
	NaCl (342 mM)		246	
	NaCl (428 mM)		445	
Nicotiana tabacum	30% relative humidity	Wilted leaves	40	Sano and Kawashima (1982)
Solanum lycopersicon	PEG 20%	Cultured cells	38	Handa et al. (1983)
	PEG 25%		244	
	PEG 30%		506	
Capsicum annuum	PEG 25%	Cultured cells	200	del Socorro Santos-Díaz and Ochoa-Alejo (1994)
Arabidopsis thaliana	NaCl (120 mM)	Rosette leaves	8–10	Chiang and Dandekar (1995)
Arabidopsis thaliana	NaCl (150 mM)	14-day-old seedlings	16	Signorelli et al. (2016))
Arabidopsis thaliana	Low Ψ_w (−1.2 MPa)	Seedlings	160	Shinde et al. (2016)
Oryza sativa	NaCl (100 mM)	Seedlings	2–10	Lv et al. (2015)
Oryza sativa	NaCl (200 mM)	Seedlings	2–3	Bertazzini et al. (2018)
Trifolium pratense	Drought (42% hydric index reduction)	Wilted leaves	139	Signorelli et al. (2013b)
Lotus japonicus	Drought (45% relative water content reduction)	Leaves	18	Signorelli et al. (2013c)
	Drought (33% relative water content reduction)	Roots	35	
Zea mays	Growth in medium at −1.6 MPa	Apical millimeter of corn root tips	50	Voetberg and Sharp (1991)
Lotus corniculatus	Drought (50% hydric index reduction)	Wilted leaves	16	Signorelli et al. (2013b)

While several *Solanaceae* showed hundredfold increases in free proline content, much lower concentrations were found in other plants

suggested to be greatest in the chloroplast, followed by the cytoplasm (Büssis and Heineke 1998).

P5CR was shown to be induced by saline stress (Verbruggen et al. 1993); however, this was challenged by Yoshiba and co-workers (1995), who found no response of *P5CR* to salt stress or dehydration and attributed proline accumulation to increased *P5CS1* expression. Therefore, P5CR is not believed to play a critical role in proline accumulation under osmotic stress. However, the biochemical properties

of P5CR suggest that under some conditions also P5CR activity might become limiting (Giberti et al. 2014).

Although proline dehydrogenase (ProDH, also known as proline oxidase or POX) is not as determinant as P5CS1 concerning proline accumulation under stress, the oscillation of gene expression in the light/dark cycles and downregulation under dehydration, saline, and osmotic stress contributes to enhanced proline accumulation (Hayashi et al. 2000; Verslues and Sharma 2010).

Other factors, such as light or reactive oxygen species (ROS), also need to be present for proline accumulation to take place. Early studies demonstrated that proline content oscillated according to light/dark cycles (Hayashi et al. 2000), and even stress-induced proline accumulation was shown to be dependent on light (Sanada et al. 1995; Díaz et al. 2005; Aleksza et al. 2017). The light dependence of proline accumulation can be attributed, at least in part, to the need of reducing power for its biosynthesis, which is generated during photosynthesis and might be transferred into the cytosol by different redox shuttles. However, later reports showed that the expression of *P5CS1* is also affected by light and is mediated by Elongated Hypocotyl 5 (HY5) (Feng et al. 2016; Lee et al. 2007). HY5 is a basic leucine zipper (bZIP) transcription factor, controlling a plethora of processes, such as development, abiotic stress, ROS, and hormone signalling, in a light-dependent manner (Gangappa and Botto 2016; Signorelli et al. 2018). Thus, the light dependence of proline accumulation is not just due to the requirement of NADPH but also to signalling reasons. This finding of the role of HY5 in mediating *P5CS1* expression also points out how important the coordination of proline metabolism with other processes is.

Similarly, the expression of the main enzymes controlling proline accumulation, P5CS1 and ProDH, was suggested to be dependent on the Respiratory Burst Oxidase Homologue (Rboh, NADPH oxidase in animals) activity (Ben Rejeb et al. 2015). In short, the authors showed that the hydrogen peroxide (H_2O_2) produced as a consequence of Rboh and SOD activity is necessary for proline accumulation to occur. The simple pharmacological scavenging of H_2O_2 was enough to attenuate proline accumulation under stress conditions (Ben Rejeb et al. 2015). This finding demonstrated that proline accumulation is also regulated, at least in part, by ROS signalling. ROS seem to be produced under most stress conditions. Thus, in a physiological context, while light can be a determinant factor for proline accumulation to take place or not, the role of ROS is probably more complex, and variable concentrations of different ROS species may influence proline biosynthesis differentially.

The production of the phytohormone abscisic acid (ABA) is induced under stress conditions in plants, and ABA acts as a signalling molecule to mediate the adaptation of the plant to the new environment. In rice, ABA is known to mediate proline accumulation (Sripinyowanich et al. 2013); however, in arabidopsis (*Arabidopsis thaliana*), proline accumulation can be either ABA-dependent or ABA-independent depending on the stress condition (Savouré et al. 1997; Zarattini and Forlani 2017). Recently, a wild variety of barley was shown to accumulate higher proline levels under drought compared to the cultivated one. The authors showed that the difference between the ancestral and the cultivated variety was one nucleotide in the

sequence of the *P5CS1* promoter, which modified an ABF (ABA-responsive elements (ABRE) binding factors) binding site, making the cultivated barley to be unresponsive to ABA (Muzammil et al. 2018). Likewise, in arabidopsis, the 5' UTR region of *AtP5CS1* contains ABF binding sites. Other cis-responsive elements (CRE), such as AP2/EREBP, ERF2, DREB/CBF, and MYB binding sites are also found in the promoter of *P5CS1* genes of different species (for detailed reviews, see Fichman et al. 2015; Zarattini and Forlani 2017). This wide variety of CRE in the promoter of the main gene responsible for proline synthesis explains why proline accumulation is a conserved response observed in a broad range of conditions in plants.

3 Osmoprotective Role of Proline

Despite the plethora of papers describing a stress-induced increase of free proline concentration, and the evidence of a statistically significant relationship between this increase and increased stress tolerance, the mechanisms by which high levels of this amino acid may benefit the cell are far from being fully understood and conclusively proven. The early hypothesis of a central osmotic role for proline, i.e. that high levels of this compound may avoid water withdrawal to the apoplast by lowering the cellular water potential (Ψ_w), seems inconsistent with the relatively low absolute concentration reached in several cases and has been recently questioned (Bhaskara et al. 2015; Forlani et al. 2018; Kavi Kishor and Sreenivasulu 2014; Ben Rejeb et al. 2014; Sharma et al. 2011; Signorelli 2016). Notwithstanding this, many authors still consider a function of proline as a "compatible osmolyte" as an established fact.

3.1 Proline as Compatible Osmolyte

A famous quote says that *if you repeat a lie often enough, people will believe it, and you will even come to believe it yourself*. In the scientific literature, we could say that if a sound hypothesis is repeated in many papers, with citation to other previous reports, at a certain point, scientists will start to believe that it has been proven somewhere in the past, and do not verify whether it fits with their experimental system or whether their results are consistent with it. This is probably true for the "osmotic role" of proline accumulation in response to hyperosmotic stress conditions. Because of early reports describing in some plant species a striking increase of its intracellular level under drought or salt stress (Table 1), from a given moment onwards, any statistically significant increase in proline concentration has been interpreted as a genuine contribution to osmotic compensation. Is this interpretation sound? Let us make some calculations. Because in a differentiated plant tissue the measurement of both the apoplastic water volume and the external water potential

(Ψ_W) is not easy (Lohaus et al. 2001), the use of a cell suspension culture may facilitate the estimates. Cells growing in the widely used Murashige and Skoog (MS) medium with 30 g L^{-1} sucrose are in osmotic equilibrium with it, at an external Ψ_W of about −0.52 MPa (Fig. 1a). Since cellular concentration (Ψ_S) usually corresponds to an osmotic pressure (Π) ranging from −0.7 to −1.2 MPa (Handa et al. 1983; Ikeda et al. 1999), this allows cell turgidity, with a turgor pressure (Ψ_P) of about 0.1 to 0.7 MPa. The addition of 25% polyethylene glycol (PEG-6000) to the culture medium, a condition that has been found permissive in many cases (i.e. plant cells can adapt to these conditions [e.g. del Socorro Santos-Díaz and Ochoa-Alejo 1994]), lowers the external Ψ_W to −2.0 MPa (Fig. 1b). To avoid water withdrawal, the cell has to increase its internal osmolyte concentrations to obtain a Π decrease of 0.8 to

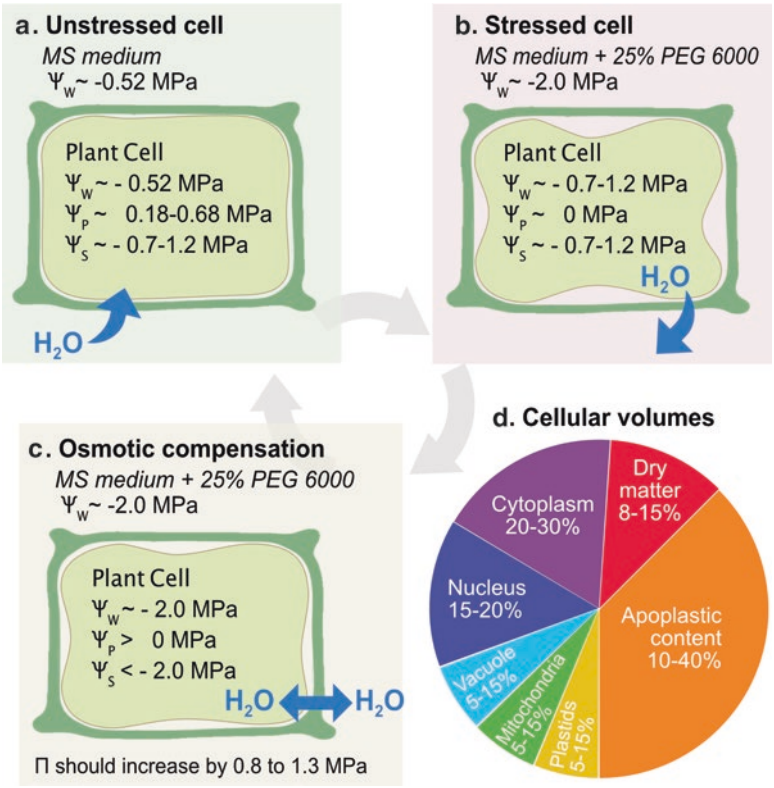

Fig. 1 Osmotic compensation in rapidly dividing cells. Suspension-cultured cells growing in MS medium are in equilibrium with an environmental water potential of about −0.52 MPa (panel **a**). The addition of an osmoticum, for instance, 25% PEG 6000, causes a dramatic decrease of the external water potential (Ψ_W) (**b**). To adapt, the intracellular concentration (Ψ_S) should increase consistently, so as to avoid loss of turgor pressure (Ψ_P) and the consequent occurrence of plasmolysis (**c**). Because only about 20–30% of the overall cell fresh weight is attributable to the cytoplasm (**d**), to obtain osmotic compensation solely by proline accumulation, an increase of cytosolic free proline concentration as high as 80–130 μmol (g fw)$^{-1}$ would be required

1.3 MPa, corresponding (at 25 °C) to an increased osmolarity of about 320–520 mmol·L^{-1} (Fig. 1c). In most studies, proline content is measured on a fresh or dry weight (fw or dw) basis following cell extraction with sulfosalicylic acid and expressed as μmol (g fw or dw)$^{-1}$ (e.g. Aleksza et al. 2017). If, as most likely, proline accumulation occurs mainly in the cytosol, the evaluation of its osmotic value would require quantitation of the cytoplasmic volume. Concerning this, we should consider that in actively proliferating cultured cells, the vacuole accounts for only 5–15% of total volume but that nucleus, mitochondria, and plastids are a significant fraction of the cell. Also, considering that the apoplastic water may represent 10–40% of water content (Binzel et al. 1987; Speer and Kaiser 1991), to a good approximation, we can consider the cytoplasmic water as 20–25% of the overall fresh weight (Fig. 1d). This would imply that if proline would be the only osmolyte produced and accumulated, to achieve osmotic compensation, its content should increase by at least 80–130 μmol (g fw)$^{-1}$. In fact, in most cases, the reported increase does not exceed 5–10 μmol (g fw)$^{-1}$ (e.g. Poustini et al. 2007), and often it is less than 1–2 μmol (g fw)$^{-1}$ (e.g. Bertazzini et al. 2018). Consistently, in some excellent and pioneer works in which most of the main components of the cellular sap have been quantified at the same time, the contribution of proline to the required increase of osmolarity was estimated as not exceeding 3–15%, despite the fact that its concentration had shown a hundredfold increase over controls (Binzel et al. 1987; Handa et al. 1983; del Socorro Santos-Díaz and Ochoa-Alejo 1994). Only in the apical millimeter of corn roots growing at a water potential of −1.6 MPa, proline accumulation reaching about 120 μmol (g fw)$^{-1}$ accounted for almost half of the osmotic adjustment (Voetberg and Sharp 1991). In wheat plants, the contribution made by proline was estimated to be equivalent to 0.07 MPa, whereas that made by K^+ and Na^+ was 0.21 and 0.45 MPa, respectively (Poustini et al. 2007). Of course, any significant input to the attainment of a suitably high cellular Ψ_s concurs to ameliorate the osmotic unbalance. However, because of the implicated values, the significance of a proline accumulation lower than 5 μmol (g fw)$^{-1}$ (approximately equivalent to 0.06 MPa Ψ_s in the cytosol) should be regarded – in our opinion – as marginal for an effective osmotic compensation.

3.2 Proline as Stabilizer for Enzymes and Membranes

Given that proline seems unlikely to fully compensate the osmotic unbalance produced under stress, the doubtless beneficial effect of higher proline levels in the cell facing hyperosmotic conditions should also rely on other possible mechanisms. Several hypotheses have been proposed. One of the most commonly accepted is a kosmotropic (= anti-chaotropic) activity of proline. In solution, proteins, and in general all macromolecules, are surrounded by highly ordered water molecules that lower entropy, allowing the attainment of proper three-dimensional folding and, in case of an enzyme, the achievement of the catalytically active conformation (Fig. 2a). Osmotic stress-induced water withdrawal from the cell or

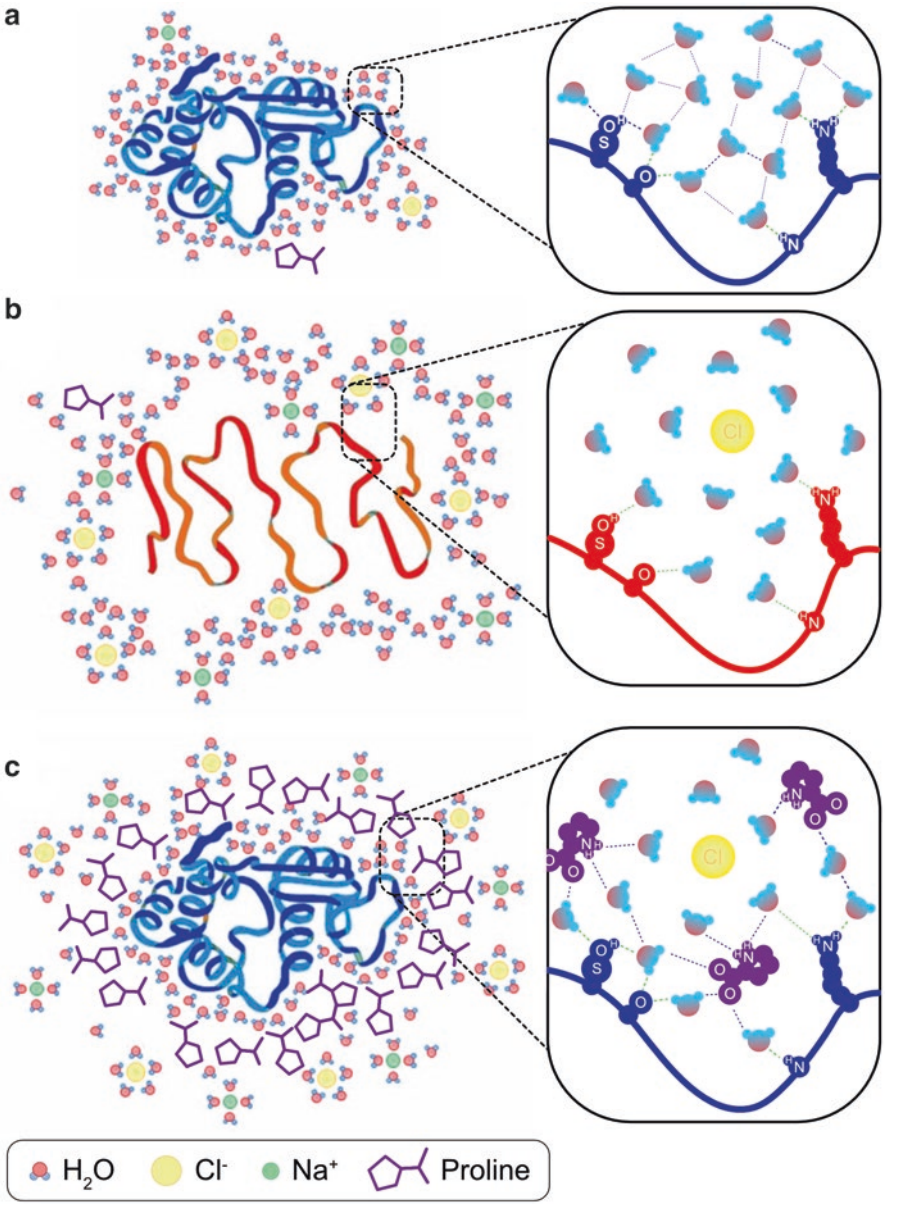

Fig. 2 Proposed mechanism for protein protection by proline. In solution, enzymes are surrounded by water molecules that interact with the hydrophilic regions of proteins, mainly through the formation of hydrogen bonds, allowing the attainment of proper three-dimensional folding and the achievement of the catalytically active conformation (panel **a**). Increased concentration of ions, and/or chaotropic substances, reorders the water molecules around them and reduces the interactions with the protein, resulting in protein unfolding and activity loss (panel **b**). Proline is believed to exert an anti-chaotropic effect and stabilize proteins by helping to maintain a proper water solvation shell around them (panel **c**). This effect may rely, for instance, on the hydrophobic interaction of the pyrrolidine ring with hydrophobic surfaces, thereby increasing hydrophilic areas, or on stabilization of the water molecules that interact with the protein through hydrogen bonds. The hydrogen bonds are represented by dashed lines. Thicker hydrogen bonds represent stronger interactions

hyperosmolarity-driven ion influxes cause a progressive increase of chaotropic substances in the cytosol, leading to protein unfolding and activity loss (Fig. 2b). Proline and other very soluble and well-hydrated molecules with little tendency to aggregate, such as the sugar α,α-trehalose, the quaternary ammonium compound glycine betaine, and 3-dimethyl-sulfonopropionate, at high concentrations exert a kosmotropic effect and stabilize the structure of macromolecules in solution by helping to maintain a proper hydration shell around them (Fig. 2c). It was postulated that proline forms aggregates by stepwise stacking and hydrophobic interaction of the pyrrolidine ring with hydrophobic surface residues of proteins, thereby increasing their hydrophilic area (Schobert and Tschesche 1978). An increased water-binding capacity of the proline-protein solution has therefore been invoked to explain alleviation of the noxious effects of water stress effects on protein activity and stability (Arakawa and Timasheff 1983, 1985). Moreover, proline has also been reported to favour protein renaturation and avoid protein aggregation by similarly trapping the folding intermediates in a supramolecular assembly (Samuel et al. 2000). A great unknown, also in this case, is whether local concentrations high enough to ensure these beneficial effects may be reached in vivo inside the cell.

On the other hand, when the phase behaviour of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine was examined in aqueous dispersions containing a range of sodium salts, the lipid phase properties exhibited a graded response analogous to that of the Hofmeister series causing protein salting out (Sanderson et al. 1991). Therefore, it is likely that at low water potential kosmotropic agents may stabilize membranes as well. Consistently, proline, betaine and trehalose were found to increase the area/molecule of three membrane phospholipids, therefore acting as stabilizer agents. In particular, data suggested that, due to its amphipathic nature, proline may interact with phosphatidylcholines through intercalation between phospholipid head groups (Rudolph et al. 1986).

4 Proline as a Potential ROS Scavenger

Abiotic and biotic stresses affect ROS homeostasis, usually resulting in an overproduction of specific ROS species. To avoid oxidative damage, plants respond by enhancing enzymatic and non-enzymatic antioxidant systems. As proline accumulates under stressful conditions, some authors hypothesized that proline protects against ROS. In good agreement with this idea, proline was shown to protect against the most potent ROS, the hydroxyl radical ($^{\bullet}OH$; Smirnoff and Cumbes 1989). Later, proline was shown to enhance photochemical activity in thylakoid membranes (Alia and Mohanty 1991). Moreover, the same group demonstrated that proline attenuates malondialdehyde (MDA) formation in cotyledons of *Brassica juncea* under saline stress (Alia and Mohanty 1993), zinc stress (Alia and Saradhi 1995), and UV stress (Saradhi et al. 1995). Based on the lower oxidative damage observed in plants treated with proline, the authors proposed that proline plays an essential role in non-enzymatic detoxification of free radicals, although no evidence of direct

reaction between proline and oxidant molecules, or their potential products, were obtained (Alia and Saradhi 1995). Proline was also shown to reduce MDA formation in thylakoids during exposure to intense light and was suggested to either react with singlet oxygen ($^1O_2$) or reduce its formation (Alia and Mohanty 1997). In 2001, the same group presented compelling evidence suggesting that proline could attenuate the singlet oxygen-mediated 2,2,6,6-tetramethylpiperidin (TEMP) oxidation, claiming they demonstrated that proline is a very effective singlet oxygen quencher (Alia and Matysik 2001). This work finished stamping the antioxidant label on proline.

In an elegant work, Hamilton and Heckathorn (2001) demonstrated that the primary cause of mitochondrial electron transport disruption by saline stress is oxidative damage in complex I and Na^+ toxicity in complex II. The authors showed that proline was unable to protect complex I, whereas non-enzymatic antioxidants, such as glutathione, tocopherol, and ascorbic acid, and enzymatic antioxidants, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT), did it. As SOD showed the highest protection of the complex I, the authors concluded that superoxide ($O_2^{\bullet -}$) was causing most of the oxidative damage of complex I. Altogether, these results challenge the idea that proline is an antioxidant and suggest that proline does not protect against superoxide. In addition, proline was shown to induce the activity of CAT, peroxidase, and polyphenol oxidase (Öztürk and Demir 2002), suggesting that some of the observations of proline amelioration of oxidative damage could be related to an enhancement of the enzymatic antioxidant machinery. The connection between proline accumulation and the oxidative damage/antioxidant response under different stress conditions was evaluated in many different plant species; however, a clear link between proline accumulation and oxidative stress was difficult to establish, as the results were opposite in many cases. This controversy might be explained by the fact that while proline accumulation is suggested to protect against the oxidative damage, vast evidence was also provided showing that excess proline can be a pro-oxidant, as it induces ROS production via mitochondrial electron leakage (Deuschle et al. 2004; Lv et al. 2011; Miller et al. 2009a). The latter is well established in animals (Donald et al. 2001; Elia et al. 2017; Liu et al. 2009; Polyak et al. 1997). In turn, proline-induced ROS generation can induce the antioxidant response, acting as priming agent, adding an extra layer of complexity. For instance, the exogenous treatment with proline induced the activity of antioxidant enzymes in cultured tobacco BY2 cells (Hoque et al. 2007; Hossain and Fujita 2010; Islam et al. 2009), but could not directly protect against superoxide or H_2O_2 (Hoque et al. 2007). Concerning endogenous proline accumulation, two arabidopsis non-proline-accumulating mutant lines, *p5cs1–2* and *p5cs1–4*, were shown to have higher H_2O_2 and lipid peroxidation upon saline stress, whereas the enzymatic antioxidant activities showed no uniform results (Szekely et al. 2008).

The lack of direct evidence about the putative antioxidant properties of proline was in part attributed to the difficulty to work with ROS, due to their high reactivity. Although proline was suggested to quench singlet oxygen (Alia and Matysik 2001),

the mechanism had not been described. In order to determine whether this quenching was chemical or physical, some of us investigated these putative mechanisms of quenching using direct real-time measurement of singlet oxygen by its luminescence at 1270 nm. Surprisingly, proline was not able to quench singlet oxygen, neither chemically nor physically (Signorelli et al. 2013b). In an attempt to reproduce the observations from Alia and Matysik (2001), we found out that the EPR signal of singlet-O_2-dependent TEMP oxidation becomes less stable in the presence of proline, explaining why the authors apparently detected lower TEMP oxidation activity, which led to the misinterpretation that proline was scavenging singlet oxygen and avoiding formation of oxidised TEMP.

Similarly, the reaction mechanisms of proline with hydroxyl radicals were investigated to assess whether hydroxyproline is a potential product of this interaction. Using computational chemistry, we found out that proline would rapidly react with a hydroxyl radical through hydrogen abstraction (H-abstraction). When the H-abstraction occurs on a C atom, it is more likely to yield P5C as a non-radical product than hydroxyproline (Signorelli et al. 2014). As P5C is converted back to proline by P5CR, we suggested a Pro-Pro cycle in which proline could scavenge two hydroxyl radicals and being regenerated with the consumption of one NADPH (Signorelli et al. 2014). One of the essential characteristics of antioxidants, such as glutathione and ascorbate, is that enzymes can recycle them. By the Pro-Pro cycle, proline was for the first time able to share this typical feature of antioxidants. When the H-abstraction on the nitrogen atom was evaluated, we found that this reaction was energetically preferred by hydroxyl radicals and would result in the decarboxylation of proline (Signorelli et al. 2015). The potential non-radical product was shown to be δ^1-pyrroline, a ūFE;-aminobutyric acid (GABA) precursor. This provided a link between proline and GABA involving non-enzymatic reactions under stress conditions (Signorelli et al. 2015). It is worth mentioning that, due to the non-enzymatic nature of the reaction, this link would never explain the concomitant accumulation of proline and GABA, which is most likely due to similar upstream regulators of their biosynthesis. As the second most reactive ROS, singlet oxygen, was not able to react with proline, we wondered whether proline was able to react with more stable ROS and reactive nitrogen species (RNS). In both in vivo and in vitro experimental conditions, we observed that proline was not able to scavenge superoxide, nitric oxide ($^{\bullet}NO$), nitrogen dioxide ($^{\bullet}NO_2$), and peroxynitrite ($ONOO^-$; Signorelli et al. 2016). This challenged the idea that proline is accumulated under stress to act as an antioxidant. There is at present no evidence to suggest that proline is more likely to react with hydroxyl radicals than most other organic molecules. Further research is needed to understand how significant the contribution of proline to hydroxyl radical scavenging is in physiological conditions. With the current evidence, we believe that the observed beneficial effects of proline on oxidative damage are more likely to be due to its direct stabilization effect on membranes and proteins (Sect. 3.2) and/or the capacity to activate the antioxidant defence (further in Sect. 6) than to a direct role as an antioxidant.

5 Effect of Proline Accumulation on Redox Balance

Proline synthesis from glutamate requires a double equivalent amount of reducing power. When needed to accumulate to high concentrations, the increased synthetic rate is therefore capable of lowering NAD(P)H availability inside the cytosol. Conversely, the mitochondrial catabolism of proline provides reducing equivalents in the form of an $FADH_2$ moiety bound to the ProDH enzyme (that is believed to transfer electrons directly to the respiratory chain) and an NADH molecule formed during the subsequent P5C oxidation (Fig. 3). As a consequence, a reciprocal dynamic relationship has been hypothesized between proline metabolism and the redox status of the cell, i.e. the possibility that any variation in proline homeostasis may result in a corresponding change in NAD(P)H/$NAD(P)^+$ ratio and (more recently) that also the opposite may be true. Moreover, as the two pathways occur spatially separated in the cytosol and in mitochondria, the interconversion of

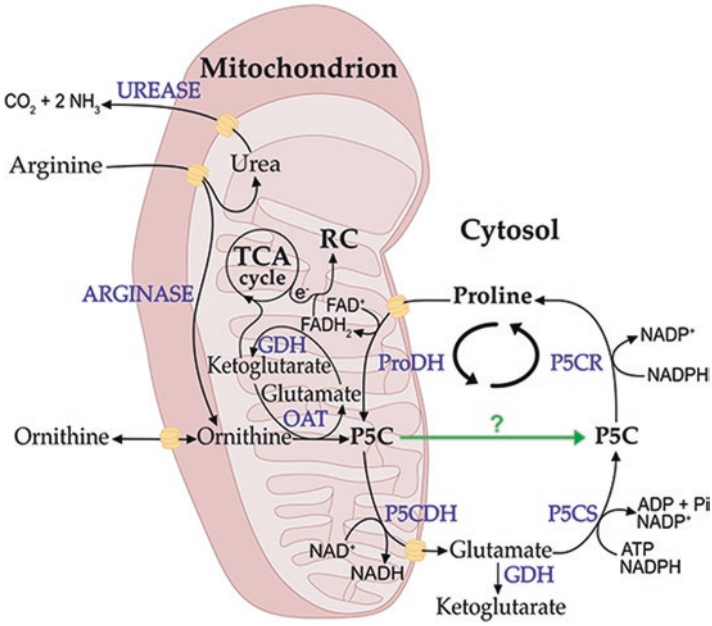

Fig. 3 The potential P5C-proline cycle. Proline synthesis in plants proceeds in the cytosol through a two-step pathway, while proline is oxidized back to glutamate in the mitochondrion. It has been hypothesized that the intermediate in both routes, P5C, may be transported through the inner mitochondrial membrane. If so, a cycle may be established in which the interconversion of P5C and proline may transfer reducing equivalents from the cytosol to the mitochondrion without the expense of cytosolic ATP, directly fuelling the respiratory chain by the activity of ProDH. Arginine catabolism might also contribute to this cycle by generating P5C through transamination between ornithine and α-ketoglutarate. GDH glutamate dehydrogenase, OAT ornithine-δ-aminotransferase, P5C δ^1-pyrroline-5-carboxylate, P5CDH P5C dehydrogenase, P5CS P5C synthase, P5CR P5C reductase, ProDH proline dehydrogenase, RC respiratory chain, TCA tricarboxylic acids

glutamate and proline could be used as a mean to transfer reducing equivalents between these cell compartments.

5.1 Proline-Glutamate Interconversions in Redox Balance

The rapid activation of the oxidative pentose phosphate pathway that has been reported in response to drought, salt, and temperature stress conditions can significantly increase the cytosolic NADPH/$NADP^+$ ratio (Esposito 2016). Moreover, as several NAD kinases were found to be calmodulin-dependent, Ca^{2+} fluxes early occurring following the exposure to many types of stress may alter the NADP(H)/NAD(H) ratio (Li et al. 2018). Because of the functional properties of the enzymes involved in proline biosynthesis, these metabolic stress responses are expected to impact proline homeostasis greatly. P5CR, which can use in vitro both NADH and NADPH as the electron donor, showing higher maximal catalytic rate but a lower affinity with NADH, has been found to be highly sensitive to changes in the ratio of phosphorylated versus non-phosphorylated pyridine dinucleotides. The NADH-dependent activity of the plant P5CR is very sensitive to $NADP^+$, being already inhibited at physiological $NADP^+$ concentrations (Giberti et al. 2014). Moreover, high proline and salt levels were found to inhibit the enzyme when NADH was the co-factor, whereas the NADPH-dependent reaction was unaffected or even stimulated (Forlani et al. 2015b; Giberti et al. 2014; Ruszkowski et al. 2015).

Concerning P5CS, which is believed to catalyse the rate-limiting step in the short anabolic pathway (Fichman et al. 2015), much less is known. However, it seems to use preferentially, if not exclusively, NADPH rather than NADH (Fichman et al. 2015; Zhang et al. 1995). Moreover, preliminary data showed an even higher sensitivity of P5CS to $NADP^+$, with 50% inhibition at a NADPH to $NADP^+$ ratio of 1.5 (Forlani 2017). As a consequence, increased NADPH/$NADP^+$ and NADP(H)/NAD(H) ratios are expected to increase the biosynthetic rate and the homeostatic level of proline inside the plant cell, without the need and before any transcriptional activation of the corresponding genes. Consistently, the Rboh inhibitor diphenyleneiodonium was found to induce high levels of proline accumulation (Shinde et al. 2016), although Rboh is necessary for the transcriptional induction of proline accumulation (Ben Rejeb et al. 2015). An unexpected effect of the impairment of very-long-chain fatty acid synthesis on proline homeostasis was shown to be mediated by effects on redox status rather than signalling functions of lipid metabolism enzymes or intermediates (Shinde et al. 2016). A recent report showed the binding of MYB-type transcription factors Phosphate Starvation Response1 and PHR1-Like1 to *P5CS1* regulatory sequences in wild-type arabidopsis plants subjected to phosphate starvation (Aleksza et al. 2017). The consequent gradual increase in proline content could also be reasonably related to a reduction in the NADP(H)/NAD(H) ratio, although this aspect was not investigated in detail.

All these results showed that the cellular redox status influences proline metabolism. Nevertheless, the opposite also holds true, as increased rates of proline

synthesis (or oxidation) may influence in turn the NAD(P)H/NAD(P)$^+$ ratio. The interconnection between high levels of proline synthesis during stress and regulation of the adenylate redox status was early hypothesized to maintain NAD(P)$^+$/NAD(P)H ratios at values compatible with metabolism under normal conditions and to reduce stress-induced cellular acidification (e.g. Hare and Cress 1997). This hypothesis has found more detailed substantiation in several recent studies. Although not definitely proven, proline production dissipating excess reducing equivalents was proposed to act as a compensatory strategy to sustain photosynthesis and prevent photoinhibition under excess light in arabidopsis mutants lacking a chloroplast NADP-dependent malate dehydrogenase (Hebbelmann et al. 2012). Trapping reducing power through enhanced proline biosynthesis has been proposed to limit the generation of ROS and the consequent cell damage (Ben Rejeb et al. 2014). Tissue-specific differences in proline metabolism and function in maintaining a favourable NADP$^+$/NADPH ratio, where proline synthesis in photosynthetic tissues regenerates NADP$^+$, while its catabolism in meristematic and expanding cells is needed to sustain growth by increased availability of energy and reducing power, were found to take place during drought adaptation in arabidopsis (Sharma et al. 2011).

5.2 The P5C-Proline Cycle

More recently, a puzzling result has been reported in an increasing number of studies: the activation under stress of genes in both the proline anabolic and the catabolic pathways. Under these conditions, only transfer of redox equivalents to mitochondria but no proline accumulation would be achieved with the expense of one cytosolic ATP per cycle, since proline is oxidized as soon as it is synthesized, and does not accumulate in the cell (or accumulates much less than expected based on the enhancement of its biosynthetic rate). In PEG-treated arabidopsis seedlings, *P5CS1* was induced about 20-fold (Sharma and Verslues 2010), but a five-fold increase was evident also for *P5C dehydrogenase* (*P5CDH*, recently proposed to be renamed as glutamate semialdehyde dehydrogenase; Korasick et al. 2019), the enzyme catalysing the second and last step in the mitochondrial oxidation of proline (Forlani et al. 1997; Forlani et al. 2015a), and for ornithine aminotransferase (OAT), the enzyme deaminating ornithine to yield P5C (da Rocha et al. 2012) (Fig. 3). In some instances, microarray and RT-PCR data showed the concurrent transcriptional activation of *ProDH* and *P5CR*. During cold acclimation in arabidopsis, the steady-state mRNA levels for *P5CSs* markedly increased after 12 h of exposure to 4 °C but then declined to basal levels after 96 h of cold treatment, while transcript level of *ProDH1* continuously increased; *P5CDH* mRNA level was unaffected and that for *P5CR* increased slightly throughout (Kaplan et al. 2007). If the product of proline oxidation in the mitochondrion, P5C, may trespass the membrane and enter the cytosol, this would cause an apparently futile cycle between glutamate and proline, feeding electrons from cytosolic NADPH to the respiratory chain (Fig. 3).

Overexpression of *ProDH* in tobacco and arabidopsis or impairment of P5C oxidation in the arabidopsis *p5cdh* mutant was reported not to change the cellular proline to P5C ratio under both normoosmotic and stress conditions, leading the authors to suggest that excess P5C produced in the mitochondrion may be reduced to proline in the cytosol (Miller et al. 2009b). This so-called P5C-proline cycle involving ProDH and P5CR, conclusively demonstrated in mammalian cells where both enzymes are localized in mitochondria (Liu and Phang 2012), has therefore been hypothesized to play a role also in plants. However, as long as P5CDH is active in mitochondria, it is very difficult to distinguish whether a glutamate-proline or a P5C-proline cycle is operative in vivo. Hyperactivity of either cycle, for instance, following the exogenous supply of proline, enhances ROS production in the mitochondrion. Consistently, *p5cdh* mutants showed hypersensitivity to exogenous proline (Deuschle et al. 2004). Metabolic cycling between glutamate and proline or P5C and proline could potentially benefit the cell and play a critical role for plant survival under stress through maintenance of the cellular redox balance, regulation of the NAD(P)H/NAD(P)$^+$ ratio, and enhancement of the oxidative pentose phosphate pathway (Hayat et al. 2012; Kavi Kishor and Sreenivasulu 2014; Lv et al. 2011; Miller et al. 2009b). Moreover, an unbalanced activity of ProDH and P5CDH could lead to direct electron transfer to O_2 and production of ROS or unspecific damage to mitochondrial components by reaction with P5C (Liang et al. 2013). At low levels, ROS act as a signal for reinstating metabolic homeostasis during stress situations (Türkan and Demiral 2009), whereas at high levels ROS can play a role in the hypersensitive reaction to pathogens (see Sect. 5.3). Conclusive evidence for the occurrence of a P5C transporter in the mitochondrial membrane has not been obtained, yet, and in arabidopsis *p5cs1-p5cs2* double mutants, arginine and ornithine could not substitute glutamate as precursor for proline (Funck et al. 2012; Mattioli et al. 2012). Therefore, the P5C-proline cycle has still to be regarded as a hypothesis in plants, and further work is required to confirm its occurrence and physiological role. However, an increasing number of data point at the activation of proline metabolism, more than the resulting homeostatic level of the free amino acid inside the cell, as the determinant for an effective stress response of the plant (e.g. Signorelli et al. 2016; Forlani et al. 2018).

5.3 Proline Catabolism and ROS Generation Under Stress

Under this perspective, some early experimental evidence about the expression of the catabolic pathway in rust-infected plants may be reconsidered and suggest a role for proline metabolism also in the plant response to biotic stress conditions. Early induction of the gene coding for P5CDH was shown in several crops following penetration of virulent, but not of avirulent fungal strains (Ayliffe et al. 2002; Mitchell et al. 2006). Moreover, induction of proline oxidation was reported in arabidopsis during incompatible plant-pathogen interactions (Cecchini et al. 2011). Therefore, the possibility that proline metabolism may be part of the process leading

to programmed cell death (PCD) during the hypersensitive defence reaction was proposed (Senthil-Kumar and Mysore 2012). However, it is still unclear which may be the active molecule, whether proline itself, P5C, or ROS produced during proline catabolism. The early activation of *ProDH* during pathogen attack was accompanied by an increase in *P5CR* but not in *P5CDH* transcripts, apparently with few changes occurring in proline and P5C levels (Cecchini et al. 2011). Therefore, also in this case, the whole picture strengthened the possible occurrence of the P5C-proline cycle, leading to ROS production. Enhanced proline oxidation in the mitochondrion leads to sustained ROS generation (Cecchini et al. 2011; Servet et al. 2012), which in turn act as second messengers in various signalling cascades and induce the expression of defence pathways conferring tolerance to either abiotic or biotic stress conditions (Miller et al. 2011; Ben Rejeb et al. 2014). Indeed, the analysis of wild-type arabidopsis plants and *p5cdh* mutants showed that the absence of P5CDH does not reduce ROS production, cell death, or pathogen resistance and suggested that the enzyme does not act synergistically with ProDH in the potentiation of such defence responses (Monteoliva et al. 2014). The whole picture is made even more complex by the presence of another pathway yielding P5C (and possibly proline), the mitochondrial catabolism of arginine via ornithine (Fig. 3). Upon treatment with exogenous proline or pathogen infection, arabidopsis wild-type and *p5cdh* plants consecutively induced the expression of *ProDH* and Pro biosynthetic genes, but while the former seemed to induce both routes, *p5cdh* mutant plants may primarily activate the ornithine route and sustain *ProDH* induction without reducing the Pro content but rather increasing it (Rizzi et al. 2015). Whatever the way to fuel the P5C-proline cycle, the concurrent induction of *P5CDH* could make the difference between compatible and incompatible plant-pathogen interactions. In incompatible interactions, low levels of P5CDH activity increases the rate of proline-P5C interconversion, which in turn leads to increased ROS production by ProDH and (directly or indirectly) to PCD. In the former, on the contrary, high levels of P5CDH lower substrate availability for the cycle, delaying PCD induction and allowing a systemic spread of the pathogen. In any case, the exact mechanisms underlying such a role of proline metabolism under biotic stress conditions still await full elucidation.

6 Effect of Proline Metabolism on Antioxidant Enzymes

Notwithstanding the role of proline catabolism in generating ROS in the mitochondrion, different reports have shown that proline accumulation correlates with an enhanced antioxidant enzymatic activity (Hoque et al. 2007, 2008; Islam et al. 2009; Kaushal et al. 2011). This effect has been mainly inferred from the capacity of proline to act as a protectant for enzymes (Ben Rejeb et al. 2014; Szabados and Savouré 2010) or from the transient ROS signals induced by proline catabolism, which result in increased expression of antioxidant enzymes (Ben Rejeb et al. 2014; Zarse et al. 2012). As mentioned before, proline accumulation was suggested to be dependent

on Rboh activity (Ben Rejeb et al. 2015). This enzyme produces superoxide in the apoplast, which is then converted to H_2O_2, and it is considered to be one of the main sources of ROS accumulation under stress and to mediate a systemic ROS signal throughout the plant (Miller et al. 2009b). Thus, it is not surprising that proline accumulation and induction of the levels of antioxidant enzymes occur in parallel, while at present it is unclear whether there is a direct effect of proline on the expression of antioxidant enzymes. In the arabidopsis mutants *p5cs1–2* and *p5cs1–4*, unable to accumulate proline, some of the antioxidant enzymes showed higher activity (CAT, glutathione peroxidase), whereas others showed lower activity (SOD, APX, and glutathione reductase) (Szekely et al. 2008). These opposite effects questioned how important the endogenous proline accumulation is for the overexpression-protection of antioxidant enzymes in vivo. Moreover, when the transcriptomic data of one of these mutants (*p5cs1–4*) was analysed and compared to wild-type plants, none of the genes coding for the aforementioned antioxidant enzymes were differentially expressed under both stress and control conditions (Shinde et al. 2016). This suggests that proline anabolism does not contribute to the regulation of antioxidant response; however, it does not exclude the effect of its catabolism. To establish whether proline catabolism regulates antioxidant response in plants, the study of the expression of antioxidant enzymes under stress conditions in wild-type and *pdh* mutant lines would be beneficial.

7 Proline as a Source of C and N During Recovery

Because of the high consumption of reducing power needed for its synthesis, the oxidation of proline to glutamate and the subsequent channelling of the latter into the TCA cycle can yield as many as 30 ATP equivalents (Atkinson 1977). Proline accumulation may therefore represent an efficient method for energy storage. Consistently, honeybees and other nectar-foraging insects preferentially utilize proline as a fuel during the initial phases of flight (Micheu et al. 2000), and experimental evidence supports the preference of bees and butterflies for nectars or sugar solutions enriched with proline (Bertazzini et al. 2010). In plants, proline oxidation was shown to be required to sustain growth even at low external water potential, since high *ProDH* expression was maintained in the root apex and shoot meristem under stress rather than being repressed (Sharma et al. 2011). A fortiori, the use of proline to fuel cell metabolism and as a source of organic nitrogen and carbon to resume growth should be highly valuable after coming back to non-stressful conditions. In fact, a rapid reactivation of *ProDH* transcription to high levels has been reported in many cases during recovery (Yoshiba et al. 1997). However, modulation of proline metabolism during recovery and its role in plant survival are still largely unexplored. Some data showed that the post-drought response is dependent on drought severity, suggesting that sustained synthesis and accumulation of proline can promote plant damage reparability by up-regulating antioxidant activity also during the recovery from stress (An et al. 2013). If not required, as following the

exogenous supply in the absence of stress, proline is promptly utilized, and its intracellular concentration rapidly lowers to homeostatic levels (e.g. Forlani et al. 2015a). Therefore, there is a need to distinguish between different cases, in order to avoid that proline may be oxidized when its accumulation is functional to withstand stress conditions. This goal could be accomplished by differential signals regulating *ProDH* transcription. Additionally, recent results in arabidopsis described the identification of a mitochondrial protein, Drought and Freezing Responsive gene 1 (DFR1), involved in the inhibition of proline degradation during drought and cold stresses. Two alternatively spliced isoforms of DFR1 were detected that are strongly induced by stress and specifically interact with ProDH and P5CDH, thereby inhibiting their activities (Ren et al. 2018).

8 Conclusions

Over the last decades, many studies have shown that proline accumulation in plants correlates with greater stress tolerance. The broad variety of *cis*-responsive elements present in the promoter of the plant *P5CS1* gene explains why proline accumulation is a conserved response observed in a wide range of conditions. Furthermore, the fact that a master regulator of light-dependent processes such as HY5 mediates *P5CS1* expression shows that proline biosynthesis is important to be coordinated with other light-dependent processes. However, the main mechanism by which proline accumulation contributes to stress tolerance remains largely elusive. It is unlikely that proline accumulation exerts its protective function just by preventing water withdrawal from the plant cells, as in many cases its contribution maximally reached 15% of the required osmotic adjustment. Instead, proline can act as a kosmotropic agent, stabilizing proteins and membranes under unfavourable conditions. More fundamental research needs to be done to understand the kosmotropic properties of proline. Recent findings showed that proline accumulation is also dependent on Rboh activity, suggesting that ROS signalling is involved in the regulation of its accumulation. This response could be important if proline accumulation attenuates oxidative damage. Currently, the most likely ways in which this can be achieved is directly by proline, protecting the antioxidant enzymes from denaturation, or by its catabolism, inducing the antioxidant response. Yet, recent findings showed that proline is unable to directly protect against most ROS and RNS. In addition, proline can contribute to regulation of the NAD(P)H/NAD(P)$^+$ ratio, and the activation of a P5C-proline (or glutamate-proline) cycle in plants appears as an effective stress response, in which cytosolic reducing equivalents can be converted into mitochondrial reducing equivalents to fuel the respiratory chain. Regarding biotic stress, proline catabolism was suggested to lead to programmed cell death during the hypersensitive defence reaction.

Overall, the latest research in the field has contributed to limit some speculations about the role of proline and its metabolism during abiotic and biotic stress in plants,

but also several new questions have arisen. Thus, future research on proline metabolism in stressed plants needs to be supported to finally understand its molecular and physiological function under stress.

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