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REVIEW ARTICLE

Proline accumulation in plants: a review

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Abstract Proline (Pro) accumulation is a common physiological response in many plants in response to a wide range of biotic and abiotic stresses. Controversy has surrounded the possible role(s) of proline accumulation. In this review, knowledge on the regulation of Pro metabolism during development and stress, results of genetic manipulation of Pro metabolism and current debate on Pro toxicity in plants are presented.

Keywords Osmotic stress · Genetic engineering · Proline toxicity · P5CS · P5CR · P5CDH · PDH

Abbreviations

ABA	Abscissic acid
ABRE	ABA responsive element
AS	Antisense
At	*Arabidopsis thaliana*
GFP	Green fluorescent protein
GSA	Glutamate semialdehyde
Nat-siRNAs	Natural silencing RNA
PPP	Pentose phosphate pathway
Pro	Proline
PDH	Pro dehydrogenase
P5C	Pyrroline-5-carboxylate
P5CDH	P5C dehydrogenase
P5CR	P5C reductase
P5CS	P5C synthase
RNAi	RNA interference
ROS	Reactive oxygen species
UTR	Untranslated region.

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Introduction

Proline (Pro) accumulation occurs in eubacteria, protozoa, marine invertebrates and plants after various stresses. In plants, Pro accumulation has been reported to occur after salt, drought, high temperature, low temperature, heavy metal, pathogen infection, anaerobiosis, nutrient deficiency, atmospheric pollution and UV irradiation (Hare and Cress 1997; Saradhi et al. 1995; Siripornadulsil et al. 2002). The level of Pro accumulation in plants varies from species to species and can be 100 times greater than in control situation. Osmotic stress, which include treatments lowering the osmotic potential component of the water potential, are by far the most studied ones because they represent a major concern in agriculture. Pro metabolism in plants has mainly been studied in response to osmotic stress.

Pro accumulation is believed to play adaptive roles in plant stress tolerance. Pro has been proposed to act as a compatible osmolyte and to be a way to store carbon and nitrogen (Hare and Cress 1997). Salinity and drought are known to induce oxidative stress. Early in vitro studies showed that Pro can be a ROS scavenger (Smirnoff and Cumbes 1989). Pro has also been proposed to function as molecular chaperone stabilizing the structure of proteins, and Pro accumulation can provide a way to buffer cytosolic pH and to balance cell redox status. Finally Pro accumulation may be part of the stress signal influencing adaptive responses (Maggio et al. 2002).

Pro accumulation during osmotic stress is mainly due to increased synthesis and reduced degradation. Although Pro

transport certainly plays an important role in Pro distribution, its role during stress has been poorly studied (Rentsch et al. 1996).

In plants, there are two different precursors for Pro. The first pathway is from glutamate, which is converted to Pro by two successive reductions catalyzed by pyrroline-5-carboxylate synthase (P5CS) and pyrroline-5-carboxylate reductase (P5CR), respectively. P5CS is a bifunctional enzyme catalyzing first the activation of glutamate by phosphorylation and second the reduction of the labile intermediate γ-glutamyl phosphate into glutamate semialdehyde (GSA), which is in equilibrium with the P5C form (Hu et al. 1992). An alternative precursor for Pro biosynthesis is ornithine (Orn), which can be transaminated to P5C by Orn-δ-aminotransferase (OAT), a mitochondrial located enzyme. Glutamate pathway is the main pathway during osmotic stress. However in young Arabidopsis plants, the ornithine pathway seems also to contribute and δ-OAT activity is enhanced (Roosens et al. 1998). The Pro degradation is the reverse process of Pro biosynthesis and catalyzed by Pro dehydrogenase (PDH) and P5C dehydrogenase (P5CDH). Pro biosynthesis occurs in the cytosol and in the plastids (like chloroplasts in green tissues) while Pro degradation takes place in mitochondria (Elthon and Stewart 1981; Rayapati et al. 1989; Szoke et al. 1992).

Regulation of Pro metabolism during development and stress

Many studies have been performed in *Arabidopsis thaliana*. *A. thaliana* is the most studied model plant because it has the smallest genome size, was the first available plant genome sequence, has a short life cycle (2 months 1/2), a small size, is easy to culture and to transform and is amenable for genetic studies.

In *A. thaliana* there are two P5CS isoenzymes which play specific roles in the control of Pro biosynthesis (Fabro et al. 2004; Székely et al. 2008). In other plant species P5CS is also encoded by two genes (Ginzberg et al. 1998; Fujita et al. 1998). P5CS represents a rate-limiting step of pro synthesis and is controlled by feed-back inhibition and transcriptional regulation (Savouré et al. 1995; Yoshiba et al. 1995; Zhang et al. 1995). P5CR is encoded by only one gene but the enzyme seems to be active in chloroplasts and cytosol (Rayapati et al. 1989; Szoke et al. 1992; Verbruggen et al. 1993).

During development of *A. thaliana* and in the absence of stress, levels of free Pro vary among plant organs, independently of the amino acid pool size. Highest Pro levels are found in flowers, especially in pollen grains, and in seeds, and lowest levels in roots. The Pro content is also dependent on plant age, leaf age, position or leaf part (Verbruggen et al. 1993; Chiang and Dandekar 1995). The levels of *P5CS* and *P5CR* transcripts and P5CR protein are correlated with the Pro content in the different organs of *A. thaliana*, except in roots. The apparent discrepancy in roots between low Pro content and high *P5CS*/*P5CR* transcripts levels can be explained by Pro export via xylem to the shoot. Pro synthesis and degradation seem to be both induced in reproductive organs and seeds. Since the two pathways take place in different cell compartments, Pro metabolism may serve to exchange redox potential (Verbruggen et al. 1996). Moreover Pro transport to flowers is also active. Pro can be a metabolic compatible solute to transfer nitrogen, carbon and reducing potential to developing flowers and seeds.

Studies of transcriptional regulation of genes involved in Pro synthesis confirmed developmental regulation. In young Arabidopsis plants, GUS analysis of *AtP5R* (Arabidopsis *P5CR* gene) promoter revealed high expression in apical meristem and young leaf, in root meristem, secondary root primordia and root vascular cylinder. In young leaf high *P5R* expression could be detected all over the leaf blade, while in old leaves, expression was restricted to the veins, hydathodes, guard cells and base of trichomes (Hua et al. 1997). In flowering plants, high *AtP5R* expression could be detected in rapidly dividing cells, such as root meristem, and cells or tissues undergoing changes in water potential, such as hydathode, guard cell, ovule, developing seed and pollen grain (Hua et al. 1997).

Arabidopsis accumulates Pro upon osmotic stress (Verbruggen et al. 1993; Yoshiba et al. 1997). During stress, the expression of *P5CS*, but not of *P5CR* gene, is well correlated with Pro content (Yoshiba et al. 1995; Savouré et al. 1995). During heat for example, *AtP5R* transcripts accumulate without further protein level enhancement or Pro accumulation (Hua et al. 2001). Post-transcriptional regulation of *AtP5R* occurs during stress. The role of the 5′UTR leader sequence of *AtP5R* in mRNA stabilisation and translation inhibition was demonstrated (Hua et al. 2001).

More recently two closely related *P5CS* genes have been identified in *Arabidopsis thaliana*. During stress *P5CS1* but not *P5CS2* gene is required for Pro accumulation (Fabro et al. 2004; Székely et al. 2008). During osmotic and salt stress there are different signalling pathways responsible for the up-regulation of *P5CS1* gene. Induction of *P5CS1* expression *in A. thaliana* depends on phospholipase C during salt stress but not during drought (Parre et al. 2007). The abscissic acid (ABA) hormone and salt stress also induce Arabidopsis *P5CS1* expression through ABA responsive (ABRE) element (Strizhov et al. 1997; Savouré et al. 1997; Abraham et al. 2003). The role of ABA in Pro accumulation was dissected by Verslues and Bray (2006). These authors used ABA biosynthetic and signalling

mutants to show dependency of Pro accumulation not only on the ABA amount but also on the plant sensitivity, or competency, to respond to the ABA. Further results by Verslues et al. (2007) support the idea that H_2O_2 is part of ABA signaling and ABA-regulated responses like Pro accumulation.

Recently Székely et al. (2008) provided first evidence about the specificity of cell type and subcellular localization patterns of P5CS1 and P5CS2 proteins in Arabidopsis. This study highlighted the non-redundancy of the two P5CS isoenzymes and the importance of compartmentalization of Pro metabolism. Depending on the developmental stage, organs and growth conditions, P5CS1- and P5CS2-GFP fusions displayed different localization in the cell (plastids, vesicles or in the cytoplasm). In mesophyll cells of mature leaves, salt and osmotic stress stimulate the import of P5CS1 to chloroplasts, while the distribution of the P5CS2 pool in the cytoplasm and chloroplasts does not change (Székely et al. 2008).

During osmotic stress, availability of atmospheric CO_2 is reduced because of increased stomatal closure and consumption of NADPH by the Calvin Cycle is decreased. Activity of P5CS in the chloroplasts can recycle $NADP^+$, the last acceptor of the photosynthetic electron transfer chain, which may reduce ROS production at the photosystem I. Also glucose-6-phosphate dehydrogenase, the first and rate-limiting enzyme of the pentose phosphate pathway (PPP) requires $NADP^+$ and is inhibited by NADPH. Phang (1985) proposed a model in which Pro/P5C interconversions modulate $NADP^+$/NADPH redox states. Interestingly the PPP takes place in the cytoplasm and in the plastids of plant cells, the two compartments where Pro synthesis takes place.

Kohl et al. (1988, 1990) have applied Phang's model to nitrogen-fixing soybean nodules, which accumulate Pro. Pro accumulates via enhanced synthesis and high Pro synthesis maintains a high NADP/NADPH ratio in the nodules. Such a high ratio can drive the PPP, which in turn supports purine biosynthesis. Purine derivatives are used as the primary transport molecule for fixed nitrogen. However in vitro activities could not support the correlation between P5CR and PPP activities (Kohl et al. 1990). Kohl et al. (1988, 1990) have proposed that Pro transfers redox potential from the plant cytoplasm to the bacteroid. This was supported by high PDH activity in bacteroids of soybean nodules. During stress the link between Pro accumulation and induction of the PPP was episodically reported but hardly demonstrated (Hare and Cress 1997).

Pro degradation is also regulated during development and stress. In *A. thaliana*, *PDH* expression is low except in flowers and young siliques, which also contain higher Pro concentrations than in roots or leaves. *PDH* expression is strongly induced by the addition of exogenously supplied Pro. During stress both transcript and protein levels of *At-PDH* are repressed during stress and induced during recovery from stress (Kiyosue et al. 1996; Peng et al. 1996; Verbruggen et al. 1996). There is thus evidence for a negative transcriptional regulator which overrides the positive effect of accumulated Pro on *AtPDH* expression (Verbruggen et al. 1996). Data on *At-PDH* expression during development and stress were confirmed by a gene reporter–promoter study published by the group of Shinozaki (Nakashima et al. 1998). Recently bZIP transcription factors involved in the up-regulation of *At-PDH* during rehydration were identified (Weltmeier et al. 2006).

Although Pro level has been thought to be regulated mainly by P5CS and PDH, the regulation by P5CDH seems to be also important. Arabidopsis *P5CDH* and *SRO5*, a gene of unknown function, form an overlapping antisense gene pair. These two genes generate convergent transcripts that overlap by 760 nucleotides (Borsani et al. 2005). *SRO5* is not expressed in plants grown under normal conditions, but its expression is upregulated by NaCl treatment (not by PEG- or mannitol- treatments, which induce osmotic stress). Upon salt treatment, the *SRO5* and *P5CDH* mRNAs can form a dsRNA that is then processed to generate 24-nt *SRO5-P5CDH* natural silencing RNS (nat-siRNAs). Those nat-siRNAs downregulate the expression of *P5CDH* by causing mRNA cleavage (Borsani et al. 2005). This in turn contributes to Pro accumulation but also causes an increase in ROS production, which is counteracted by the SRO5 protein. This is a nice example of regulation of two genes in a converse manner.

Genetic manipulation of Pro metabolism

As much as one-half of the irrigated areas of the world are affected by high salinity. Therefore there is a high interest to improve plant osmotolerance in agriculture. This has been achieved by the genetic manipulation of osmolytes, transcriptions factors and more recently of the cytokinin hormone (Seki et al. 2007; Rivero et al. 2007). Engineered transgenic plants have also been useful tools to address fundamental questions.

Figure 1 summarizes different examples of genetically modifications of the Pro metabolic pathways. First Pro transgenic plants were published by the group of Desh Pal Verma (Kishor et al. 1995). Tobacco plants overexpressing mothbean *P5CS* gene, coding for the first enzyme of pro biosynthesis under the activity of a constitutive promoter, synthesized 10–18-fold more Pro than control plants and were better salt stress tolerant. Surprisingly the osmotic potential of P5CS transgenics was not lower than in control plants. Removal of feed-back inhibition of P5CS resulted in higher Pro accumulation and protection of plants from

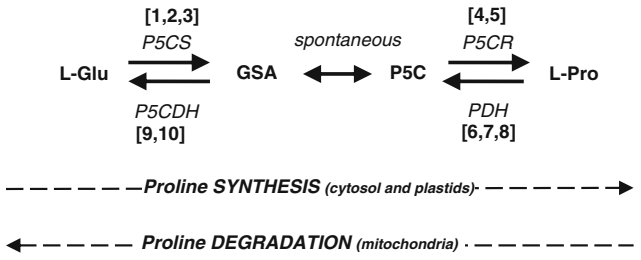

Fig. 1 Genetic manipulations of Pro metabolic pathways in plants. The main precursor of Pro synthesis is L-glutamic acid (L-Glu). L-Glu is first reduced to glutamate semialdehyde, which spontaneously cyclizes to pyrroline-5-carboxylate (P5C), by P5C synthase (P5CS). The second reduction, of P5C to Pro, is catalyzed by P5C reductase (P5CR). This pathway is found in the cytosol and in plastids. Pro is catabolized to Glu in mitochondria by Pro dehydrogenase (PDH) and P5C dehydrogenase (P5CDH). Examples of genetic manipulations of Pro metabolic pathway and impact on osmotolerance: **[1]** Overexpression of mothbean *P5CS* in tobacco plants. Those transgenics were better salt tolerant (Kishor et al. 1995); **[2]** Overexpression of *P5CS* in antisense orientation in Arabidopsis (Nanjo et al. 1999a) and **[3]** *p5cs1* insertional mutation (Székely et al. 2008) resulted in reduced osmotolerance; **[4]** Overexpression of soybean *P5CR* gene in tobacco (Szoke et al. 1992) did not modify osmotolerance; **[5]** Overexpression of Arabidopsis *P5CR (AtP5R)* gene in soybean improved drought and heat stress (de Ronde et al. 2000, 2004); **[6]** Overexpression of *PDH* did not change osmotolerance in Arabidopsis, except in the presence of exogenously supplied Pro. In these conditions PDH-sense lines were better osmotolerant than WT. **[7]** Higher salt tolerance was observed in Arabidopsis plants overexpressing *PDH* in antisense orientation by Nanjo et al. (1999b), but not by Mani et al. (2002). Study of modifications of Pro catabolism in Arabidopsis in relation to P5/GSA and/or Pro toxicity: **[6]** Overexpression of *PDH* decreased sensitivity to externally supplied Pro, **[7]** decrease of PDH activity by antisense strategy (Mani et al. 2002) or **[8]** knock-out mutation (Nanjo et al. 2003) increased sensitivity to Pro; **[9]** *P5CDH* overexpression decreased sensitivity to externally supplied Pro (Deuschle et al. 2004); **[10]** *p5cdh* knock-out mutants were hypersensitive to Pro (Deuschle et al. 2004)

osmotic stress (Hong et al. 2000). On the contrary *P5CS* antisense Arabidopsis lines that were impaired in their capacity to synthesize Pro were hypersensitive to osmotic stress (Nanjo et al. 1999a). P5CS antisense lines showed morphological abnormalities of epidermal and parenchymatous cells, underlying the role of pro as major constituent of cell wall proteins (Nanjo et al. 1999a). Similarly *p5cs1 Arabidopsis* insertion mutant showed reduced salt tolerance (Székely et al. 2008). Furthermore analysis of Arabidopsis *p5cs* insertion mutants confirmed a role in vivo for Pro in ROS scavenging, which was first postulated by Smirnoff and Cumbes in (1989). Enzymes of the ROS-scavenging glutathione-ascorbate cycle showed significantly lower activities in the *p5cs1* mutants compared to wild type under salt stress suggesting that Pro accumulation is implicated in the control of either stability or activity of enzymes in the glutathione-ascorbate cycle (Székely et al. 2008).

However high pro levels are not always correlated with osmotolerance. Mutants displaying higher Pro accumulation can be salt hypersensitive (Lui and Zhu 1997).

Effects of genetic manipulation of Pro synthesis can be plant species specific. Overexpressing soybean *P5CR* gene in transgenic tobacco did not increase osmotolerance (Szoke et al. 1992). Different results were observed in soybean by a team of South Africa. The *A. thaliana* gene encoding P5CR was overexpressed in soybean in the sense and antisense orientation into a heat shock cassette containing an inducible heat shock promoter (de Ronde et al. 2000). Soybean production in South Africa is affected by frequent periods of drought. Field trials in South Africa with P5CR transgenic soybean lines supported improved drought performance and higher heat tolerance compared to wild type cultivars (de Ronde et al. 2004). It is therefore possible that crops with genetically manipulated Pro synthesis will be commercialised in the future.

Pro degradation was also manipulated. Overexpression of *PDH* in *Arabidopsis thaliana* did not result in morphological abnormalities, probably because Pro homeostasis relies on regulated transport between cell compartments (Nanjo et al. 1999b; Mani et al. 2002). Pro degradation depends on prior Pro transport to mitochondria. The normal phenotype of PDH-sense plants is in contrast with the results of the team of Jim Phang in human cells where overexpression of Pro oxidase induced Pro-dependent and mitochondria-mediated apoptosis (Hu et al. 2007).

Decreasing Pro catabolism by PDH antisense strategy did not alter plant development (Nanjo et al. 1999b; Mani et al. 2002). A slight decrease in seed germination was observed in PDH RNAi tobacco plants (Ribarits et al. 2007). Higher tolerance to salt stress or to drought was sometimes observed in some PDH antisense lines but not always (Nanjo et al. 1999b; Mani et al. 2002). Differences in observed tolerance between laboratories can lie in the levels of Pro dehydrogenase inhibition and corresponding increase in Pro content, as well as applied stress conditions. Furthermore PDH sense lines, overexpressing *PDH* under a constitutive promoter, showed lower Pro accumulation during osmotic stress but no change in osmotolerance. However these PDH-sense lines were better osmotolerant than WT in the presence of exogenously supplied Pro. In the latter conditions, chromatography after radiolabelled Pro supply showed that degradation in glutamate was increased (Mani et al. 2002). These results suggest that Pro can be a good source of energy during stress too, and that the second step of the oxidation pathway is not rate-limiting.

Pro toxicity

Pro accumulation is a common response of plants to stress. However external supply of pro in control conditions is toxic. There is a debate whether Pro or its degradation

product P5C is the cause of toxicity (Hellman et al. 2000; Deuschle et al. 2001; Mani et al. 2002; Ayliffe et al. 2002).

Application of both Pro and P5C cause cell death in plants (Deuschle et al. 2004). Data of Deuschle et al. (2004) support that Pro toxicity is mediated by GSA/P5C accumulation. Exogenously applied P5C increases ROS production, reduces growth and induces a number of stress-responsive genes. Furthermore *P5CDH* overexpression decreased sensitivity to externally supplied Pro while *p5cdh* knock-out mutant was hypersensitive to Pro (Deuschle et al. 2004).

Other data in mutants with impaired Pro catabolism clearly support that Pro toxicity is not, or not only mediated by P5C/GSA. Pro external concentrations higher than 10 mM are toxic to wild-type Arabidopsis plants. In PDH-antisense plants that displayed a lower Pro catabolism Pro toxicity was observed at lower Pro concentrations than in wild-type (Mani et al. 2002). PDH-sense transgenic plants, with a higher Pro catabolism capacity, displayed wild-type Pro sensitivity. *Pdh* knock-out mutants were even more sensitive to Pro than PDH antisense plants, most probably because the mutation results in a more severe inhibition of Pro catabolism (Nanjo et al. 2003). Transcriptomic analysis of *pdh* mutants treated with Pro revealed the repression of genes involved in photosynthesis and genes involved in the synthesis of cell-wall associated proteins, which are believed to account for Pro toxicity.

Hypersensitivity of *pdh* KO and of PDH-AS plants to Pro is against the theory that not Pro but only P5C causes toxicity.

Conclusion

In short, Pro accumulation is a common physiological response to various stresses but is also part of the developmental program in generative tissues like pollen for example. Transgenic approaches have confirmed the beneficial effect of Pro overproduction during stress. However consensus was not achieved on the exact roles of Pro accumulation. Classical gain or loss of function strategies could not bring clear answers, probably because Pro also displays the essential role of being a protein component. Stresses like drought or salt stress have multiple targets and Pro is also believed to play different roles. Moreover the balance between biosynthesis and degradation of Pro is also thought to be essential in the determination of the osmoprotective and developmental functions of Pro.

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