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Multiple Roles of Proline in Plant Stress Tolerance and Development

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Abstract The recent progresses in the research on proline will be described, focusing on plants and covering proline metabolism and signal transduction as well as the role of this imino acid in stress response. Furthermore, the recently described developmental role of proline in flowering and reproduction will be illustrated and discussed.

Keywords Flowering, development, proline, P5CS1, stress

Subject codes L24027–L24043

1 Introduction

For many years, proline has been known to be involved in the response to a number of environmental stresses, particularly salt and drought stress. The accumulation of proline upon osmotic stress is well documented in a large number of different organisms, including protozoa (Kaneshiro et al. 1969; Poulin et al. 1987), eubacteria (Csonka 1989), marine invertebrates (Burton 1991), and algae (Schobert 1977; Brown and Hellebust 1978), as well as in

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different plant species (Verbruggen et al. 1993; Yoshiba et al. 1995; Peng et al. 1996; Nakashima et al. 1998; Mattioli et al. 2008; Székely et al. 2008). However, a general agreement on the precise role of proline in the response of plants to stress is still lacking and several hypotheses have been proposed on the significance of the accumulation of proline caused by stress (for a review, see Kavi Kishor et al. 2005).

An increasing body of evidence seems now to indicate that proline is also involved in flowering and development (Mauro et al. 1996; Nanjo et al. 1999a; Samach et al. 2000; Trovato et al. 2001; Mattioli et al. 2008; Székely et al. 2008), and it has been proposed that this developmental role of proline is uncoupled from its role in stress response (Mattioli et al. 2008).

2 Proline metabolism

2.1 Proline biosynthesis

Proline biosynthesis in higher plants may proceed either via the glutamate or the ornithine pathway. The former converts glutamate into proline in a two-step pathway and is thought to be the most important biochemical route under physiological conditions and under nitrogen shortage or osmotic stress, whereas the latter leads to proline via the deamination of ornithine and is thought to act mainly under supra-optimal nitrogen conditions (for a review, see Delanauney and Verma 1993).

2.1.1 The glutamate pathway. The synthesis of proline from glutamate was initially characterized in bacteria (Leisinger 1987) and found to be similar in a wide range of prokaryotic and eukaryotic organisms (Baich 1969; Krishna and Leisinger 1979; Adams and Frank 1980; Csonka 1989). In this pathway, illustrated in Fig. 1, the synthesis of proline starts with the ATP-dependent phosphorylation of L-glutamate, which is converted into γ-glutamyl-phosphate (γ-GP) by the enzyme γ-glutamyl-kinase (γ-GK). Subsequently, γ-GP is reduced to glutamic-γ-semi-aldehyde (GSA) by glutamic-γ-semi-aldehyde-dehydrogenase (GSADH), and GSA spontaneously cyclizes to pyrroline-5-carboxylate (P5C) that is converted into L-proline by the enzyme pyrroline-5-carboxylate-reductase (P5CR).

A similar proline pathway from glutamate was hypothesized to take place in plants on the basis of indirect evidence (Stewart 1981). However, the isolation and characterization of the cDNA encoding pyrroline-5-carboxylate-synthetase (P5CS) – a bifunctional enzyme catalyzing the first two steps of proline synthesis from glutamate (Hu et al. 1992) – challenged this hypothesis revealing the existence of a divergent pathway between plants and others organisms. The proline metabolic pathways taking place in higher plants are shown in Fig. 2. The gene encoding P5CS was first isolated by means of a

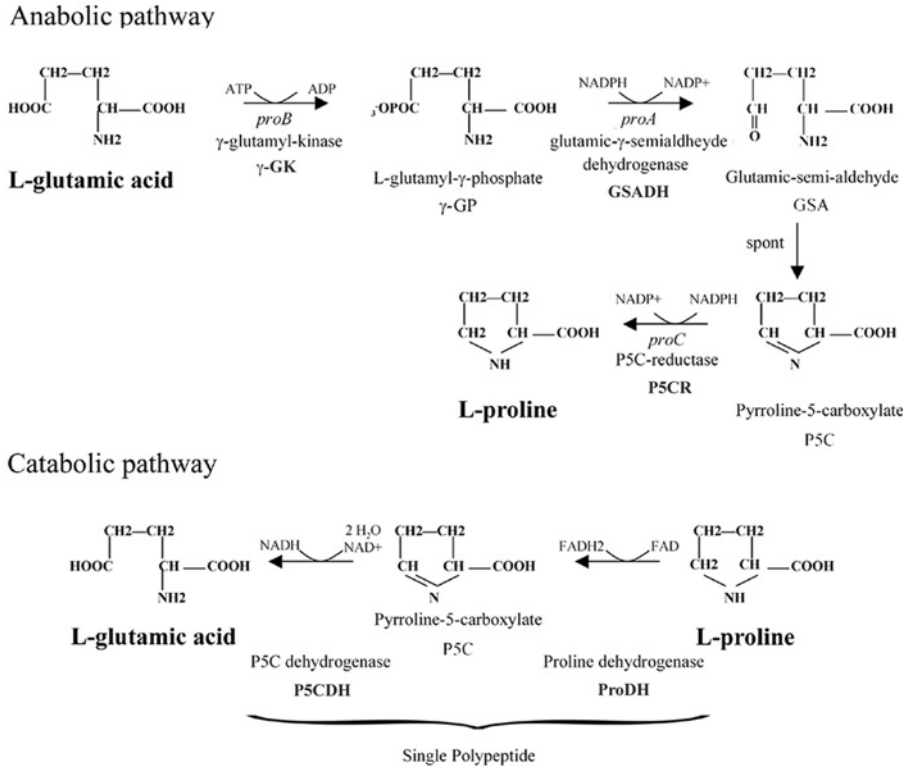

Fig. 1 Pathway of proline biosynthesis and degradation in bacteria.

functional complementation strategy where proline-deficient *E.coli* mutants (*proB, proA* and *proB*A, in Fig. 1) were transformed with a *Vigna aconitifolia* (mothbean) cDNA library (Hu et al. 1992). Surprisingly, all complementing clones harbored the same 2417 bp-long cDNA encoding a putative 73.2 kDa protein exhibiting similarity with both γ-GK and GSADH. A bioinformatic analysis performed on the deduced amino acid sequence, revealed 33.3% identity (55.3% similarity) in the amino terminal domain to the bacterial γ-GK, and a 35.7% identity (57.9% similarity) in the carboxy terminal domain to the GSADH. Both *proB*, and *proA* auxotrophic mutants could grow in proline-deficient media after transformation with this mothbean cDNA; in addition, enzymatic assays performed on the complementing mothbean protein expressed in a *proBA E.coli* mutant confirmed the bifunctional nature of this enzyme, which exhibited both γ-GK and GSADH activity in vitro (Hu et al. 1992). As in bacterial γ-GK, the γ-glutamyl-kinase domain of plant P5CS is subjected to feedback regulation by proline, although to a lesser extent compared to the bacterial enzyme (Hu et al. 1992). Genes encoding *P5CS* were subsequently isolated from others species, such as *Arabidopsis* (Savouré et al. 1995; Yoshiba et al. 1995), and rice (Igarashi et al. 1997). Despite these early reports suggest-

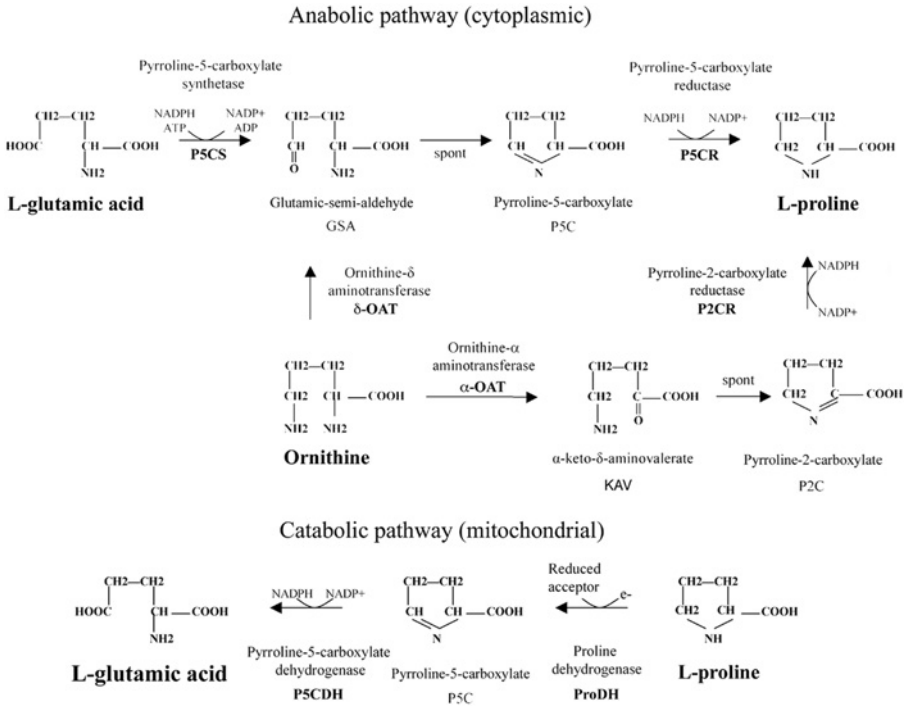

Fig. 2 Pathway of proline biosynthesis and degradation in higher plants.

ing the presence of a single gene coding for *P5CS,* two paralog *P5CS* genes (*P5CS1* and *P5CS2*) were later described in different species (Fujita et al. 1998; Ginzberg et al. 1998; Armengaud et al. 2004) including *Arabidopsis* (Strizhov et al. 1997). The two genes are highly homologous, sharing 82% of nucleotide sequence identity and coding for proteins with 94% identical amino acids, but are differentially regulated (Strizhov et al. 1997; Savouré et al. 1995; Ábrahám et al. 2003; Székely et al. 2008). It is not clear how widespread the duplication of *P5CS* is among plant species; when present, it seems that only one of the two genes responds to environmental stimuli and is involved in the plant response to abiotic stresses, particularly of osmotic type. In *Arabidopsis*, it is well established that *P5CS1* is induced by abscisic acid (ABA) (Yoshiba et al. 1995; Ábrahám et al. 2003) as well as by drought and salt stress (Savouré et al. 1995; Yoshiba et al. 1995), while the expression of *P5CS2* was observed in dividing cells in culture (Strizhov et al. 1997), in meristematic and reproductive tissues (Székely et al. 2008), and in response to biotic stress such as plant-pathogen incompatible interactions (Fabro et al. 2004). In *Medicago truncatula*, on the contrary, *P5CS2* seems involved in stress response, while *P5CS1* was found to be insensitive to stress but to accumulate specifically in reproductive tissues.

The subcellular localization of the P5CS proteins is generally believed to be cytosolic, consistent with the lack of recognizable motifs in their amino

acid sequence. Recently, however, fusions of the green fluorescence protein (GFP) with either P5CS1 or P5CS2 have been shown to localize not only in the cytoplasm but also in chloroplasts. In *Arabidopsis* embryo cells, the P5CS1 protein was found to segregate in subcellular bodies while P5CS2 was detected in the cytosol (Székely et al. 2008).

The last step of proline biosynthesis from glutamate is similar in both prokaryotes and eukaryotes, and consists in the reduction of P5C to proline catalyzed by P5CR (compare Figs. 1 and 2). This enzyme has been purified from different sources, including barley (Krueger et al. 1986), tobacco (LaRosa et al. 1991) and soybean (Chilson et al. 1991), and its biochemical properties have been found to be similar to those of bacterial P5CR. The first gene encoding plant P5CR was cloned by functional complementation of a proline auxotrophic *E.coli* mutant (*proC)* with an expression library from soybean (Delauney and Verma 1990), and showed 39% identity (52% similarity) to bacterial P5CR. The subcellular localization of plant P5CR was determined in soybean and tobacco, by means of antibodies raised against soybean P5CR, and found to be mainly cytosolic. About 15% of the activity, however, was detected in the plastid fraction (Skoze et al. 1992).

Compelling evidence indicates that P5CS is the rate-limiting enzyme of proline synthesis in plants. This evidence derives primarily from the tight correlation observed between the level of *P5CS* expression and proline accumulation, particularly during salt-stress conditions (Hu et al. 1992; Savouré et al. 1995; Yoshiba et al. 1995; Peng et al. 1996; Igarashi et al. 1997; Strizhov et al. 1997), and has been confirmed later by the analysis of plants overexpressing *P5CS* (Kavi Kishor et al. 1995), *P5CS*-antisense (Nanjo et al. 1999b) and *p5cs* mutants (Mattioli et al. 2008; Székely et al. 2008).

In contrast, P5CR is not rate-limiting (LaRosa et al. 1991; Skoze et al. 1992), and the expression of the *P5CR* gene is not correlated with proline accumulation, even under stress conditions (Yoshiba et al. 1995). Some authors, however, claim significant induction of *P5CR* expression upon stress (Verbruggen et al. 1993; Savouré et al. 1997).

2.1.2 The ornithine pathway. As shown in Fig. 2, the synthesis of proline in higher plants may proceed not only from glutamate but also from ornithine (Mestichelli et al. 1979; Adams and Frank 1980; Chiang and Dandekar 1995), which can be transaminated either to GSA by the enzyme δ-ornithine-aminotransferasi (δ-OAT), or to α-keto-δ-aminovalerate (KAV) by the enzyme ornithine α-aminotransferase (α-OAT; Adams and Frank 1980; Mestichelli et al. 1979). In the first biochemical route, GSA spontaneously cyclizes to P5C, which is converted into proline by P5CR. In the second pathway, KAV spontaneously cyclizes to pyrroline 2-carboxylate (P2C), which is converted into proline by P2C reductase (P2CR) (Fig. 1). Despite early reports claiming the prominence of α-OAT and 2PCR in the synthesis of proline from ornithine

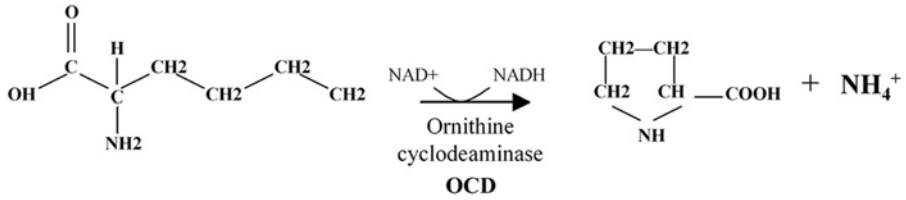

Fig. 3 The biosynthesis of proline from ornithine as catalyzed by ornithine-cyclodeaminase (OCD). OCD is an uncommon enzyme found in some eubacteria and archaea that catalyzes a direct NAD^+-dependent conversion of ornithine to proline. The *Agrobacteriun rhizogenes rolD* oncogene encodes an OCD (Trovato et al., 2001).

(Duranton and Wurtz 1965; Mestichelli et al. 1979), current evidence suggests that in plants, δ-OAT may be the only enzyme responsible for the conversion of ornithine into proline, casting doubts on the existence of the alternative P2C pathway. The transamination of ornithine to GSA has been characterized and the gene encoding δ-OAT has been cloned in mothbean (Delauney et al. 1993), *Arabidopsis* (Roosens et al. 1998), and *Medicago truncatula* (Armengaud et al. 2004). Analysis of δ-OAT in mothbean revealed low levels of expression under normal or stressed conditions, and high levels under supra-optimal nitrogen conditions. Since expression of *P5CS* shows an opposite pattern, the ornithine pathway is thought to be the main route of proline synthesis under excess nitrogen (Delauney et al. 1993). In *Medicago truncatula* (Armengaud et al. 2004), however, and in young *Arabidopsis* leaves (Roosens et al. 1998) induction of δ-OAT mRNA by osmotic stress has been reported, suggesting that both the glutamate and the ornithine pathways may contribute to proline accumulation under stress conditions.

The subcellular localization of δ-OAT has not been determined in plants, but the enzyme is thought to be mitochondrial because its N-terminal domain contains a putative mitochondrial transit peptide, and because its aminoacid sequence shares significant similarity with human OAT which has been localized in mithochondria (Inana et al. 1986).

A further, unusual pathway leading from ornithine to proline is the direct conversion of the former into the latter and ammonia (Fig. 3), catalyzed by the NAD^+-dependent enzyme ornithine cyclodeaminase (OCD). This reaction is active in plants infected by the soil bacterium *Agrobacterium rhizogenes* (for a review on *A. rhizogenes*, see Meyer et al. 2000) as well as in plants expressing the *A. rhizogenes* oncogene *rolD*, which encodes a functional OCD (Trovato et al. 2001).

2.2 Proline catabolism and the regulation of proline levels

In eukaryotes, the catabolism of proline takes place in mitochondria (Boggess et al. 1975; Rayapati and Stewart 1991) with the conversion of proline into

P5C – catalyzed by the enzyme proline dehydrogenase (ProDH) – which is further converted into glutamate by the enzyme pyrroline-5-carboxylate dehydrogenase (P5CDH) as shown in Fig. 2. In plants, ProDH has been localized in the inner mitochondrial membrane, while P5CDH has been found in the matrix (Elthon and Stewart 1981). In most bacteria, the single protein PUTA (Proline UTilization) catalyzes both steps of proline catabolism (Fig. 1).

The gene encoding *ProDH* has been independently isolated in *Arabidopsis* by differential screening of a cDNA library prepared from dehydrated plants (Kiyouse et al. 1996), by functional complementation of a Δ*put1* yeast lacking *ProDH* (Peng et al. 1996), and by PCR screening (Verbruggen et al. 1996). The deduced amino acid sequence of ProDH is very similar to other ProDH proteins, such as yeast PUT1 or Drosophila SLUGGISH-A, and its N-terminal sequence presents the characteristic feature of a mitochondrial signal peptide.

The only gene encoding a *bona fide* plant P5CDH has been cloned in *Arabidopsis* by functional complementation of a Δ*put2* yeast mutant, defective in *P5CDH* (Deuchle et al. 2001). The full-length cDNA encoded a protein with no significant similarity with the yeast P5CDH or any other known (non-plant) P5CDH. The *Arabidopsis* P5CDH has a putative mitochondrial signal peptide and a calculated molecular weight of 60.1 KDa, similar to the experimentally determined molecular mass of purified potato P5CDH (Forlani et al. 1997).

Plant cells are capable of rapidly accumulating proline and quickly degrading it when needed. As mentioned in the section on proline biosynthesis, during osmotic stress plants rapidly accumulate high levels of proline (for a review, see Delanauney and Verma 1993) following induction of the biosynthetic gene *P5CS* and downregulation of *ProDH* (Hu et al. 1992; Savouré et al. 1995; Yoshiba et al. 1995; Peng et al. 1996; Kiyosue et al. 1996; Verbruggen et al. 1996; Igarashi et al. 1997; Strizhov et al. 1997). When the stress is relieved, the concentration of proline rapidly drops (Kiyouse et al. 1996; Nakashima et al. 1998; Peng et al. 1996). Accordingly, the expression of the proline catabolic gene *ProDH* is downregulated by osmotic stress and upregulated by proline (Kiyouse et al. 1996; Peng et al. 1996; Verbruggen et al. 1996).

The reason for the quick drop in proline concentration when stress is relieved is that excess proline is toxic, due to its conversion product P5C that causes the production of reactive oxygen species (ROS) and induction of apoptosis and programmed cell death (PCD) when accumulated in excessive amounts (Hellmann et al. 2000; Székely et al. 2008).

Contrary to proline biosynthesis, which may follow different routes, the catabolic pathway involving ProDH and P5CDH seems to be the only way for plants to degrade excess proline. *Arabidopsis pdh* mutants (lacking a functional ProDH) are unable to eliminate excess exogenous proline (Nanjo et al. 2003). Consistently, *p5dh* mutants (lacking a functional P5CDH) are hypersensitive to exogenous proline, while plants overexpressing *P5CDH* have a decreased sensitivity to proline (Deuchle et al. 2001) as overproduction of

P5CDH increases the rate of degradation of P5C thus preventing cell death (Hellmann et al. 2000; Deuchle et al. 2001).

Fabro et al. (2004) recently reported accumulation of proline at or around the sites of hypersensitive reaction (HR) in plants infected by incompatible pathogens. 73% amino acid identity was found between the *Arabidopsis* P5CDH enzyme and the protein encoded by *LuFIS1*, a flax gene of unknown function upregulated during susceptible pathogen interactions (Ayliffe et al. 2002). This suggests that the flax protein is a P5CDH and that this enzyme is involved in hypersensitive response. A competition has been hypothesized at the site of pathogen attack between accumulation of proline that increases P5C concentration and triggers programmed cell death in the host plant (thus isolating the pathogen) and pathogen-induced upregulation of *P5CDH* to prevent P5C accumulation (Fabro et al. 2004; Skézely et al. 2008).

In addition to transcriptional control, an intriguing novel post-transcriptional regulatory mechanism mediated by a natural small interfering antisense RNA (nat-siRNA), has been recently found to fine-tune the levels of *P5CDH* mRNA (Borsani et al. 2005). A 24 bp-long nat-siRNA was detected in *Arabidopsis* under salt stress conditions and was shown to be correlated with *P5CDH* degradation. The generation of this nat-siRNA is driven by the formation of a complementary pair of transcripts between *P5CDH* and *SRO5* – a partially overlapping gene of unknown function – induced under salt stress, and has been found to be dependent on the proteins DICER-LIKE 2 (DCL2), RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) , SUPPRESSOR OF GENE SILENCING 3 (SGS3), and NUCLEAR RNA POLYMERASE D 1a (NRPD1A). The existence of this post-transcriptional level of regulation further demonstrates the importance of rapidly adjusting *P5CDH* levels to stress conditions, thus preventing accumulation of excess P5C in the cell. P5C is thought to induce apoptosis also in animal systems. Over-accumulation of proline caused by defects of ProDH, for example, leads to neuronal and behavioral disorders such as the ones observed in Drosophila defective in SLUGGISH-A (Hayward et al. 1993), and in mice bearing mutations in *ProDH* (Gogos et al. 1999). Excess P5C caused by overexpression of *ProDH* or P5C treatment, on the other hand, has been shown to induce apoptosis in human cell lines (Maxwell and Davis 2000).

3 The role of proline in osmotic stress tolerance

Osmotic stresses, such as drought, cold and salt stress, are caused by excessive accumulation of salt in the soil, either directly, because of salinization, or indirectly, because of water loss. As a consequence, the soil water potential progressively decreases, hampering and eventually halting the gradient of water flow from roots to apical shoot. The resulting osmotic stress may cause

stomatal closure, reduced photosynthesis rate, and growth inhibition. Another consequence of osmotic stress is the production of ROS and the accumulation of toxic ions, such as Na^+ or Cl^-, within the cell, causing severe damage to membrane structures, proteins, nucleic acids and lipids (for a review see, Apel and Hirt 2004).

A response to osmotic stress widespread in plants consists in the accumulation of compatible osmolytes – proline, glycinebetaine, prolinebetaine, glycerol, mannitol and sorbitol etc. – which are thought to protect cells against stress damage (for a review, see Hare and Cress 1997). These compounds are small uncharghed molecules highly soluble in water at physiological pH, which can therefore accumulate at high concentration in the cytosol of plant cells without causing any damage to cellular structures, because they are generally excluded from the hydration sphere of macromolecules (Low 1985). Once accumulated, compatible osmolytes are thought to reduce the cellular water potential below external values, driving (or preserving) water in the cell, thus maintaining turgor pressure high enough to sustain growth.

Among these plant compatible osmolytes (or plant protectants), proline is considered of major importance, as it has been reported to accumulate in a large number of species in response to stresses such as excess salinity, drought, cold, nutrient deficiency, heavy metals, pathogen infections and high acidity (for a review, see Hare and Cress 1997). In addition, high concentrations of proline have been observed in halophytic plants grown in saline environments (Briens and Larher 1982; Treichel 1986; Kant et al. 2006), in the root apical region of plants growing at low water potentials (Voetberg and Sharp 1991; Versules and Sharp 1999), in suspension cultures of plant cells adapted to water or NaCl stress (Handa et al. 1986; Rhodes et al. 1986) and in flowers and fruits of a large number of species under normal physiological conditions (Chiang and Dandekar 1995; Schwacke et al. 1999). Furthermore, the accumulation of proline under stress conditions seems an adaptative response common to organisms as diverse as eubacteria, marine invertebrate, protozoa, and algae (for a review, see Delauney and Verma 1993).

However, although a clear-cut correlation between proline accumulation and osmotolerance is well-established in bacteria (Csonka 1989), and despite the widespread occurrence of proline accumulation in plants subjected to osmotic stresses, it is not yet clear whether this accumulation would confer osmotolerance to plants as it does to bacteria.

A vast literature has been focusing over the years on the relation between proline and stress, but the existing data do not allow to clearly assess whether proline accumulation is a true adaptative response of plants to stress, or rather a secondary effect of it.

Numerous authors reported a positive correlation between proline accumulation and adaptation to stress (Rhodes et al. 1986; Chiang and Dandekar 1991; Khol et al. 1991; Kavi Kishor et al. 1995; Nanjo et al. 1999a; Hong

et al. 2000; Székely et al. 2008), while others found no significant correlation (Hanson et al. 1979; Bhaskaran et al. 1985; Chandler and Thorpe 1987; Moftah and Michel 1987; Liu and Zhu 1997; Maggio et al. 2002; Mani et al. 2002). Increased tolerance to osmotic stress, for example, has been reported by Kavi Kishor et al. (1995) in tobacco plants overexpressing mothbean *P5CS*, but in a later work the same group found no difference between the leaf osmotic potential of these transgenic plants and that of wild type controls (Blum et al. 1996). Later, Hong et al. (2000) found increased osmoprotection in tobacco plants transgenic for a mutated version of P5CS, lacking feedback inhibition, while Mani et al. (2002) observed that *pdh* Arabidopsis mutants accumulated proline without increasing osmotolerance.

An interesting paper by Kant et al. (2006) investigated the molecular basis of the salt resistance of the halophytic model species *Thellungella halophila,* and found a clear correlation between osmotolerance, increased proline levels, and tight control of Na^+ uptake (Kant et al. 2006). As a number of toxic ions, particularly Na^+ and Cl^-, tend to accumulate within the cell upon salt stress, it is crucial for the plant cell to maintain low levels of toxic Na+ ions in the cytoplasm, by pumping them out into the vacuole or outside the cell. The compartmentation of Na^+ is sustained by specific transporters, such as the plasma membrane SOS1 Na^+/H^+ antiporter (Shi et al. 2000) – whose unstable mRNA has been recently shown to be specifically stabilized during osmotic stress (Chung et al. 2008) – and it is accompanied by the accumulation of compatible osmolyte in the cytosol, to balance the osmotic potential of cytosol and vacuole.

A possible explanation to reconcile the contradictory reports and weak correlations between proline and osmotolerance in plants, compared to prokaryotic cells, may lie in the complex nature of osmotolerance and on the compartmentation of plant cells compared to bacteria.

Consistent with this idea, a cytosolic localization has been reported for P5CS1 in Arabidopsis mesophyll leaves under non-stress conditions, while under salt stress, P5CS1 appears translocated to and mainly detected in chloroplasts (Székely et al. 2008).

In addition to acting as a compatible osmolyte and stabilizing enzymes (Low 1985), membranes (Mansour 1998) and subcellular structures (Kandpal 1985), proline has been proposed to act as a scavenger of reactive oxygen species (ROS) by forming stable adducts with these latter (Floyd and Nagy 1984; Smirnoff and Cumbes 1989; Smirnoff 1993). Accordingly, *Arabidopsis p5cs1* knockout mutants have a severe reduction of stress-induced proline synthesis, are salt-stress hypersensitive, and exhibit ROS accumulation (Székely et al. 2008). However, according to Hare and Cress (1997) the amount of proline produced in response to stress might not be adequate for proline to function as an osmolyte and/or form adducts with ROS. These authors propose instead that the cycle of proline synthesis in the cytosol and proline oxydation in mitochon-

dria may provide a redox buffer system that assists specific metabolic pathways during and after osmotic stresses (Hare and Cress 1997). According to this hypothesis, proline accumulation may reduce stress-induced cellular acidification – thanks to the production of NADP+ (Fig. 2) – and support the oxidative pentose phosphate pathway – which is dependent on NADP+ and inhibited by NADPH – while during relief from stress proline oxidation may prime oxidative respiration providing energy to the cell during the phase of stress recovery: the oxidation of one molecule of proline yields 30 ATP equivalents (Atkinson 1977) and is therefore well suited to sustain high energy-requiring processes.

4 Signal transduction in proline metabolism

In spite of the extensive work on proline ever since this imino acid was first reported to accumulate in plants (Kemble and MacPherson 1954), little is still known on the regulation of its metabolism. Proline accumulation is generally believed not to be a primary response to stress and was found to be dependent on *de novo* protein synthesis (Stewart et al. 1986; Verbruggen et al. 1993), suggesting the existence of a signaling cascade controlling proline biosynthesis (Hare et al. 1999). The study of the regulation of the genes *P5CS* and *ProDH* has contributed to understanding important aspects of the upstream signaling cascade. It has been clearly shown, for example, that different signaling pathways regulate *P5CS1* upon cold and osmotic stress (Yoshiba et al. 1995; Igarashi et al. 1997; Hare et al. 1999). As of this latter, it was shown that ABA induces *P5CS1* (and probably *P5CS2*), but the analysis of the response of mutants defective in either ABA biosynthesis or ABA responsiveness suggested the existence of both ABA-dependent and ABA–independent signaling pathways (Yoshiba et al. 1995; Savouré et al. 1997; Igarashi et al. 1997; Strizhov et al. 1997; Ábrahám et al. 2003). Bioinformatic analysis of the *P5CS1* and *P5CS2* promoters showed the presence of cis-acting ABA responsive elements (ABRE) in the former but not in the latter (Hare et al. 1999), and failed to identify any dehydratation-responsive element (DRE/C) in either promoter (Hare et al. 1999).

A signaling cascade based on phospholipase–induced second messengers, has been recently proposed to mediate stress-induced proline synthesis (Knight et al. 1997; Thiery et al. 2004; Parre et al. 2007). In plants as in animals, extracellular stimuli can activate phospholipase C (PLC) that specifically hydrolyzes phosphatidylinositol 4,5-biphosphate to generate inositol 1,4,5-triphosphate (IP_3) and 1-2-diacylglycerol (DAG). These latter two molecules act as second messengers to release intracellular caged Ca^{++} and activate protein kinase C (PKC), respectively.

Recently, the use of the aminosteroid U73122 (a specific inhibitor of PLC) allowed the essential role played by an IP_3-dependent calcium signaling chain

in proline accumulation under salt stress (Parre et al. 2007) to be pinpointed: U73122 specifically inhibited proline accumulation in *Arabidopsis* seedlings treated with NaCl, but did not affect seedlings treated with mannitol. The PLC signaling pathway affecting proline biosynthesis was found to involve calcium as a second messenger, as 2-aminoethoxydiphenyl borate (an inhibitor of the IP_3-dependent calcium signaling pathway) specifically prevented accumulation of proline upon salt stress (Parre et al. 2007). A transient increase of free calcium concentration ($[Ca^{2+}]$cyt) upon osmotic stress has been detected in *Arabidopsis* seedlings overexpressing the calcium-sensing protein aequorin; this increase was specifically inhibited by calcium-chelators such as EGTA, or by calcium-channel blockers such as lanthanum or varapimil, suggesting the involvement of extracellular calcium in this response. However, since neither EGTA nor lanthanum could completely abolish the increase of $[Ca^{2+}]$cyt induced by osmotic stress, the release of additional Ca^{++} from intracellular stores has been hypothesized (Knight et al. 1997). Interestingly, also the expression of *P5CS1* and its induction upon osmotic stress were inhibited by EGTA, lanthanum and verapamil (Knight et al. 1997). This evidence points to a role of calcium signaling in the response of plant cells to stress and to *P5CS1* stress-induced expression. However, calcium alone was unable to induce *P5CS1* expression in the absence of stress suggesting that additional signaling factors may be required (Knight et al. 1997). Among these factors, phosphatidic acid (PA) has been suggested to play a major role in water stress signaling (Munnik 2001; Testerink 2004). Although PA may derive from the activity of PLC, phospholipase D (PLD) has been recently shown to be the major determinant in the generation of PA. In *Arabidopsis*, PLD consists of a family of heterogeneous enzymes which have been characterized and divided into six groups on the basis of sequence similarity and biochemical properties. All PLDs share the common property to hydrolyze membrane lipids to generate phosphatidic acid (PA) and a free-head group. The different classes of PLDs exhibit large differences in biochemical properties and requirements, particularly in terms of Ca^{2+} concentrations and substrate lipids, and are involved in different physiological processes (Wang 2005). Among these, a PLD, was shown to act as a negative regulator of proline biosynthesis in *Arabidopsis,* since 1-butanol (a specific inhibitor of PLD) can induce *P5CS1* expression and stimulate proline accumulation even in the absence of osmotic stress, thus enhancing osmo-tolerance (Thiery et al. 2004).

5 The role of proline in plant development

Apart from its well established roles in protein synthesis and in the response of plant cells to environmental stresses, it is increasingly evident that proline also plays a role in plant development, particularly in the reproductive phase of Arabidopsis.

The first hint of a possible role for proline in flowering and reproduction came from the measurements of proline content, which revealed strong accumulation of this imino acid in floral organs and siliques of different plant species under (unstressed) physiological conditions (Vansuyt et al. 1979; Venekamp and Koot 1988; Mutters et al. 1989; Walton et al. 1991; Chiang and Dandekar 1995; Schwacke et al. 1999). For example, Chiang and Dandekar (1995) reported that proline accumulates in *Arabidopsis* reproductive tissues to up to 26% of the total amino acid pool while in vegetative tissues represents only 1–3%. Schwacke et al. (1999) observed that the content of free proline in tomato flowers was 60-fold higher than in any other organ analyzed. Different authors reported upregulation of both proline biosynthesis (*P5CS, P5CR*) and transport genes (*ProT*) in reproductive tissues of different plant species (Verbruggen et al. 1993; Savouré et al. 1995; Schwacke et al. 1999), suggesting a possible role of proline in flower and reproductive development. Kavi Kishor et al. (1995) reported that constitutive overexpression of *P5CS1* in tobacco plants enhances flower development under drought-stress, while Nanjo et al. (1999a) showed that antisense expression of *P5CS1* inhibits bolting – a fast stem elongation occurring at flower transition – in *Arabidopsis*.

Very recently, Székely et al. (2008) analyzed *Arabidopsis* mutants defective in either *P5CS1* or *P5CS2* and found that *p5cs2* (but not *p5cs1*) mutants are embryo lethal and could be partially rescued by exogenous proline, pointing to an involvement of proline (and a specific role for *P5CS2*) in embryo development.

Although a role for proline in plant development is widely accepted, its precise role and mechanism of action is still a matter of debate. An obvious function of proline in development may be, at least in some cases, that of protecting developing cells from osmotic damage, especially in those developmental processes, such as pollen development and embryogenesis, in which tissues undergo spontaneous dehydratation. Similarly to the osmotic stress caused by environmental factors, the natural desiccation gradually achieved in some reproductive tissues may have deleterious effects on the cell machinery, which are likely to be counteracted by proline accumulation. Accordingly, higher levels of proline have been found (Chiang and Dandekar 1995) in tissues with relatively low water content (seeds and siliques) compared to tissues with high water content (rosette leaves) The correlation between proline accumulation and water content, however, is not very tight, – florets, for example, have been reported by the study of Chiang and Dandekar (1995), as the organs with the highest proline concentration – and leaves space for alternative roles of proline in plant development to be found.

In analogy with what has been mentioned in a previous section, the accumulation of proline in flowers may provide reducing equivalents to sustain mitochondrial oxidative phosphorylation and generate ATP equivalents to assist the rapid metabolic activities required during bolting and embryo devel-

opment. Other energy-requiring developmental processes where it has been pointed out that proline may play a role, include pollen tube elongation – in petunia and tomato proline has been found to be the most abundant amino acid and proposed to represent the main nitrogen and carbon source to support pollen tube elongation (Bathurst 1954; Zhang et al. 1982; Schwacke et al. 1999); maize root elongation – proline was found to accumulate specifically in the elongation zone of the maize root at low water potential (Voetberg and Sharp 1991; Verslues and Sharp 1999; Spollen et al. 2008); and root growth – proline has been proposed to sustain the fast elongation of hairy roots induced by *A. rhizogenes* (White et al. 1985; Trovato et al. 2001).

A positive correlation between proline and cell elongation, however, might also be explained in terms of protein synthesis, as hydroxyproline-rich glycoproteins (HRGPs, extensins, and arabinogalactan proteins), are important structural constituents of the plant cell wall thought to play a key role in the regulation of cell division, cell wall self assembly, and cell extension (Munoz et al. 1988; Showalter 1993; Majewska-Sawka 2000). Accordingly, an increase in the amount of proline could affect the rate of biosynthesis of hydroxyproline-rich glycoproteins and sustain physiological processes related to cell elongation, including bolting (Nanjio et al. 1999), pollen tube elongation (Zhang et al. 1982) and primary root elongation (Verslues and Sharp 1999). In support of this hypothesis, Nanjo et al. (1999) found decreased proline and hydroxyproline content in the cell wall protein fraction of antisense-P*5CS1* transgenic Arabidopsis impaired in bolting and flowering.

Proline has also been proposed to positively control flower transition in different species. *Arabidopsis* plants harboring a *35S-P5CS1* construct were found to flower earlier than wild type, and a peak of *P5CS1* overexpression and proline accumulation was shown to precede visible flower transition; in addition, *Arabidopsis p5cs1* knock-out mutants, containing a T-DNA insertion into *P5CS1* were shown to have reduced proline levels and to be late-flowering (Mattioli et al. 2008). Interestingly, *AtP5CS2* was found to be an early target of CONSTANS, a transcriptional activator involved in *Arabidopsis* flower transition (Samach et al. 2000). Ectopic expression of the *A. rhizogenes* oncogene *rolD*, encoding the unusual proline-biosynthetic enzyme ornithine-cyclodeaminase (Trovato et al. 2001), was found to induce early flowering in tobacco (Mauro et al. 1996) and in tomato (Bettini et al. 2003). An involvement of proline in flower transition was also suggested in *Sinapis alba* (Bernier et al. 1981) and kiwifruit (Walton et al. 1991).

Very recently, in a genome-wide screen aimed at discovering regulators of multiple stresses in *Arabidopsis*, the *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* gene was shown to be a central regulator of salt, osmotic and heat stress, suggesting a cross-talk between these processes and those regulated by the circadian clock like flower transition (Kant et al. 2008). In addition, *EARLY FLOWERING 4 (ELF4),* a circadian gene involved in coordinating the

switch from vegetative to reproductive growth, was also identified in the same screen as a key regulator of multiple stress responses, further corroborating functional correlation between stress and flowering. It is tempting to speculate that flowering and reproduction may be part of a survival strategy to escape stressful conditions.

It should be pointed out, however, that the accumulation of proline upon salt stress (or in the above described energy requiring developmental processes) leads to a relatively high concentration of the imino acid – up to 10-fold the normal concentration (Chiang and Dandekar 1995; Savouré et al. 1995), while the increase in proline concentration involved in flower transition is relatively modest – only up to 2–3fold (Mattioli et al. 2008), suggesting that in flower transition proline may act as a signal molecule.

The possible multiple roles of proline as an osmolyte (or as a source of energy, or as a ROS scavenger) at high concentration and as a signal molecule at low concentration, are reminiscent of the multiple roles of glucose that serves as a carbon and energy source, and has been shown to act also as a signaling molecule controlling cell growth and gene expression in a wide range of organisms including plants (Moreno et al. 2005; Cho et al. 2006). The glucose receptor hexokinase-1 discriminates between low and high concentrations of glucose, triggering growth at low concentration and growth repression at high concentration (Moreno et al. 2005).

Proline has been shown to be able to induce gene expression (Hellmann et al. 2000), and a role for proline as a signal would also be consistent with the role played by other amino acids in regulating gene expression and in signal transduction (for a review, see Fafournoux et al. 2000). In flower transition, proline may act as a signal coupling metabolic status to cell growth, in analogy to the role played by other amino acids in the TOR (target of rapamycin) pathway in yeast (Beck and Hall 1999; Dann and Thomas 2006), or in the mTOR pathway in mammals (Beugnet et al. 2003; Dann and Thomas 2006). The existence of a TOR kinase pathway has been recently demonstrated also in plants, where it appears to respond not only to nutrient availability, but also to osmotic stress (Mahfouz et al. 2006). Accordingly, the modulation of intracellular proline may be perceived by the plant as a signal to trigger adaptative responses such as flower setting.

6 Summary and conclusions

In plants, proline is involved in the response to numerous environmental stresses and in different developmental processes. The accumulation of proline in response to different stresses is a well-established fact, and different roles for proline as an osmolyte and/or as an energy source and/or as an ROS scavenger have been proposed. However, none of these roles has been incontrovertibly

demonstrated to date, and the very (adaptative) significance of proline accumulation has been questioned. An involvement of proline in flower transition, in flower and embryo development and in other developmental processes has been pinpointed: in flower transition proline may act as a signal molecule, while during flower, embryo and other developmental processes it may support the energetic needs of rapidly dividing or elongating cells.

Numerous questions related to proline remain still unanswered. A particularly exciting area of future work will be dissecting the signaling cascades in the stress responses and developmental processes proline has been associated with, and identifying and elucidating any possible cross-talk.

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