Proline Accumulation in Plants: Roles in Stress Tolerance and Plant Development

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Abstract

Proline accumulation occurs in a wide range of plant species in response to various kinds of environmental stresses. A large body of evidence suggests that a positive correlation occurs between proline accumulation and plant stress tolerance. In this chapter, we will discuss the metabolism of proline accumulation and its role in stress tolerance in plants. Existing literature indicates that despite acting as an osmolyte, proline also plays important roles during stress as a metal chelator and an antioxidative defence molecule. Moreover, when applied exogenously at low concentrations, proline enhanced stress tolerance in plants. However, some reports point out adverse effects of proline when applied at higher doses. Role of proline in seed germination, flowering and other developmental programmes is also presented in this chapter.

9.1 Introduction

Plants are subjected to various kinds of abiotic and biotic stresses throughout their life cycles which include salinity, drought, temperature extremes, heavy metal, infection by pathogens, nutrient deficiency and UV radiation (Hare and Cress 1997; Saradhi et al. 1995; Siripornadulsil et al. 2002). A general response of plants to various kinds of stresses is the accumulation of compatible osmolytes such as proline, glycine betaine, proline betaine, glycerol, mannitol and sorbitol, etc. which protect cells against damage caused by stress (Hare and Cress 1997). These small uncharged molecules at physiological pH are highly soluble in water, which allows them to accumulate at

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elevated concentration in the cytosol of plant cells without harming cellular structures (Low 1985; Trovato et al. 2008). These osmolytes help plants to cope up with stress conditions by adjusting cellular osmotic pressure, contributing in ROS detoxification, protection of membrane integrity and stabilization of enzymes/proteins (Ashraf and Foolad 2007; Bohnert and Jensen 1996; Hayat et al. 2012). Among them, proline plays a pivotal role (Hare and Cress 1997) and accumulates in a large number of species under salinity (Yoshiba et al. 1995), drought (Choudhary et al. 2005), cold, nutrient deficiency (Hare and Cress 1997), heavy metals (Schat et al. 1997), pathogen attack (Fabro et al. 2004; Haudecoeur et al. 2009; Sreedevi et al. 2013; Rehman et al. 2014) and high acidity (Hare and Cress 1997). Proline is a proteinogenic amino acid with the α-amino group present as a secondary amine and is essential for primary metabolism (Szabados and Savoure 2009; Verslues and Sharma 2010). Different kinds of organisms including protozoa, eubacteria, marine invertebrates and also various plant species under stress conditions have been found to accumulate the amino acid proline (Kaneshiro et al. 1969; Poulin et al. 1987; Csonka 1989; Burton 1991; Brown and Hellebust 1978; Verbruggen et al. 1993; Yoshiba et al. 1995; Peng et al. 1996; Nakashima et al. 1998; Mattioli et al. 2008).

9.2 Proline Metabolism

Intracellular proline levels in plants are governed by biosynthesis, catabolism and transport between cells and different compartments of cell (Szabados and Savoure 2009). In higher plants, proline biosynthesis occurs either via the glutamate or the ornithine pathway. The former involves the synthesis of proline from glutamic acid via intermediate pyrroline-5-carboxylate (P5C) in the cytoplasm or chloroplast and is the key biochemical pathway under physiological conditions and under nitrogen insufficiency or osmotic stress (Delanauney and Verma 1993; Trovato et al. 2008; Verslues and Sharma 2010). The biosynthesis of proline from this pathway was first found in bacteria (Leisinger 1987) and subsequently in numerous prokaryotic and eukaryotic organisms (Krishna and Leisinger 1979; Adams and Frank 1980; Csonka 1989). The core enzymes in this reaction are pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) (Sekhar et al. 2007; Fig. 9.1). P5CS is encoded by two genes, whilst P5CR is encoded by only one in the majority of plant species (Strizhov et al. 1997; Armengaud et al. 2004; Verbruggen et al. 1993). In another pathway, proline synthesis occurs via deamination of ornithine which is transaminated to P5C by ornithine-delta-aminotransferase (OAT) (Verbruggen and Hermans 2008; Fig. 9.1). It has been reported that the ornithine pathway occurs during supra-optimal nitrogen conditions and seedling development (Roosens et al. 1998; Armengaud et al. 2004).

Plant cells have a potential to accumulate proline speedily and degrade it quickly when needed (Trovato et al. 2008). During proline biosynthesis, conversion product P5C may cause the production of reactive oxygen species (ROS) and induction of apoptosis and consequently a programmed cell death when undue amounts are accumulated (Hellmann et al. 2000; Székely et al. 2008). Therefore, plants need to degrade it quickly as soon as the stress is relieved. Proline catabolism occurs in mitochondria via the incessant action of proline dehydrogenase (PDH) or proline oxidase (POX) producing P5C from proline and P5C dehydrogenase (P5CDH), which converts P5C to glutamate (Szabados and Savoure 2009; Fig. 9.1). PDH is encoded by two genes, whereas only one P5CDH gene is known in *Arabidopsis* and tobacco (*Nicotiana tabacum*) (Kiyosue et al. 1996; Verbruggen et al. 1996; Ribarits et al. 2007). The interconversion of proline and glutamate is sometimes known as the “proline cycle” (Verslues and Sharma 2010). Unlike proline biosynthesis, the catabolic pathway involving PDH and P5CDH seems to be the only route for plants to degrade excess proline. *Arabidopsis pdh* mutants (lacking a functional PDH) are not capable of eliminating excess proline when supplied exogenously (Nanjo et al. 2003). Similarly, *p5cdh* mutants (lacking a functional P5CDH) are hypersensitive to exoge-

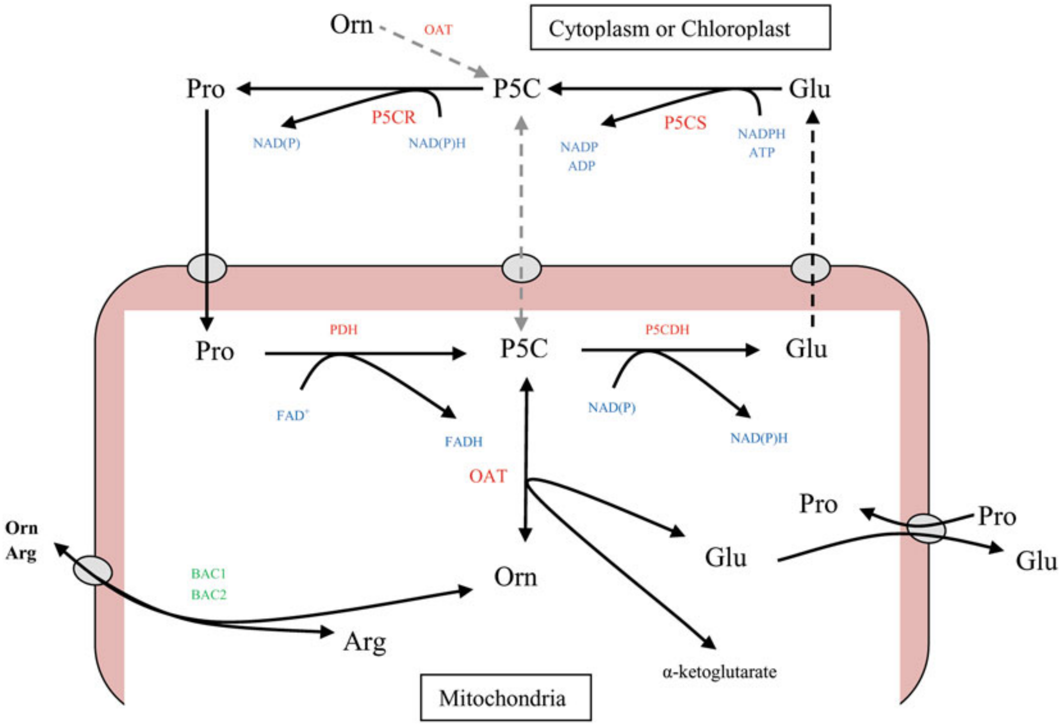

Fig. 9.1 The core pathways of proline metabolism in higher plants Pro biosynthesis from Glu occurs in the cytoplasm and/or chloroplast via two enzymatic steps. Pro catabolism to Glu occurs in the mitochondria also by two enzymatic steps. Both Pro biosynthesis and catabolism use common intermediate P5C (formed by spontaneous cyclization of glutamic-5-semialdehyde produced by P5CS or PDH). *Solid lines* represent known metabolic or transport steps, whilst *dashed lines* represent proposed but not demonstrated steps. Carriers BAC1 and BAC2 may also influence proline metabolism by movement of Arg or Orn. OAT had been proposed to be a cytoplasmic enzyme that functioned as an alternative route to proline, but recent evidence has placed it in the mitochondria. Arg, arginine; BAC, basic amino acid carrier; Glu, glutamate; Orn, ornithine; Pro, proline (Adapted from Verslues and Sharma (2010)

nously supplied proline, whilst plants overexpressing *P5CDH* are less sensitive to proline (Deuschle et al. 2001) as overproduction of P5CDH boosts the rate of degradation of P5C thus thwarting cell death (Hellmann et al. 2000; Deuschle et al. 2001).

Depending upon environmental conditions, proline can be synthesized in different subcellular compartments (Szabados and Savoure 2009). Housekeeping biosynthesis of proline takes place in the cytosol, and in *Arabidopsis* it is controlled by the *P5CS2* gene (Székely et al. 2008; Szabados and Savoure 2009), which is active in dividing meristematic tissues, such as shoot and root tips, and inflorescences (Madan et al. 1995; Deuschle et al. 2001; Tripathi and Gaur 2004). Both P5CS genes are active in floral shoot apical meristems and contribute proline for flower development (Csonka and Hanson 1991). Proline synthesis in chloroplasts is controlled by the stress-induced *P5CS1* gene in *Arabidopsis* (Székely et al. 2008; Savoure et al. 1995; Strizhov et al. 1997; Szabados and Savoure 2009) (Fig. 9.1).

9.3 Role in Stress Tolerance

Although plants exhibit species-specific differences in proline accumulation, there have been mixed results regarding the relationship between proline accumulation and stress tolerance in plants. In some cases, proline accumulation was found higher in stress-tolerant than in stress-sensitive plants. For example, in the roots of salt-tolerant alfalfa plants, proline accumulation increased dramatically under saline stress, whilst salt-sensitive plants responded slowly (Fougere et al. 1991; Petrusa and Winicov 1997). Similarly, salt-tolerant ecotypes of *Agrostis stolonifera* concentrated more proline under salt-stressed conditions than salt-sensitive ecotypes (Ahmad et al. 1981). Significant enhancement in salt tolerance was also found at the cellular level in some plant species. For example, in vitro studies with *Brassica juncea* revealed that elevated levels of free proline were found in salt-tolerant calli under salt stress compared with non-stressed calli (Madan et al. 1995; Gangopadhyay et al. 1997). Similarly, salt-adapted calli of *Citrus limon* had considerably greater proline concentration compared with salt-sensitive calli (Piqueras et al. 1996).

Proline accumulation also occurs in plants in response to drought stress. For example, water deficit rice plants accumulated high amounts of proline in leaves (Hsu et al. 2003) which was attributed to improved contents of the precursors for proline biosynthesis, including glutamic acid, ornithine and arginine. In the case of wheat, rate of proline accumulation and utilization was also considerably higher in the drought-tolerant cultivar than drought-sensitive cultivar under the conditions of drought stress (Nayyar and Walia 2003). Moreover, activities of proline biosynthetic enzymes P5CR and OAT increased primarily in tolerant lines in *B. juncea* plants grown under stress environment; however, the activity of enzyme which degrades proline “proline oxidase” decreased in all lines (Madan et al. 1995).

Drought, cold and salt stress causes excessive accumulation of salts in the soil which directly (because of salinization) or indirectly (because of water loss) results in osmotic stress (Trovato et al. 2008). As a result, the soil water potential gradually decreases, which hinders and finally stops flow of water gradient from roots to apical shoot. The resulting osmotic stress causes closure of stomata, reduction in photosynthesis rate and growth inhibition (Trovato et al. 2008). Within the cell, osmotic stress results in the production of ROS and the accumulation of toxic ions such as Na^+ or Cl^-, which can severely damage the membrane structures and other cell components (Apel and Hirt 2004). To maintain low levels of Na^+ ions in the cytoplasm, plants possess specific transporters such as the plasma membrane SOS1 Na^+/H^+ antiporter which pump out Na^+ ions into the vacuole or outside the cell (Shi et al. 2000). The mRNA of this antiporter has been shown to be specifically stabilized during osmotic stress (Chung et al. 2008) and is escorted by the accumulation of compatible solutes in the cytosol, to balance the osmotic potential of cytosol and vacuole. This was further supported by the cytosolic localization of P5CS1 in *Arabidopsis* mesophyll leaves under non-stress conditions, whilst under salt stress, P5CS1 is translocated to and mainly spotted in chloroplasts (Székely et al. 2008).

Moreover, the rate of the Calvin cycle is reduced during stress conditions, preventing oxidation of NADPH and restoration of $NADP^+$. The flow of electrons in the electron transport chain gets suppressed because of inadequate electron acceptor $NADP^+$, which leads to produce singlet oxygen in the PSI reaction centre and results in the accumulation of ROS (Chaves et al. 2009). Proline biosynthesis is a reductive pathway and uses NADPH to reduce glutamate to P5C and P5C to proline and generates $NADP^+$ that can be further used as electron acceptor in the oxidative pentose phosphate pathway (Trovato et al. 2008). Furthermore, during the phosphorylation of glutamate, ADP is produced from ATP. An increasing rate of proline biosynthesis in chloroplasts during stress conditions causes to maintain the low $NADPH/NADP^+$ ratio, which results to continue the flow of electrons between photosynthetic excitation centres, stabilizes the redox balance, and protects an important photosynthetic apparatus from photoinhibition (Hare and Cress 1997). During the time of stress recovery, proline oxidation leads to oxidative respiration which provides energy to the cell: the oxidation of one molecule of proline capitulates 30 ATP equivalents (Atkinson 1977) and is therefore well suitable to keep up high-energy-requiring processes (Trovato et al. 2008). The important role of proline biosynthesis for maintaining $NADP^+$ pools during stress conditions was further confirmed when in transgenic soybean plants, inhibition of proline biosynthesis and NADPH-NADP$^+$ conversion by antisense P5CR led to drought hypersensitivity, whilst overexpression of P5CR resulted in drought tolerance (De Ronde et al. 2004).

In mitochondria, proline performs distinctive protective functions. After relief from stress, proline pools contribute a reducing potential for mitochondria through the oxidation of proline by PDH and P5CDH, providing electrons for the respiratory chain and consequently supplying energy to resume growth (Hare and Cress 1997; Kishor et al. 2005). Proline stabilizes mitochondrial respiration by protecting Complex II of the mitochondrial electron transport chain during salt stress (Hamilton and Heckathorn 2001). The P5C-proline cycle discovered in recent times can send electrons to the electron transport chain of mitochondria without producing glutamate and, under some conditions, can cause generation of more ROS in the mitochondria (Miller et al. 2009). Thus, proline catabolism plays a key role in regulating cellular ROS balance and can also control various other regulatory pathways (Hayat et al. 2012).

Proline accumulation has also been suggested to activate alternative detoxification pathway by protecting and stabilizing ROS scavenging enzymes. In salt-stressed tobacco cells, proline has been shown to increase the activities of methylglyoxal detoxification enzymes; enhance peroxidase, glutathione-S-transferase, superoxide dismutase and catalase activities; and increase the glutathione redox state (Hoque et al. 2008; Islam et al. 2010). Accumulation of P5CS1 and P5CR in chloroplasts during the conditions of salt stress indicates that under such adverse conditions, glutamate-derived proline biosynthesis is elevated in plastids, where photosynthesis takes place (Székely et al. 2008).

However, the correlation between proline accumulation and abiotic stress tolerance in plants is not always clear. Accumulation of proline in the leaves was believed to be a symptom of salt injury rather than a sign of salt tolerance in rice plants grown under salinity (Lutts et al. 1999). Similarly, assessment of proline accumulation and distribution in two sorghum genotypes differing in salt tolerance indicated that proline accumulation occurred due to salt stress and not because of salt tolerance (de-Lacerda et al. 2003).

9.4 Response of Plants to Exogenously Applied Proline

A large body of evidence suggested that exogenous application of proline to plants can also increase their stress tolerance. For example, exogenously applied proline provided osmoprotection and promoted growth in various plant species grown under salt-stressed conditions (Csonka and Hanson 1991; Yancey 1994). Exogenous application of proline (30 mM) on salt-stressed rice seedlings neutralized the adverse effects of salinity on the growth of premature plants (Roy et al. 1993). Ali et al. (2007) reported that the exogenous proline sprayed at seedling and/or at vegetative stage of *Zea mays* enhanced its growth under the conditions of water deficiency conditions.

Application of exogenous proline protected cell membranes from salt-induced oxidative stress by increasing activities of different antioxidative enzymes (Yan et al. 2000). Exogenous application of 10 mM proline has been reported to facilitate the growth of salt-stressed tobacco suspension cells (Okuma et al. 2000). Exogenous application of proline enhanced the activities of superoxide dismutase and peroxidase in soybean cell cultures maintained under salinity (Yan et al. 2000; Hua and Guo 2002). The increase in the activities of superoxide dismutase and peroxidase is believed to contribute increased salt tolerance in plants (Yan et al. 2000; Hua and Guo 2002). Kaul et al. (2008) using in vitro studies showed that exogenously applied l-proline proved to be an effective free radical (particularly ROS) scavenger. Hong et al. (2000) suggested that the role of proline as a free radical scavenger in relieving stress is more appreciable than its role as an osmolyte.

Under the conditions of trace metal stress, proline accumulation induces the formation of phytochelatins which chelate with metals like cadmium (Cd), thus alleviating metal toxicity (De Knecht et al. 1994). Xu et al. (2009) found that proline treatment caused reduction in ROS level and protected the membrane integrity of callus cells of *Solanum nigrum* when exposed to high concentrations of Cd, eventually regenerating its shoots. Exogenously applied proline proved to protect the activity of glucose-6-phosphate dehydrogenase and nitrate reductase against inhibition by Cd and Zn (Sharma et al. 1998). This protection was due to the formation of a proline-metal complex (Sharma et al. 1998). Similar complex-forming characters of proline were reported by Farago and Mullen (1979) where complex formation occurred between proline and Cu in metal-tolerant *Armeria maritima*. Proline pretreatment also minimized the toxicity of Hg^{2+} in rice (*Oryza sativa*) by scavenging ROS, such as H_2O_2 (Doke 1997).

Despite the fact that exogenous application of proline to plants subjected to abiotic stresses usually prevents or recuperates the deleterious effects caused by stress, on the other hand, application of high concentrations of proline to plants may inhibit their growth or may have detrimental effects on cellular metabolisms of plants (Ehsanpour and Fatahian 2003; Nanjo et al. 2003). For example, exogenous application of proline caused damage to ultrastructures of chloroplast and mitochondria in *Arabidopsis* plants (Hare et al. 2002), which may subsequently cause a significant increase in ROS in these organelles resulting a significant decrease in photosynthetic electron acceptor pools (Hare et al. 2002). Thus, the broadly accepted hypothesis that proline as an inert compatible solute is accumulated to high levels with least effects on cellular metabolism was questioned (Hare et al. 2002). Application of 20–33 Mm proline to cell cultures of *Vigna radiata* under salt (NaCl) stress alleviated the adverse effects, but concentrations of 50 mM or above proved inhibitory for the growth of both salt-stressed and non-stressed cell cultures (Kumar and Sharma 1989). In this study, contents of Na^+ and Cl^- declined in cells when the concentration of proline reached optimal, but they started increasing with further addition in proline concentration. Similarly, Roy et al. in 1993 determined in rice that 30 mM proline was the most helpful concentration in improving germination and seedling growth under saline conditions, whilst higher concentrations (40 or 50 mM) caused reduced seedling growth and lowered K^+/Na^+ ratio in the leaves. Thus, despite of its much accepted positive role, the toxic effects of proline at high concentrations may cause problems (Ashraf and Foolad 2007). From the above examples, it is therefore very important to determine optimal concentrations of exogenous proline when using as a tolerance-inducing agent.

9.5 Role in Flowering and Development

An increasing body of evidence indicates that proline is also involved in flowering and development (Mauro et al. 1996; Nanjo et al. 1999; Samach et al. 2000; Trovato et al. 2001; Székely et al. 2008; Mattioli et al. 2008, 2009; Lehmann et al. 2010), and it is suggested that the developmental role of proline is not coupled with its role in stress response (Mattioli et al. 2008; Trovato et al. 2008). Existing literature suggests that proline may play a role in flowering and development both as a metabolite and as a signal molecule (Mattioli et al. 2009).

Accumulation of proline in undeveloped seeds of *Vicia faba* indicates that proline might be playing an important role in the development of generative organs (Venekamp and Koot 1984). Numerous studies reported high-proline contents in *Arabidopsis* seeds (Chiang and Dandekar 1995; Schmidt et al. 2007), although data from other plant species are meagre. However, the genes which encode enzymes of proline metabolism are expressed during seed development of different species of plants, including *Arabidopsis*, *Solanum lycopersicum*, *Medicago truncatula* and *Oryza sativa* (Verbruggen et al. 1993; Fujita et al. 1998; Armengaud et al. 2004; Hur et al. 2004). The antisense repression of *AtP5CS1* postponed the emergence of radicle during the seed germination of *Arabidopsis*, indicating that proline synthesis restores the NADP$^+$ pool and consequently activates the oxidative pentose phosphate pathway (Hare et al. 2003).

Various scientists also reported considerable levels of proline in the reproductive organs of different plant species (Chiang and Dandekar 1995; Schwacke et al. 1999; Mattioli et al. 2012), which creates the possibility that this amino acid could be also accumulated under non-stressed conditions for developmental functions. For example, proline accounts about 26 % of the total amino acid pool in the reproductive tissues such as florets, pollen, siliques and seeds of *Arabidopsis*, whilst in the case of vegetative tissues, it only represents 1–3 % (Chiang and Dandekar 1995). Schwacke et al. (1999) analysed various organs of tomato for proline accumulation and found that the amount of this amino acid in tomato flowers was 60 times higher than other organs. Similar physiological accumulations of free proline have been reported at different concentrations, in reproductive organs of other plant species (Chiang and Dandekar 1995), and in most cases the amount of this amino acid is higher than required demand of protein synthesis. Mattioli et al. (2012) found that the development of the male gametophyte in *P5CS1* and *P5CS2* mutants of *Arabidopsis thaliana* was severely compromised, thereby providing genetic evidence that proline is required for pollen development and fertility.

The findings of Mattioli et al. (2008, 2009) raised the possibility that low proline levels contained in apical meristems may signal the most favourable conditions for the plant to flower, whilst higher levels of proline might be read as a stress signal by the plant and induce adaptive responses. In a similar way, low concentration of glucose prompted growth stimulation in yeast whilst at high concentration suppressed the growth (Cho et al. 2006).

The possible role of proline in flower and reproductive development is supported by upregulation of genes involved in proline biosynthesis (*P5CS*, *P5CR*) and transport (*PT*) in reproductive tissues of various plant species (Verbruggen et al. 1993; Savoure et al. 1995; Schwacke et al. 1999). It has been reported that constitutive overexpression of *P5CS1* caused early flowering in transgenic plants under normal (Mattioli et al. 2008) or salt stress conditions (Kishor et al. 1995), whilst Nanjo et al. (1999) found that decreased expression of *P5CS1* in antisense *Arabidopsis* plants inhibits bolting and delayed flowering. Besides bolting, there are other developmental processes in which proline is suggested to play a role, including elongation of pollen tube. In petunia and tomato, the amino acid proline has been found in abundance and suggested as the key nitrogen and carbon source for carrying pollen tube elongation (Zhang et al. 1982; Schwacke et al. 1999), the elongation of the hairy roots in dicotyledonous plants infected by *Agrobacterium rhizogenes* (Trovato et al. 2001) and the elongation of the maize primary root in which proline was found to accumulate particularly in the elongation zone of the maize root at low water potential (Voetberg and Sharp 1991; Spollen et al. 2008), indicating that proline might be generally used by the plant cell in developmental processes involving rapid cell growth as an energy source (Mattioli et al. 2009).

A positive correlation between proline and cell elongation can also be described in terms of protein synthesis, as hydroxyproline-rich glycoproteins (HRGPs, extensins and arabinogalactan proteins) are important structural components of the plant cell wall which play an important role in the regulation of cell division, cell wall self-assembly and cell extension (Snowalter 1993; Majewska-Sawka and Nothnagel 2000). Nanjo et al. (1999) found declined proline and hydroxyproline levels in the cell wall protein fraction of antisense *P5CS1*transgenic *Arabidopsis* impaired in bolting, further supporting the hypothesis that proline content and cell elongation in plants are positively related with each other.

Although the accumulation of proline in reproductive organs has been frequently reported in various plant species, its exact role is still not clear. It is proposed that proline protects developing cells from osmotic damages, especially during pollen development and embryogenesis where tissues experience spontaneous water loss (Trovato et al. 2008; Mattioli et al. 2009). Similarly, the desiccation process that occurs in reproductive tissues due to osmotic stress caused by environmental factors may critically damage the plant cell, and it is likely to be thwarted by proline accumulation (Trovato et al. 2008). Accordingly, higher levels of proline have been found (Chiang and Dandekar 1995) in tissues with low water content as compared to tissues with high water content. The correlation between proline accumulation and water content is not always tight. Florets, for example, have been depicted by Chiang and Dandekar (1995) as the organs with the highest proline content, in spite of their quite high water content.

The oxidation of a single molecule of proline provides 30 ATP equivalents (Atkinson 1977), making this amino acid well fit for sustaining high-energy-requiring processes. Therefore, it is believed that proline has a major role in sustaining the plant reproduction, a highly metabolically requiring process, by providing energy (Micheu et al. 2000).

9.6 Conclusion

Proline accumulation in response to various kinds of environmental stresses and in some developmental processes is a well-established fact, and its role in such processes has been confirmed by various transgenic approaches. Various kinds of functions for proline as an osmolyte and/or as an energy source and/or as an ROS scavenger and/or stress reliever have been proposed. However, the very (adaptative) function of proline accumulation has been questioned. The balance between proline biosynthesis and its degradation is suggested to be very important in determining its osmoprotective and developmental role. When proline was applied exogenously to plants exposed to stress environment, it resulted in improved growth and other physiological attributes of plants. Exogenously applied proline scavenges the ROS generated in plants subjected to various environmental stresses. Low doses of exogenous proline proved to protect plants from salinity, drought, heavy metal and temperature stress. However, higher concentrations may cause toxicity.

An involvement of proline in developmental processes, viz. seed germination, flower transition, flower development and other developmental processes, has been recognized. Proline may act as a signal molecule during flower transition, whilst during flowering and other developmental processes it provides energetic needs for rapidly dividing or elongating cells.

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