

Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: Its implications in plant growth and abiotic stress tolerance

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Dramatic accumulation of proline due to increased synthesis and decreased degradation under a variety of stress conditions such as salt, drought and metal has been documented in many plants. Similarly, a decrease in the level of accumulated proline in the rehydrated plants is due to both down regulation of proline biosynthetic pathway enzymes and upregulation of proline degrading enzymes. But, the role of proline during plant development and the molecular basis of the effect of proline accumulation during stress and upon relief of stress are still largely obscure. Here, we summarize the genes governing the proline biosynthetic pathway, its degradation and regulation. Sequentially, we provide an account on transgenics raised so far to engineer the overproduction of osmolyte proline. Also, the identification of specific cellular pathways involved in proline biosynthesis and metabolic changes occurring in transgenic plants developed for proline enhancements are discussed. Further, emphasis is also made on an untouched area of signal transduction of proline biosynthetic pathway.

UNDERSTANDING the molecular responses of plants exposed to different abiotic stresses is of much importance as they give hope for genetically modifying crops to cope with these stresses in a better way. There are many cellular mechanisms by which organisms ameliorate the effects of environmental stresses; for instance, accumulation of compatible osmolytes such as proline is one such phenomenon. The accumulation of free proline has been studied in a number of taxa subjected to hyperosmotic stress conditions for over 45 years. The accumulation of proline under abiotic stress conditions accounts for few millimolar concentrations, depending on the species and the extent of stress^{1,2}. Very high accumulation of cellular proline (up to 80% of the amino acid pool under stress and 5% under normal conditions) due to increased synthesis and de-

creased degradation under a variety of stress conditions such as salt and drought has been documented in many plant species¹⁻⁷. In Arabidopsis, proline can account for up to 20% of the free amino acid pool after sodium chloride stress. Although proline is known to confer osmotic tolerance during stress conditions, its specific role during plant growth is not completely clear. The biosynthetic pathway of proline diverged between bacteria and plant systems^{8,9}. Genes encoding most of the enzymes associated with the synthesis and degradation of proline were cloned and partially characterized, but the factors regulating the expression of these enzymes are largely unidentified. In the last decade, several attempts were made to increase the level of proline accumulation in plants by transferring the genes associated with the biosynthetic pathway. Tolerance to abiotic stress, especially to salt and improved plant growth, was observed in a variety of transgenics that were engineered for overproduction of proline¹⁰⁻¹⁵. Proline seems to have diverse roles under osmotic stress conditions, such as stabilization of proteins, membranes and subcellular structures¹⁶, and protecting cellular functions by scavenging reactive oxygen species¹⁷. Here, we discuss the advances made in recent years about the cellular pathways involved in proline biosynthesis and its regulation. Metabolic engineering of plants for proline overproduction and stress tolerance is also reviewed and the impact of higher accumulation of proline on primary metabolism during plant development and stress tolerance is critically evaluated from transgenic studies. We further deal with an important aspect of signalling events associated with proline accumulation in plants.

Genes that encode proline biosynthetic pathway enzymes

The role of proline and sulphur metabolism during osmotic stress tolerance in plants has been emphasized recently¹⁸. Accumulation of proline could be due to *de novo* synthesis

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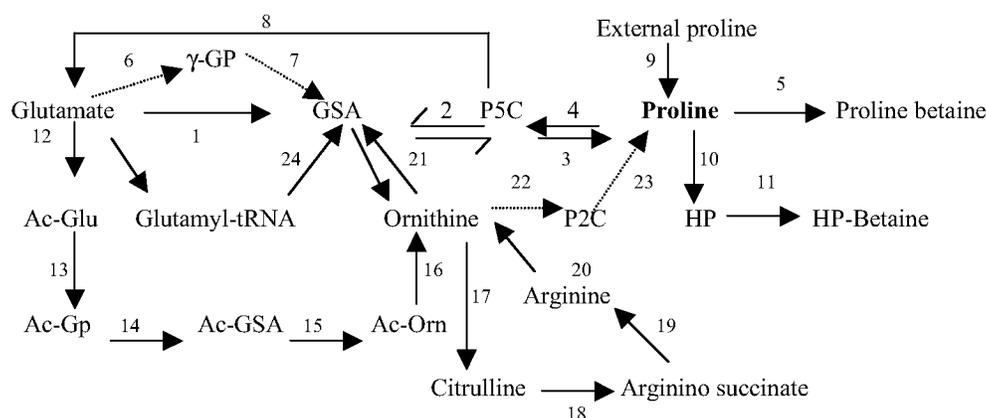


Figure 1. Network of proline biosynthesis and degradation pathways in plants and lower organisms. The proline biosynthetic pathway characterized in plants is depicted as straight arrowhead (\longrightarrow) and in lower organisms as dashed arrowhead ($\cdots\cdots\longrightarrow$). Numbers given for individual reactions represent the individual genes catalysing specific reactions of proline metabolism; the corresponding names are listed in Table 1. Further, abbreviations of the gene list are also indexed in Table 1.

or decreased degradation or both¹⁹. Proline is synthesized not only from glutamate, but also from arginine/ornithine (Figure 1). The enzymes involved in the proline biosynthetic pathway of both prokaryotic and eukaryotic systems, including higher plants as well as the network of pathways are shown in the Figure 1 and Table 1. In the following sections, the mode of proline biosynthesis via glutamate, which is diverged between *Escherichia coli* and higher plants and also the alternate pathway of proline production via ornithine are discussed.

Pathway of proline biosynthesis via glutamate

In bacteria, proline biosynthetic pathway starts with the phosphorylation of glutamate, which gets converted to γ -glutamyl phosphate and then to glutamic- γ -semialdehyde (GSA) by the enzymes γ -glutamyl kinase and glutamic- γ -semialdehyde dehydrogenase respectively. GSA gets converted to pyrroline 5-carboxylate (P5C) by spontaneous cyclization. On the other hand, glutamate is directly catalysed to GSA by pyrroline 5-carboxylate synthetase (P5CS) in plants and other eukaryotes²⁰. P5C is then reduced to proline by P5C reductase (P5CR) in both prokaryotes and eukaryotes.

P5CS: Mothbean (*Vigna aconitifolia*) cDNA clone encoding P5CS was first isolated by a functional complementation technique using *E. coli* mutants²⁰. The 2417-base pair sequence contains a single major open reading frame that encodes a polypeptide of 73.2 kDa. P5CS is a novel bifunctional enzyme (EC 2.7.2.11/1.2.1.41) that catalyses the first two steps of proline biosynthesis in plants. It is a rate-limiting enzyme in the pathway and exhibits both γ -glutamyl kinase as well as glutamic- γ -semialdehyde dehydrogenase activities. The two enzymatic domains of

P5CS correspond to the ProB and ProA proteins of *E. coli* and contain a leucine zipper in each domain. While the amino terminal domain of the P5CS protein showed 33.3% identity and 55.3% similarity to the bacterial ProB protein, the carboxyl end exhibited 35.7% identity and 57.9% similarity to the ProA protein of *E. coli*. The leucine zippers may facilitate inter- or intramolecular interaction of this unique protein. The leucine zippers may also maintain the tertiary structure of the two domains of this enzyme and homodimer or heterodimer formation may occur through the leucine zippers to allow proper and continuous association of both the domains²⁰. Besides, P5CS contains a phosphorylation site, the function of which is not yet known. In plants and other eukaryotes, the enzyme is feedback-regulated by proline, but is less sensitive to feedback inhibition when compared to bacterial enzymes. The γ -glutamyl kinase activity of one tomato P5C synthetase isoform is 70–250 times more sensitive to feedback inhibition by proline than the recombinant P5C synthetase²¹. In bacteria, the feedback regulation was removed by site-directed mutagenesis, which involves a nucleotide substitution of A for G, resulting in a change from aspartic acid to asparagine residue. Plant P5CS is less sensitive to end-product inhibition, though aspartate residue is present in this enzyme.

A cDNA clone encoding P5CS was later isolated from rice and characterized²². The expression of *OsP5CS* mRNA and content of proline under salt-stress conditions were compared between a salt-tolerant cultivar, Dee-gee-woo-gen and a salt-sensitive line, IR-28. The expression of P5CS and the accumulation of proline in Dee-gee-woo-gen steadily increased, whereas in IR-28 lines, it increased slightly. Initially, a single P5CS gene was cloned, sequenced and its chromosomal location was found in Arabidopsis²³. *AtP5CS* encoded a protein of 717 amino acids showing

Table 1. Genes involved in the network of proline biosynthesis and degradation pathway

Genes of proline biosynthetic pathway	E.C. number	Prosites signature	Localization	Organism
Pyroline-5-carboxylate synthetase (P5CS)	2.7.2.11	Glutamate-5-kinase	Unknown (may be chloroplastic)	Plants
Spontaneous cyclization		No hit in EXPASY	Unknown	
Pyroline-5-carboxylate reductase (P5CR)	1.5.1.2	Delta-1-pyrroline-5-carboxylate reductase	Cytosolic; chloroplastic	Plants
Proline dehydrogenase (PDH)	1.5.99.8	Aldehyde dehydrogenase-g and c active site	Mitochondrial inner membrane	Plants
Proline methyltransferase		No hit in EXPASY	Unknown	
γ -Glutamyl kinase (γ -GK)	2.7.2.11	Glutamyl kinase	Cytosolic	Prokaryotes
Glutamic- γ -semialdehyde dehydrogenase (GSDH)	1.2.1.41	Gamma-glutamyl phosphate reductase	Mitochondrial inner membrane	Plants
Pyrroline-5-carboxylate dehydrogenase (P5CDH)	1.5.1.12	No hit in EXPASY	Mitochondrial; cytosolic	
Proline permease		No hit in EXPASY	Cell membrane	
Prolyl hydroxylase	1.14.11.2	Thioredoxin family site	Unknown	Plants
Hydroxyproline-methyl transferase		No hit in EXPASY	Unknown	
Acetyl-CoA: glutamate <i>N</i> -acetyl transferase (Ac GACT)	2.3.1.35	Hydrolytic activity on acetyl-L-ornithine	Chloroplastic	Plants
<i>N</i> -Acetylglutamate kinase (Ac GK)	2.7.2.8	<i>N</i> -Acetylglutamate 5-phosphotransferase active site	Mitochondrial	Plants
Acetyl glutamic- γ -semialdehyde dehydrogenase (Ac GSD)	1.2.1.38	<i>N</i> -acetyl-gamma-glutamyl-phosphate reductase active site	Chloroplastic	Prokaryotes
Acetyl ornithine aminotransferase (Ac OAT)	2.6.1.11	Aminotransferases class-III	Chloroplastic	Prokaryotes
Acetyl ornithine deacetylase (Ac Oam DH)	3.5.1.16	ArgE/dapE/ACY1/CPG2/yscS family signature 1	Chloroplastic	Prokaryotes
Ornithine carbamoyl transferase (OCT)	2.1.3.3	Aspartate and ornithine carbamoyl-transferases signature	Mitochondrial; Cytosolic	Plants
Arginino succinate synthetase (ASS)	6.3.4.5	Argininosuccinate synthase signature 1	Cytosolic	Plants
Arginino succinate lyase (ASL)	4.3.2.1	Fumarate lyases signature	Cytosolic	Plants
Arginase (ARG)	3.5.3.1	Arginase family signatures	Mitochondrial	Plants
Ornithine- δ -aminotransferase (δ -OAT)	2.6.1.68	Amino group transfer	Mitochondrial	<i>Trichomonas vaginalis</i>
Ornithine- α -aminotransferase (α -OAT)	2.6.1.68	Amino group transfer	Mitochondrial	<i>T. vaginalis</i>
Pyroline-2-carboxylate reductase (P2CR)	1.5.1.1	Pyroline-2-carboxylate reductase signature	Unknown	Plants
Glutamate tRNA reductase		No hit in EXPASY	Unknown	

Genes of proline metabolism isolated from plants as well as from lower organisms are represented. Further, E.C. number, prosites signature, probable localization as well as the source of organism from which the corresponding gene was isolated are indexed.

high identity with the P5CS of *Vigna*. High homology was found at the N-terminus to bacterial and yeast γ -glutamyl kinase and at the C-terminus to bacterial γ -glutamyl phosphate reductase. The authors suggested putative ATP- and NAD(P)H-binding sites in the AtP5CS protein²³. *AtP5CS* gene was mapped at the bottom of the chromosome two in *Arabidopsis*. Later, two *P5CS* genes were shown to be present in *Arabidopsis thaliana*, *Medicago sativa* and *Lycopersicon esculentum*²⁴⁻²⁶. *AtP5CS1* gene of *Arabidopsis* is expressed in most organs, differentiated tissues and is primarily upregulated by dehydration, high salinity and abscisic acid (ABA) treatment²⁷, but it cannot be detected in dividing cell cultures in the absence of stress. On the other hand, *AtP5CS2* (EC not yet assigned) is expressed in dividing cell cultures and its induction to stress is dependent on protein synthesis²⁴. Similarly, there are two *Pro* loci in the nuclear genome of tomato and the cDNA clones, *tomPRO1* and *tomPRO2*, specifying P5CS were isolated²⁶. *tomPRO1* resembled a prokaryotic polycistronic

operon that directs the synthesis of γ -GK and GSA dehydrogenase as two separate peptides, whereas *tomPRO2* encoded a full length P5CS^{26,28}. Sequence comparison of these two genes with known proline biosynthetic pathway genes suggested that *tomPRO1* is similar to prokaryotic P5CS loci, whereas *tomPRO2* is closely related to other eukaryotic P5CS genes. Studies further revealed that there was a more than threefold increase in the mRNA levels of *tomPRO2*, while *tomPRO1* message was not detectable in response to NaCl stress. These studies on *L. esculentum* clearly showed that transcriptional regulation of these genes for P5CS is not important for the osmotic or pollen-specific regulation of proline synthesis²⁶. Restriction fragment-length polymorphism mapping revealed that the location of these two loci is in the nuclear genome. Such divergences of the biochemical functions of P5C synthetase isoforms were also observed in *Medicago sativa*²⁵ (GenBank entries X98421; X98422). The two P5CS genes of *Medicago truncatula* showed regulation at transcript

level that differed according to organs and in response to osmotic stress. *MtP5CS1* steady-state transcript levels in different organs were well correlated with proline levels, but transcript abundance was unaffected by osmotic stresses²⁹. In contrast, *MtP5CS2* transcripts were poorly detected in all organs, but strongly accumulated in shoots of salt-stressed plants. *P5CS1* and *P5CS2* genes were oppositely regulated during NaCl stress in *Arabidopsis* and *M. sativa*. While higher transcript accumulation of *P5CS1* was noticed in *Arabidopsis*, *P5CS2* was higher in *M. sativa*^{24,25}. It appears that *MtP5CS1* is a good candidate for the transcriptionally regulated proline level in reproductive tissues of *M. truncatula*. Also, post-transcriptional regulation appeared to play a minor role in proline biosynthesis regulation in this plant. Armengaud *et al.*²⁹ clearly showed that *MtP5CS1* acts as a developmental housekeeping enzyme and may be responsible for the supply of proline to the reproductive organs and *MtP5CS2* acts as a shoot-specific osmoregulated isoform. Thus, transcriptional regulation of proline biosynthesis in *M. truncatula* revealed both developmental and environment-specific features. It is clear from the literature that the regulation of each P5CS transcript varies from one species to another. Also, it is worth noting that proline accumulation in developing grapevine fruit occurs independently of changes in the levels of Δ^1 -pyrroline-5-carboxylate synthetase mRNA or its protein. The developmental accumulation is clearly distinct from the osmotic stress-induced accumulation of proline. Also, the accumulation is dependent on the decrease in steady-state levels of proline dehydrogenase protein³⁰. This suggests that other physiological factors are important for the regulation of proline in grapevine fruit.

Promoter of an *Arabidopsis* *P5CS* gene was isolated and analysed using β -glucuronidase (GUS) reporter gene in transgenic plants³¹. While GUS expression was rapid and was only within 3 h period under NaCl stress, it was noticed for 24 h under dehydration stress. The *P5CS* gene promoter isolated from *Arabidopsis* contained two transcription start sites. Dehydration stimulated the transcription predominantly from the downstream site³¹. Abscisic acid (ABA) failed to induce the *P5CS* gene expression though ABA is known to increase during dehydration stress. Further, glutamine, proline and cold showed no induction or repression of *P5CS* gene expression. Perhaps, proline regulates its own biosynthesis at the enzyme level and its inability to induce *P5CS* gene expressions is a big bottleneck in the regulation of proline metabolism. Though sub-cellular location of this enzyme is still not exactly known, it is predicted to be cytosolic (Table 1).

P5CR: *P5CR* or *P5R* (pyrroline-5-carboxylate reductase) was the first gene to be cloned in the proline pathway by functional complementation of a *proC* mutation in *E. coli* with an expression library of soybean root nodule cDNA, and was found to be osmoregulated³². The gene is 1.2 kb in length and encodes a 28,586 Mr polypeptide with 39%

amino acid identity to the bacterial P5CR sequence. Genomic analysis showed that there are 2–3 copies of the *P5CR* gene in the genome of soybean. *P5CR* gene was later isolated from *Pisum sativum*³³, *A. thaliana*³⁴ and kiwifruit³⁵. *AtP5CR* was previously named *AtP5C1* and the cDNA includes 2574-bp 5' flanking region³⁴. P5CR transcripts as well as P5CR activity were reported to increase under stress in *Arabidopsis*³⁴. This sequence contains an open reading frame encoding a polypeptide of 28,626 D (276 amino acids) with a calculated isoelectric point of 8.64. A comparison of the *AtP5CR* primary and secondary structures with those of six other organisms revealed that not only is the primary structure conserved, but also greater similarity exists in the secondary structures. P5CR from soybean was purified to homogeneity as a monomeric protein of 29 kDa by overexpression of this clone in *E. coli*. Accumulation and developmental regulation of transcript encoding P5C reductase in *Arabidopsis* suggest that it plays an important role in proline synthesis in rapidly dividing cells and/or in cells undergoing changes in osmotic potential³⁶. Expression of *AtP5CR* is high in cells or tissues that experience developmentally programmed osmotic adjustment, such as guard cells, hydathodes, pollen grains and developing seeds. Its expression is also high in apical meristems, root meristems and lateral root primordia³⁷. The enzyme P5CR was purified from spinach (*Spinacia oleracea*) leaves. Two isoenzymes were resolved by anion exchange chromatography and designated as P5CR-1 and P5CR-2. Only P5CR-2 was purified from intact chloroplasts. This indicates differential distribution of the isoenzymes of P5CR in spinach³⁸. The *P5CR* gene is not identified in the chloroplast genomes³⁹, indicating that P5CR-2 is synthesized in the cytosol and transferred into chloroplasts through a transport mechanism in the chloroplast membrane. Both P5CR-1 and P5CR-2 are homopolymers with an apparent molecular mass of 310 kDa, consisting of 10 to 12 subunits of about 28.5 kDa. The two isoenzymes had much lower affinity for NADH than for NADPH and were inhibited by free ATP and Mg^{2+} ions. Murahama *et al.*³⁸ showed the effects of NaCl and $MgCl_2$ on both isoforms were inhibitory. In contrast, P5CR activity of both pea chloroplasts and etiolated pea shoots increased twofold⁴⁰ in the presence of 10 mM $MgCl_2$ or 100 mM KCl. No difference in the effects of cations between the P5CR-1 and P5CR-2 activities was found in spinach. It appears that the effect of salts on P5CR-1 and P5CR-2 activities was not due to anion, but high cation concentrations³⁸. Interestingly, P5CR-2 was more stable to heat treatment at 40°C than P5CR-1 isoform in spinach. Genomic DNA hybridization studies suggested the presence of two to three copies of the *P5CR* gene per haploid genome in pea and soybean^{33,40,41}.

It was shown that a 69 bp fragment of the *AtP5CR* promoter (P69) located between –120 and –51 with respect to transcription start is necessary for tissue-specific expression of it in *Arabidopsis*⁴². It was also demonstrated that P69

is necessary and sufficient to activate a minimal cauliflower mosaic virus 35S promoter in a tissue-specific manner⁴². Although both P5CS and P5CR transcripts were induced under stress conditions, P5CR activity may not have a significant role in enhancing proline accumulation (see section on transgenics). While P5CS utilizes both ATP and NADPH, most P5CR enzymes (EC 1.5.1.2) use NADPH and NADH as reductants⁴³. Hua *et al.*³⁷ concluded that P5CR activity might be involved in the metabolic regulation of cellular redox potential by affecting the level of reduction of NADPH. Studies on the subcellular location of P5CR using Western blot assay revealed that the enzyme is localized in cytosol (Table 1) and plastid fractions in different parts of the plant^{33,41}. The location of this enzyme in plastids suggests that it may assist in counteracting photoinhibitory damage of RuBisCo enzyme under adverse conditions.

Pathway of proline biosynthesis via arginine/ornithine

In plants, proline is synthesized not only from glutamate but also from arginine/ornithine^{44,45}. Arginine gets converted to ornithine by the enzyme arginase. In bacteria, ornithine is degraded to α -keto- δ -aminovalerate by the enzyme ornithine α -aminotransferase (α -OAT), which then spontaneously gets cyclized to pyrroline 2-carboxylate (P2C). P2C is finally catalysed to proline by P2C reductase (Figure 1). This pathway has not yet been found in plant systems. In plants however, GSA is derived directly from ornithine by the enzyme ornithine δ -aminotransferase (δ -OAT).

Ornithine δ -aminotransferase: Proline auxotroph mutants of *E. coli* were electroporated with *V. aconitifolia* cDNA expression library, and ornithine and proline prototrophy was restored. This novel strategy called 'trans-complementation' facilitated the isolation of cDNA clones encoding δ -OAT. In plants, δ -OAT (EC 2.6.1.13) transaminates ornithine directly to GSA, and subsequently gets converted to proline⁹ via P5C. Identity of *Vigna* OAT gene was confirmed by sequence homology to mammalian and yeast δ -OATs as well as to a family of bacterial and fungal ω -aminotransferases. When sequence comparisons were made with α -OATs, no homology was noticed. The mothbean 1559 base pair OAT clone encodes a polypeptide of 48.1 kDa. The mothbean OAT enzyme was expressed in *E. coli* and the native enzyme behaved as a monomer by gel-filtration on a Superose-6 high performance liquid chromatography column⁹ with a native molecular mass of about 50 kDa. Our computer-based homology modelling studies confirmed the monomeric nature of this protein (Sekhar and Kavi Kishor, unpublished). The K_m for ornithine was found to be 2 mM and 0.75 mM for α -ketoglutarate. Optimal activity of OAT was observed at a pH of 8.0. Surprisingly, the amino acid valine inhibited the activity

of this enzyme. This clearly shows that valine binds to the enzyme more strongly than L-ornithine. Activity of OAT was reduced by 60% with the removal of pyridoxal phosphate from the reaction mixture. Under different physiological conditions, the levels of mRNA in *V. aconitifolia* for P5CS and δ -OAT were monitored and their levels of expression compared. Both salt stress and nitrogen starvation induced P5CS mRNA levels and decreased the mRNA levels of OAT. On the other hand, OAT levels increased in plants supplied with excess nitrogen. The results indicated clearly that glutamate pathway is predominant for proline synthesis during osmotic stress and ornithine pathway operates when plants are supplied with high nitrogen⁹.

Further, δ -OAT cDNA clones were isolated from *Arabidopsis* and its expression was studied during salt stress⁴⁶. The N-terminal residues of *Arabidopsis* enzyme exhibited several common features with a mitochondrial transit peptide. It was observed that younger plants exhibited more OAT than the older plants. Therefore, the authors opined that it is a necessity to dispose-of an easy recycling product, glutamate. In young seedlings of *Arabidopsis*, proline content, P5CS mRNA, δ -OAT activity and OAT mRNA increased under salt stress conditions. Contrary to the results obtained from *V. aconitifolia*⁹, in *Arabidopsis* the ornithine pathway together with the glutamate pathway appeared to be playing an important role in proline accumulation during osmotic stress. Their results further showed that δ -OAT activity did not change in 4-week-old seedlings of *Arabidopsis* though P5CS mRNA was still induced. It could be inferred from these observations that increased proline levels are due to the activity of glutamate pathway. Located in the mitochondria (Table 1), this enzyme thus appears to play an important role in proline metabolism.

Proline catabolism

Proline dehydrogenase

Many organisms accumulate proline far in excess of the demands of protein synthesis. Proline catabolism is repressed under osmotic stress^{1,47,48}, but once the stress is withdrawn, proline is oxidized to P5C by proline dehydrogenase (PDH; EC 1.5.99.8), also known as proline oxidase, the first enzyme in the proline degradation pathway. P5C is then converted back to glutamate by the enzyme P5C dehydrogenase (P5CDH; 1.5.1.12). Thus, both PDH and P5CDH form two important enzymes in the degradation of proline to glutamate in higher organisms. The enzyme PDH is bound to the inner membrane of mitochondria. This is important for the cells since oxidation of proline generates NADP/NADPH cycling or redox balance¹⁹. A nuclear gene encoding mitochondrial proline dehydrogenase enzyme was isolated from *Arabidopsis*⁴⁸. Sequence analysis of an *Arabidopsis* cDNA clone, ERD5 (for early responsive to dehydration stress) revealed that it encodes a

protein that has identity with the products of the yeast *PUT1* (for proline utilization) gene and *Drosophila* slugish-A gene (34.5% over 255 amino acids). The amino acid sequence identity of the ERD5 protein is similar to those of proline oxidases of yeast and *Drosophila*. Further, the highly conserved regions at the C-terminus found in these proteins are also found in the PutA protein of *E. coli*. In bacteria, PutA is a multifunctional protein that functions not only as a proline dehydrogenase, but also as a P5C dehydrogenase and as a repressor of *put* genes⁴⁹. Transcripts of *ERD5* could not be detected when Arabidopsis plants were dehydrated for 10 h, but the transcripts accumulated after rehydration. The product of *ERD5* is localized in the mitochondrial fraction and accumulated in response to proline in cultured cells. The results suggest that this gene is upregulated during rehydration or by proline but down-regulated by dehydration in Arabidopsis, with no net accumulation of free proline⁴⁸. This corroborates the view that cycling between proline and its precursors, glutamate and ornithine attains redox balance during stress. The amino acid sequence of the ERD5-encoded protein also includes a putative signal peptide for mitochondrial localization at the N-terminus. This indicates the mitochondrial location of PDH in Arabidopsis (Table 1).

P5C dehydrogenase

Bogges *et al.*⁵⁰ studied the characteristics of the enzyme Δ^1 -pyrroline-5-carboxylate dehydrogenase (P5CDH; EC1.5.1.12; the second enzyme involved in proline catabolic pathway) in barley. Later, sub-mitochondrial location and electron transport characteristics of the enzymes involved in proline oxidation were recorded by Elthon and Stewart⁵¹. In cultured cells of *Nicotiana plumbaginifolia* exposed to NaCl, two Δ^1 -pyrroline-5-carboxylate dehydrogenase isoforms were expressed and were differentially modulated during the culture growth cycle⁵². Surprisingly, both accumulation of proline as well as high activity of one of the isoforms of P5C dehydrogenase were noticed during exponential growth of the cultured cells. However, whether the activation of this isoform can be caused by dehydration or not, and also whether it can be induced by proline synthesized from glutamate or ornithine or by both is not yet known. The enzyme P5C dehydrogenase was also purified from cultured cells of potato⁵³. A 1600-fold purification of the enzyme was achieved with a recovery of one-third of the initial activity. Biochemical properties of this enzyme revealed that it is a α -4 tetramer with subunits of an apparent molecular mass of about 60 kD and had a mildly acidic isoelectric point value. The enzyme may be located both in the mitochondria and also in the cytosol (Table 1). This enzyme had Michaelis constant values of 0.11 and 0.46 mM for NAD⁺ and P5C respectively, and was inhibited by the anion chloride⁵³. This finding strengthens the fact that hyperosmotic stress negatively modulates proline oxidation in

plants. Though the enzyme is purified, the corresponding gene has not yet been cloned from plant systems.

Intermediates of proline biosynthesis and catabolism

It was found that intermediates of proline biosynthesis and catabolism such as glutamine and P5C could increase the expression of several osmotically regulated genes in rice⁵⁴ such as *salt* and the dehydrin *dhn4*. Also, gene induction by P5C did not depend on the *de novo* protein synthesis and respiration. Plants treated with P5C and its analogue 3,4-dehydroproline consumed less O₂, displayed more NADH levels, but reduced NADPH levels and accumulated several osmolytes associated with dehydrated plants of rice. The findings support the proposal put forward by Hare and Cress⁴³, that a signal derived from the proline metabolism may control gene expression under osmotic stress. It is clear from these experiments that P5C or the intermediates of proline metabolism are influencing some of the responses during osmotic stress^{54,55}. Stimulation of phosphoribosyl pyrophosphate and purine nucleotide production by pyrroline-5-carboxylate was also reported in human erythrocytes⁵⁶. P5C in plants is converted back to glutamate^{54,55} by P5C dehydrogenase, an enzyme predicted to be present in the cytosol and mitochondrial matrix (Table 1). This is an important enzyme and isolation of this cDNA clone would be a valuable contribution to the understanding of proline catabolism and salt stress. Once this enzyme is cloned, it can reveal if it is also suppressed under salt stress and de-repressed when plants are rehydrated like PDH, but the gene encoding this enzyme has not yet been cloned and studied from higher plants, though it was cloned from humans⁵⁷. The overall study on proline catabolism suggests that the decline in proline is regulated at the genetic level.

Proline uptake and inter-organ transport during stress

Transport of amino acids is regulated not only by endogenous but also by environmental signals in plants. Water deficit like drought and salt stress, affects long-distance transport and results in massive changes in partitioning of carbon and nitrogen. Meristems, developing tissues, and reproductive organs usually import amino acids to support growth and development. Plant amino acid transporters are classified into two superfamilies; the amino acid, polyamine, and choline transport superfamily and the amino acid transporter family (ATF) superfamily⁵⁸⁻⁶⁰. Amino acid transporter superfamily has five sub-classes of transporters that have been described so far. These include the amino acid permeases, the lysine, histidine transporters, the proline transporters (ProT) with two members examined so far, the putative auxin transporters and a new member of the family,

which transports aromatic amino acids, neutral amino acids, arginine and auxin^{59,60}. In bacteria, the uptake of proline as well as glycine betaine is osmotically regulated and osmotic strength of the medium triggers the uptake of these osmolytes^{61,62}. Existing evidence shows that proline permease may be located in cell membranes (Table 1). Exogenous supply of proline to osmotically stressed callus of rice increased the growth of callus *in vitro*⁶³. This indicates that proline transporters exist in plants. While proline biosynthetic pathway is well characterized in plants, the uptake and its transport are not well understood¹. It was reported that in alfalfa, proline transport processes play an important role in adaptation to osmotic stress⁶⁴. From Arabidopsis, eight different amino acid transporter clones were isolated and characterized using yeast mutants^{65,66}. Two of these encoded specific proline transporters (*ProT*) are distantly related to the amino acid permease gene family. Though *ProT1* was expressed in all organs of the plants, highest levels were found in the floral stalk phloem that enters the carpels and were down-regulated after fertilization. This is consistent with the evidence that proline synthesis and degradation play an important role in flowering and seed set^{34,36}. *ProT* may be playing a specific role of supplying proline to pollengrains⁶⁷. In contrast, mRNA levels of *ProT2* were observed throughout the plant, but their expression was strongly induced by water or salt stress⁶⁵. This suggests that the gene may be distributing nitrogen during water stress unlike the members of amino acid permease gene family, the expressions of which are generally suppressed under similar conditions. Within plant superfamilies of amino acid transporters, the transporters of proline transport proline but not other amino acids^{59,60,65}. High proline concentrations were reported in the phloem sap of drought-stressed alfalfa⁶⁴. It is proposed that proline synthesis might be more in roots, but most of the production may be exported to shoot tissues²⁹. This is again consistent with the observation of active expression of both *ProT1* and *ProT2* in roots. Also, Verslues and Sharp⁶⁸ presented evidence for the transport of proline to the root tips of maize, where it accumulates during stress. In general, proline export or transport is increased under salt-stress conditions as evident by the accumulation of *ProT2* transcript, while transport of broad specificity amino acids is suppressed^{59,60,65-67}. Arabidopsis amino acid transporters AAP3 ProT2 were also identified as γ -amino butyric acid (GABA, a stress-induced amino acid) transporters at pH 4. The transport of GABA, L-proline and D-proline simultaneously by ProT2 as a function of pH, provided evidence that the zwitterionic state of GABA is an important parameter in substrate recognition. Also, ProT2-mediated transport was inhibited by proline and quaternary ammonium compounds⁶⁹. In *L. esculentum*, proline transporters *LeProT1*, *T2* and *T3* that are homologous to Arabidopsis were isolated⁶⁷. Analysis of the free amino acids in different tissues revealed that proline content was 60 times higher in pollen than in any other organ. It was

also observed that *LeProT1* supplies proline to both mature and germinating pollen under normal conditions. Expression of *LeProT1* in a yeast mutant demonstrated that *LeProT1* transports proline and γ -amino butyric acid with low affinity and glycine betaine with high affinity. Thus, *AtProT2* and *LeProT1* are considered to mediate proline transport by cotransport of H⁺, similar to amino acid permeases (AAPs), as these transport activities show pH-dependence^{65,67,69}. Direct uptake and competition studies demonstrated that *LeProT1* constitutes a general transporter for compatible solutes^{67,69}. A cDNA encoding a *ProT* was isolated and characterized from rice also⁷⁰. ProT protein of rice had 68.8% homology to the ProT1 protein of Arabidopsis and 59.6% homology to that from tomato. Salt stress did not induce this porter in rice and Southern blot analysis revealed that *OsProT* has a gene family. Igarashi *et al.*⁷⁰ also found that OsProT specifically transported L-proline in a transport assay. Recently, Andreasson *et al.*⁷¹ found that proline and the toxic proline analogue azetidine-2-carboxylic acid are efficiently imported into yeast cells by four amino acid permeases, including two nitrogen-regulated permeases.

Transgenics for enhancing proline accumulation and abiotic stress tolerance

Tobacco plants were first transformed with the *P5CS* gene isolated from *V. aconitifolia* under the influence of tobacco mosaic virus 35S promoter. These transgenic plants produced a high level of the enzyme and synthesized 10–18-fold more proline than the corresponding control plants. Overproduction of proline enhanced root biomass and also plants tolerated NaCl stress in the glasshouse conditions (Table 2). Since proline production increased several folds in transgenics, it suggests that the activity of P5CS in the pathway is the rate-limiting step. Exogenous supply of nitrogen further enhanced proline production in transgenic tobacco¹⁰. Studies using purified P5CS enzyme indicated that *Vigna* P5CS is feedback inhibited to 50% by 5 mM proline *in vitro*²⁰. Substrate nitrogen as well as the end-product of the pathway, i.e. proline, thus control the activity of the enzyme P5CS. A clear correlation exists between the induction of the gene for Δ^1 -pyrroline-5-carboxylate synthetase and the accumulation of proline in *A. thaliana* under osmotic stress²³. It appeared that the feedback regulation of P5CS is lost in plants under stress conditions. Transgenic tobacco plants expressing a wild-type form of *V. aconitifolia* P5CS and a mutated form of the enzyme (P5CSF129A), whose feedback inhibition by proline was removed by site-directed mutagenesis were used to compare proline levels^{10,18,21}. Tobacco plants expressing mutated form of P5CSF129A accumulated nearly twofold more proline than those expressing *V. aconitifolia* wild-type P5CS. Further increase in proline was observed in tobacco plants treated with 200 mM NaCl

Table 2. Transgenics developed from proline biosynthetic pathway genes that conferred abiotic stress tolerance

Gene	Species	Phenotypic effects of transgenic plants	Reference
Pyrroline-5-carboxylate synthetase	Tobacco	Increased biomass production and enhanced flower and seed development under salinity stress	10
Pyrroline-5-carboxylate synthetase (mutated P5CS 129A)	Tobacco	Preventing feedback regulation of P5CS increased twofold more proline accumulation in transgenics	21
Pyrroline-5-carboxylate synthetase	Rice	Increased biomass production under drought and salinity stress	11
Pyrroline-5-carboxylate synthetase	Arabidopsis	Antisense plants showed hypersensitivity to osmotic stress and show morphological changes during non-stress condition	77
Pyrroline-5-carboxylate synthetase	Rice	Reduced oxidative stress under osmotic stress	12
Pyrroline-5-carboxylate synthetase	Rice	Transgenic rice plants showed better root growth and biomass development during 200 mM NaCl treatment	109
Pyrroline carboxylate synthetase	Rice	Stress-inducible expression of <i>P5CS</i> gene in rice seedlings showed significant higher tolerance to drought and salt stress	110
Pyrroline-5-carboxylate synthetase	Wheat	Wheat transgenic plants showed enhanced proline levels and conferred salt tolerance	13
Pyrroline-5-carboxylate synthetase	Chlamydomonas	Transgenic algae express <i>Vigna P5CS</i> and had 80% higher free proline than wild type cells and conferred tolerance to toxic heavy metals	72
Pyrroline-5-carboxylate synthetase	Yeast	Reduced growth under non-stress and the same promoted growth under mild stress	78
Pyrroline-5-carboxylate synthetase	Carrot	Tolerance to salt stress	14
Pyrroline-5-carboxylate synthetase (<i>OsP5CS2</i>)	Rice	Enhanced salt and cold stress tolerance	111
Pyrroline-5-carboxylate synthetase (<i>P5CSF129</i>)	Citrus	Drought tolerance	115
Pyrroline-5-carboxylate reductase	Tobacco	Enhanced P5CR activity in transgenics did not yield significant increase in proline level	74
Pyrroline-5-carboxylate reductase	Soybean	Antisense plants produced low number of seeds	75
Pyrroline-5-carboxylate reductase	Soybean	Enhanced heat and drought stress	116
Proline dehydrogenase	Arabidopsis	Altered levels of proline dehydrogenase conferred salt and freezing tolerance	112
Proline dehydrogenase	Arabidopsis	Antisense plants showed hypersensitivity to proline	76
Proline dehydrogenase	Tobacco	Antisense plants showed increased proline content	113
Ornithine- δ -aminotransferase	Tobacco	Overexpression increased proline biosynthesis and osmotolerance	15
Ornithine- δ -aminotransferase	Rice	Overexpression increased proline 5–15-fold of that in non-transgenic control plants during osmotic stress and transgenic plants showed improved yield under stress conditions	114

solution. These results clearly indicate that the feedback regulation of P5CS plays a role in controlling the proline levels in plants under both normal and stress conditions. In plants that overproduced proline¹⁰, salt-stress tolerance was significantly higher and the plants withstood NaCl up to 200 mM. Elevated levels of proline caused by overexpression of mothbean P5CS in transgenic rice conferred enhanced tolerance to salt stress¹¹. Similarly, *P5CS* gene was introduced into wheat using *Agrobacterium*-mediated gene transfer via indirect pollen system¹³. Salinity test of these transgenic wheat plants indicated that overproduction of proline results in increased tolerance to salt stress (Table 2). Also, introduction of mothbean *P5CS* gene via *Agrobacterium* into carrot resulted in enhanced salt tolerance¹⁴. Transgenic cell lines of carrot exhibited six-times increased degree of tolerance to 250 mM NaCl. *Vigna P5CS* gene was also transferred into the green microalga *Chlamydomonas reinhardtii*, where it was overexpressed⁷². It was shown that transgenic algae expressing the mothbean *P5CS* gene had 80% higher free-proline levels than wild-type cells. These transgenics grew more rapidly in toxic cadmium

concentrations (100 μ M) and bound fourfold more cadmium than wild-type cells. Recently, *OsP5CS* was introduced into rice and the gene in transgenics was found to be salt-inducible and is also essential for salt and cold tolerance (Table 2). Promoter of the *P5CS* gene was also characterized in transgenic Arabidopsis subjected to water stress³¹. The biochemical basis for NaCl tolerance was investigated using ¹³C-NMR imaging in both wild-type and mutant lines of *Nicotiana glauca*⁶⁸ that overproduced proline. The mechanism of proline accumulation via glutamate pathway was determined in the mutant lines using ¹³C-NMR with [5-¹³C] glutamate as a proline precursor. Under salt-stress conditions, mutant lines accumulated more proline compared to wild-type plants. The studies also revealed that the enzymatic activities of the ornithine pathway and also proline catabolism via proline oxidation were not affected in the mutant lines. Feedback inhibition of P5CS enzyme by proline could not be detected in the mutant due to a mutation. This gene mutation ought to have led to considerable reduction in the product inhibition in the RNA mutant lines of tobacco⁷³. The expression of

soybean *P5CR* gene in transgenic tobacco plants enhanced P5CR activity fifty-fold. But the enhanced P5CR activity in transgenic tobacco plants did not yield significant increase in proline levels⁷⁴. Further, these transgenic results confirm that enhanced *in vivo* activity of P5CR is limited by the lack of substrate P5C. Further, soybean plants were transformed with a *P5CR* gene construct in an antisense direction controlled by an inducible heat shock promoter (IHSP)⁷⁵. Reduction of the *P5CR* gene expression in antisense lines of soybean plants resulted in a decline in proline synthesis as well as protein synthesis. Antisense lines of transgenic soybeans did not withstand the osmotic stress due to decline in proline synthesis and accumulation. Low proline synthesis and accumulation in the transgenics resulted in lower seed production than in control plants, indicating that the antisense *P5CR* gene also negatively influenced seed production in soybean⁷⁵. Hence, co-expression of *P5CS* and *P5CR* genes under the control of stress-inducible promoter might result in enhanced proline accumulation during stress and may bring down the retardation effect of plant growth. The results obtained in *Arabidopsis* provide hints that ornithine as well as glutamate pathways might play together an important role in proline accumulation during osmotic stress conditions⁴⁶. To prove this hypothesis, *Arabidopsis* δ -OAT gene fused with CaMV35S promoter was overexpressed in *Nicotiana glumbaginifolia* via *Agrobacterium* transformation¹⁵ (Table 2). Overexpression of the δ -OAT cDNA in the transgenic lines was linked not only to an increase in δ -OAT enzyme activity, but also in higher proline content than the control plants, which in turn resulted in higher biomass accumulation and a higher germination rate under osmotic stress conditions.

In addition to proline synthesis, proline catabolism and transport are thought to control endogenous proline accumulation in plants. To elucidate the function of proline degradation, antisense transgenic *Arabidopsis* plants were generated for proline dehydrogenase (*ProDH*) gene⁷⁶. The PDH transgenic plants did not show significant levels of osmotic stress tolerance. But, exogenous application of proline increased the tolerance to osmotic stress and proline was converted to glutamate in PDH-sense plants. Antisense suppression of proline degradation in transgenic *Arabidopsis* plants accumulated proline at higher levels than wild-type plants and conferred tolerance to high salinity and freezing (Table 2). This also indicated that the enzyme ProDH plays a key role not only in proline degradation but also in controlling proline levels in *Arabidopsis*.

Metabolic implications of proline accumulation in plants during stress and plant development

In addition to the documentation of proline accumulation during stress conditions, free proline accumulation was also noticed during various stages of plant development.

Its accumulation was dependent upon the developmental stage of the plant and type of plant organs^{77,78}. Studies indicate that proline plays an important role during development of plants, especially in flowers and also in pollen serving as a readily accessible source of energy⁵⁵. It is known⁵⁷ that oxidation of one molecule of proline yields 30 ATPs. In this context, it is important to know how proline accumulation influences other energy-related pathways and carbon metabolism during stress and upon relief of stress conditions or rehydration. The proline biosynthesis mediates increased NADP⁺/NADPH ratio; this change in ratio affects carbon flux through oxidative pentose phosphate pathway (OPPP)³⁶. This in turn provides precursors in the form of erythrose-4-phosphate to synthesize phenylpropanoids or secondary metabolites during stress conditions^{19,78}. This consequently leads to changes in the physical properties of cell wall and lignin accumulation. On the other hand, the changed carbon flux through OPPP leads to synthesis of nucleotides³⁶ and in turn accelerates cell division upon relief of stress. The aforementioned changes in plant metabolism due to proline accumulation could be better studied using transgenic plants with altered expression of proline levels.

Transgenic plants that were engineered to overproduce osmolytes exhibit impaired growth in the absence of stress. It was hypothesized that growth reduction may result from proline accumulation. To examine this possibility, intracellular proline levels were manipulated by expressing mutated derivatives of *tomPRO2* (1-pyrroline-5-carboxylate synthetase, P5CS, from tomato) in *Saccharomyces cerevisiae*. This was carried in the presence and absence of a functional proline oxidase (PDH), followed by selection and screening for increased accumulation of proline in the absence of stress. It was observed that the level of proline accumulation and the amount of growth are inversely correlated in cells grown under normal osmotic conditions⁷⁸. In addition, the intracellular concentration of proline also resulted in an increase of ploidy level, vacuolation and altered accumulation of several different transcripts related to cell division and gene expression control⁷⁸. Further, it was proposed that proline might act as an energy source during stress conditions and therefore could be a key signalling/regulatory molecule capable of activating multiple responses that are part of the adaptation process⁷⁹. In *A. thaliana*, expression of antisense P5CS inhibited proline production and made plants hypersensitive to osmotic stress⁷⁷. It was further proved that antisense transgenics have a negative impact on inflorescence development and in general showed morphological abnormalities of vascular differentiation due to change in cell-wall structural proteins⁷⁷.

Role of proline during stress conditions

Proline is a compatible osmolyte, is not charged at neutral pH and is highly soluble in water. Moreover, at high con-

centrations, it has little or no perturbing effect on macromolecule-solvent interactions⁸⁰. Circumstantial evidence exists today for the role of compatible solutes as osmotic balancing agents and protection of subcellular structures⁸¹. Accumulation of compatible solutes results in an increase in cellular osmolarity that can drive influx of water or reduce the efflux. This provides the turgor that is necessary for cell expansion. Under osmotic or dehydration stress conditions, membrane integrity must be maintained to prevent protein denaturation. Proline may interact with enzymes to preserve protein structure and activities. Protection of structural and functional integrity of M₄ lactate dehydrogenase by proline has been reported⁸². Indeed, proline has been shown *in vitro* to reduce enzyme denaturations caused due to heat, NaCl stress, etc.⁸³. Presence of high concentrations of proline and/or betaine gives better protection against the biologically unfavourable consequences of dehydration-induced thermodynamic perturbations⁸³. Sodium chloride curtailed carboxylase activity of Rubisco and enhanced the oxygenase activity⁸⁴, but not quite surprisingly, salt-stress induced oxygenase activity was suppressed by proline even at a concentration of 50 mM NaCl. The above findings potentiate our view that proline plays a critical role in protecting photosynthetic activity under stress. Hamilton and Heckathorn⁸³ studied the effect of NaCl on mitochondrial function and its protection by different molecules. They found that while complex I is protected by antioxidants and small heat shock proteins, complex II is protected by proline and betaine under NaCl stress conditions. Proline synthesis is involved in pH and also in redox regulation⁸⁵. Proline acts as a reserve source of carbon, nitrogen and energy during recovery from stress (see above and also Zhang *et al.*³¹). How does proline protect the proteins? Chandalavada *et al.*⁸² demonstrated that most probably, proline has a property of forming hydrophilic colloids in aqueous media with a hydrophobic backbone interacting with the protein. On the other hand, effects of proline might be involved on the hydration layer surrounding phospholipids and possibly its interaction between phospholipid head groups⁸⁶. Proline regulates cytosolic acidity and maintains NAD⁺/NADH ratios, enhances photosystem II-mediated photochemical activity in isolated thylakoid membranes and prevents the photoinhibitory loss of photochemical activity by producing a reduction in lipid peroxidation^{84,85}. Proline is also involved in protecting thylakoid membranes against free radical-induced photodamage⁸⁴. Proline biosynthesis from glutamate would regenerate NADP⁺ that is needed to support the oxidative steps of the pentose phosphate pathway operating in the nodules⁸⁷. High concentrations of NADP⁺ are necessary for pentose phosphate pathway for regeneration of NADPH and to supply ribose-5-phosphate for the synthesis of purines. Therefore, proline accumulated under stress conditions might serve as a sink for excess reductants providing the NAD⁺ and NADP⁺ necessary for maintenance of respiratory and photosynthetic processes. While proline synthesis gene-

rates NADP⁺, its degradation produces NADPH. Thus, a cycle of proline synthesis and its degradation is essential for buffering cellular redox potential in the cytosol as well as in plastids. Redox cycling is also important in plant antioxidant defence mechanisms under stress conditions^{19,85}. Since glutathione and ascorbate need to be maintained in a reduced state, the NADPH generated from pentose phosphate pathway may serve this purpose. Among various compatible solutes, proline is the only molecule that has been shown to protect plants against singlet oxygen and free radical induced damages⁸⁸. Since proline can act as a singlet oxygen quencher⁸⁹, and as a scavenger of OH[•] radicals, it is able to stabilize proteins, DNA as well as membranes⁸³⁻⁹⁵. Hydroxy-radical scavenging activity was measured for sorbitol, mannitol, myo-inositol and proline and it was found that proline is an effective hydroxy radical scavenger^{88,95}. Thus, proline is not only an important molecule in redox signalling, but also an effective quencher of reactive oxygen species formed under salt, metal and dehydration stress conditions in all plants, including algae⁹⁶. Activities of the enzymes catalase, peroxidase and polyphenoloxidase were promoted by proline *in vivo*⁹¹. However, the ability of proline to activate the enzymes may suggest a limited conformational change.

Effectors that affect proline accumulation

Though proline accumulation is merely a stress response, it is dependent upon the availability and the interactions of a number of effectors. Endogenous levels of such effectors might vary under different stress conditions and hence can exert their influence on the synthesis and degradation of proline. It was found that proline metabolism is highly responsive to a number of carbohydrates, especially when the internal concentrations exceed a critical level, which may occur because of dehydration. In fact, accumulation of total and reducing sugars under salt-stress conditions was recorded in different plant systems^{5,97,98}. Exogenously supplied carbohydrates such as mannitol, sorbitol, meso-inositol, erythritol (compatible solutes), glucose, fructose and sucrose induced proline accumulation in callus cultures of rice (Amrutha and Kavi Kishor, unpublished) and also a range of sugars in the leaf discs of *Brassica napus*⁹⁸. However, the amplitude response to the above sugars or sugar alcohols is less compared to NaCl or PEG-grown tissues⁹⁸. Accumulation of proline was provoked in rape leaf discs incubated in NaCl, but intriguingly, transfer of tissues to sucrose solution further enhanced the accumulation. In sharp contrast to these results, sucrose-induced proline accumulation was strongly suppressed by transfer to NaCl⁹⁸. It appears, therefore, that sucrose behaves like a positive effector for proline accumulation. While proline did not influence the expression²³ of P5CS, glycine betaine had a negative effect on stress-induced proline response⁹⁸. Also, glycine betaine inhibition of proline accumulation

in *Brassica* leaf discs was associated with betaine accumulation in the plant tissues as in the case of *Bacillus subtilis*⁶². However, whether the inhibition of proline accumulation by betaine is due to suppression of P5CS or increased activity of proline dehydrogenase or by both, remains to be elucidated.

Hormonal regulation of proline metabolism

Plant growth regulators such as ABA, indole-3-butyric acid (IBA), kinetin but not gibberellic acid (GA) imitated and initiated proline accumulation like salt and water stresses elicited responses in seedlings of *Guizotia abyssinica*⁵. But the amount of proline accumulation was far less compared to NaCl-induced level. Benzylaminopurine (BAP) induced an increase in proline as well as the transcript-encoding PEPCase in *M. crystallinum*⁹⁹. Further, the activity of this enzyme was differentially affected depending on whether BAP was applied to root or shoot tissues. When NaCl plus kinetin and NaCl plus ABA were added together to the seedlings of niger, both of them stimulated proline accumulation in an additive fashion unlike GA and IBA. This suggests that phytohormones and salts may be the independent initiators of a sensing pathway that triggers proline synthesis⁵. Nonetheless, it is not clear whether all of them share a convergent induction of proline synthesis. It is also poorly understood if any of the above hormones can enhance the transcript levels for proline synthesis in niger under stress. NaCl-induced growth inhibition was alleviated by exogenous supply of GA and ABA, but not by IBA and kinetin in seedlings of niger⁵. While ABA levels increase during stress, cytokinin levels tend to decline though cytokinins are antagonistic to ABA. Since roots are the sites of cytokinin synthesis, their transport to shoots may become a bottleneck and this may alter the gene expression levels. Cytokinins did not affect the accumulation of *AtP5CS1* mRNA in roots, but reduced it in leaves. Contrary to these observations, BAP induced *AtP5CS2* transcript in leaves but not in roots²⁰. The above findings indicate that cytokinins elicit their effects at the level of gene expression. The role of ABA as a signal molecule during stress is well characterized^{100,101}, but not the roles of auxins and cytokinins. Auxin and cytokinin-regulated, stress-inducible genes may perhaps contribute to a better understanding of auxin and cytokinin-mediated signal transductions and a facile elucidation of the mechanisms of salt-stress tolerance.

Signal transduction events of proline biosynthetic pathway genes

Although the importance of proline accumulation conferring hyperosmotic stress tolerance has been demonstrated well, the regulatory molecules as well as the molecular signals involved in the expression of proline biosynthetic

genes are not understood. This subject needs comprehensive studies at physiological, molecular and genetic levels to explore the signal transduction events of proline synthesis and degradation. Nonetheless, an attempt is made in this article to summarize the events with the available data. Based on the current knowledge of proline metabolism, regulation of P5CS and PDH transcripts represents the rate-limiting steps in proline biosynthesis and degradation respectively. The levels of transcripts encoding P5CS are highly stress (NaCl)-inducible^{20,27}. After 24 h exposure of *Arabidopsis* plants to 4°C, but not to 40°C for at least 24 h, accumulation of *AtP5CS1* transcript was noticed. The same could not be observed with 10h of treatment. Transcriptional activity of *AtP5CS2* promoter could not be induced after 4°C incubation^{27,31} at 48 h. It appears therefore, that different pathways regulate Arabidopsis P5CS transcript accumulation under cold and osmotic stress. The signals during salt stress appear to be mediated by ABA that can bring about the expression of stress-related genes and subsequently the synthesis of organic osmolytes. In some plants like Arabidopsis causal link between ABA and proline accumulation was suggested and it was shown that exogenous application of ABA increases the level of *AtP5CS1* and *AtP5CS2* transcripts^{24,101}. Genes that respond to water or salt stresses do not respond to the exogenous supply of ABA, indicating that both ABA-dependent and ABA-independent signal transduction pathways exist¹⁰². Proline accumulation appears to be mediated by both ABA-dependant and ABA-independent signalling pathways¹⁰¹⁻¹⁰⁶. Analysis of the promoter elements in the proline biosynthetic pathway genes such as *AtP5CS1*, *AtP5CS2* and *AtP5CR* indicated that a *cis*-acting ABA responsive element sequence is found in *AtP5CS2* upstream region³¹. The involvement of ABA in *P5CS* gene expression was reviewed by Hare *et al.*¹⁰⁴, who showed the regulation of *P5CS* gene expression in Arabidopsis ABA-insensitive mutants (*abi* mutants). Further, evidence was shown for the role of calcium in ABA-mediated gene induction of P5CS during drought and salinity¹⁰⁷. These authors also suggested that calcium alone is not sufficient for induction of P5CS transcript. These observations indicate additional signalling factor/s required for expression of *AtP5CS* gene in proline metabolism during stress, which might be upstream components of the proline biosynthesis signal transduction pathway. Recently, evidence was provided for the involvement of phospholipase D in the regulation of proline metabolism as an upstream component in *A. thaliana*¹⁰⁸. Moreover, these authors have shown that phospholipase D is regulated by calcium. Based on these results it becomes clear that phospholipase D is the other signalling component involved along with calcium in the regulation of proline biosynthesis¹⁰⁸. The data also indicated that the application of primary butyl alcohols enhanced the proline responsiveness of seedlings to mild hyperosmotic stress. Higher proline responsiveness was observed to hyperosmotic stress when phospholipase D was abolished.

Further, it was demonstrated that phospholipase D signalling for proline biosynthesis is similar to *RD29A* gene expression, but different from that of the ABA-dependant *RAB18* gene expression¹⁰⁷. The data also suggest that phospholipase D plays positive and negative roles in hyperosmotic stress signal transduction in plants, contributing to a precise regulation of ion homeostasis and plant salt tolerance. The type of phospholipase D molecule involved in negative regulation of proline metabolism needs to be identified in the near future. Several plant protein kinases were activated^{104,106} during osmotic stress adaptation, therefore, it appears that in cell-mediated signalling, protein phosphorylation is central and is implicated. It seems likely that ABA-mediated *P5CS* gene expression might work through pathway I as expressed by Hare *et al.*¹⁰⁴. However, it is not clear if plants use MAP kinase cascades to regulate the biosynthesis of proline and other osmolytes or whether they regulate other stress proteins. During prolonged dehydration or salt stress, *P5CS* gene is activated and the gene encoding PDH is suppressed. Revealing the signal transduction cascades of this reciprocal regulation of *P5CS* and *PDH* genes might decipher interesting results in near future.

Conclusion

Understanding the biosynthesis, degradation, transport, roles of proline during stress and the signalling events that regulate stress-induced accumulation is vital in developing plants for stress-tolerance. It is of paramount importance to identify stress-regulated promoter elements associated with the proline biosynthetic pathway genes and also their regulation. This will pave the way to develop transgenics with genes driven by stress-inducible promoters, which could be devoid of growth retardation due to accumulation of proline under non-stress conditions. Also, there is urgent need to identify signalling components heralding the events related to proline biosynthesis and degradation and their coordination in gene expression events under stress as well as during stress recovery. Several mutants that were generated over the years, especially in *Arabidopsis*, will provide a means to assess the exact role of proline during stress and also to elicit the signalling processes mainly related to stress relief. As we propose here, it is important to understand the role of proline clearly in stress and plant development and its implications on metabolism using available genetically engineered plants of proline biosynthetic pathway. In this context, it is important to use genomic approaches to study the available transgenic plants to identify the metabolic pathways influenced due to changes in proline levels. Upstream events of the proline biosynthesis signal transduction pathways and the transcription factors that induce proline biosynthetic genes and their characterization can perhaps unravel the complex molecular mechanisms of proline accumulation and its relation to redox signalling during salt stress tolerance.

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