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 transgenic 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 stress⁵⁷. 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 decreased 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 systems⁸–⁹. 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 proline⁸–¹³. 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 species⁷. 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 recently¹⁶. Accumulation of proline could be due to de novo synthesis

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or decreased degradation or both. 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 catalyzed to GSA by pyrroline 5-carboxylate synthetase (P5CS) in plants and other eukaryotes. 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 mutants. 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 domains. 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 synthetase.

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 characterized. 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 Arabidopsis. Atp5CS encoded a protein of 717 amino acids showing...
Table 1. Genes involved in the network of proline biosynthesis and degradation pathway

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

Genes of proline metabolism isolated from plants as well as from lower organisms are represented. Further, E.C. number, proline 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 protein. 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. AtP5CS1 gene of Arabidopsis is expressed in most organs, differentiated tissues and is primarily upregulated by dehydration, high salinity and abscisic acid (ABA) treatment, 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 synthesis. Similarly, there are two Pro loci in the nuclear genome of tomato and the cDNA clones, tomPRO1 and tomPRO2, specifying P5CS were isolated. 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. 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 synthesis. 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 (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 stresses\textsuperscript{29}. 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\textsuperscript{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.\textsuperscript{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\textsuperscript{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 protein\textsuperscript{30}. 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 plants\textsuperscript{1}. 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 site\textsuperscript{31}. 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 subcellular 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 osmoregulated\textsuperscript{32}. 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\textsuperscript{33}, A. thaliana\textsuperscript{34} and kiwifruit\textsuperscript{35}. AtP5CR was previously named AtP5C1 and the cDNA includes 2574-bp 5’ flanking region\textsuperscript{34}. P5CR transcripts as well as P5CR activity were reported to increase under stress in Arabidopsis\textsuperscript{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 potential\textsuperscript{36}. 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 primordia\textsuperscript{37}. 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 spinach\textsuperscript{38}. The P5CR gene is not identified in the chloroplast genomes\textsuperscript{39}, 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\textsuperscript{2+} ions. Murahama et al.\textsuperscript{39} showed the effects of NaCl and MgCl\textsubscript{2} on both isofoms were inhibitory. In contrast, P5CR activity of both pea chloroplasts and etiolated pea shoots increased twofold\textsuperscript{40} in the presence of 10 mM MgCl\textsubscript{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 concentrations\textsuperscript{38}. Interestingly, P5CR-2 was more stable to heat treatment at 40°C than P5CR-1 isoforn 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\textsuperscript{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\textsuperscript{35}. It was also demonstrated that P69
is necessary and sufficient to activate a minimal cauliflower mosaic virus 35S promoter in a tissue-specific manner. 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 reductants. Hua et al. 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. The location of this enzyme in plastids suggests that it may assist in counteracting photo-inhibitory 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. Arginine gets converted to ornithine by the enzyme arginase. In bacteria, ornithine is degraded to α-keto-δ-aminooxalate 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 proline 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 α-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 column 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 Km for ornithine was found to be 2 mM and 0.75 mM for α-ketoglutarate. Optimal activity of OAT was observed at 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 nitrogen.

Further, δ-OAT cDNA clones were isolated from Arabidopsis and its expression was studied during salt stress. 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, 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, 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 (PS5DHD; 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 balance. A nuclear gene encoding mitochondrial proline dehydrogenase enzyme was isolated from Arabidopsis. 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 P5C dehydrogenase (PSCDH; 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 Stewart. In cultured cells of Nicotiana plumbaginifolia exposed to NaCl, two Δ1-pyrroline-5-carboxylate dehydrogenase isoforms were expressed and were differentially modulated during the culture growth cycle. Surprisingly, both accumulation of proline as well as high activity of one of the isoforms of PSC 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 potato. 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 6-4 tetramer with subunits of an apparent molecular mass of about 60 kDa 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 chloride. 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.

Intermediate of proline biosynthesis and catabolism

It was found that intermediates of proline biosynthetic and catabolism such as glutamine and P5C could increase the expression of several osmotically regulated genes in rice 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 Cress, 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. Stimulation of phosphoribosyl pyrophosphate and purine nucleotide production by pyrrolidine-5-carboxylate was also reported in human erythrocytes. P5C in plants is converted back to glutamate 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 stress tolerance. 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 humans. 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) superfamily. Amino acid transporter superfamily has five sub-clades 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. 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. 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. 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 understood. It was reported that in alfalfa, proline transport processes play an important role in adaptation to osmotic stress. From Arabidopsis, eight different amino acid transporter clones were isolated and characterized using yeast mutants. Two of these encoded specific proline transporters (ProT) are distant ly related to the amino acid permease gene family. Though ProT 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. ProT may be playing a specific role of supplying proline to pollen grains. In contrast, mRNA levels of ProT2 were observed throughout the plant, but their expression was strongly induced by water or salt stress. 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. High proline concentrations were reported in the phloem sap of drought-stressed alfalfa. It is proposed that proline synthesis might be more in roots, but most of the production may be exported to shoot tissues. This is again consistent with the observation of active expression of both ProT and ProT2 in roots. Also, Verslues and Sharp 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. 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 zwiterionic state of GABA is an important parameter in substrate recognition. Also, ProT2-mediated transport was inhibited by propylene and quaternary ammonium compounds. In L. esculentum, proline transporters LeProT1, T2 and T3 that are homologous to Arabidopsis were isolated. 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. Direct uptake and competition studies demonstrated that LeProT1 constitutes a general transporter for compatible solutes. A cDNA encoding a ProT was isolated and characterized from rice also. ProT protein of rice had 68.8% homology to the ProT 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. also found that OsProT specifically transported L-proline in a transport assay. Recently, Andreasson et al. 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 tobacco. Studies using purified P5CS enzyme indicated that Vigna P5CS is feedback inhibited to 50% by 5 mM proline in vitro. 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 Δ1-pyrroline-5-carboxylate synthetase and the accumulation of proline in A. thaliana under osmotic stress. 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. 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.
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 proline, 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 stress. Similarly, P5CS gene was introduced into wheat using Agrobacterium-mediated gene transfer via indirect pollen system. 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 tolerance.

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 overexpressed. It was shown that transgenic algal 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 μM) and bound fourfold more cadmium than wild-type cells. Recently, *OsP5CS* was introduced into rice and the gene in transgensics 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 stress. The biochemical basis for NaCl tolerance was investigated using 

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

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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 PS5. Further, soybean plants were transformed with a P5CR gene construct in an antisense direction controlled by an inducible heat shock promoter (HSP70). 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 plumbaginifolia 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 organs77,78. 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 known77 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)19. This in turn provides precursors in the form of erythrose-4-phosphate to synthesize phenylpropanoids or secondary metabolites during stress conditions19,77. 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 nucleotides20 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 osmolutes 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 conditions59. 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 control59. 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 process57. In A. thaliana, expression of antisense P5CS inhibited proline production and made plants hypersensitive to osmotic stress57. 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 proteins57.

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-
concentrations, it has little or no perturbing effect on macromolecule-solvent interactions\textsuperscript{60}. Circumstantial evidence exists today for the role of compatible solutes as osmotic balancing agents and protection of subcellular structures\textsuperscript{81}. 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 

- **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\textsuperscript{65,68}. Exogenously supplied carbohydrates such as mannitol, sorbitol, mesoinositol, 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*. However, the amplitude response to the above sugars or sugar alcohols is less compared to NaCl or PEG-grown tissues\textsuperscript{65}. 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 NaCl\textsuperscript{65}. It appears, therefore, that sucrose behaves like a positive effector for proline accumulation. While proline did not influence the expression\textsuperscript{65} of P5CS, glycine betaine had a negative effect on stress-induced proline response\textsuperscript{65}. 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*. 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*. 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*. 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 synthesis. 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 the exogenous supply of GA and ABA, but not by IBA and kinetin in seedlings of niger. 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 roots. 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, 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 hypersosmotic 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. 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 10 h of treatment. Transcriptional activity of *AtP5CS2* promoter could not be induced after 4°C incubation 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 osmoles. 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. 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 exist. Proline accumulation appears to be mediated by both ABA-dependent and ABA-independent signalling pathways. 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 region. The involvement of ABA in P5CS gene expression was reviewed by Hare et al., 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 salinity. 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*. 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 biosynthesis. The data also indicated that the application of primary butyl alcohols enhanced the proline responsiveness of seedlings to mild hypersosmotic stress. Higher proline responsiveness was observed to hypersosmotic 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 expression\textsuperscript{107}. 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\textsuperscript{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 \textit{et al.}\textsuperscript{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.

\textbf{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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Received 6 February 2004; revised accepted 18 September 2004