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Metabolic and molecular-genetic regulation of proline signaling and its cross-talk with major effectors mediates abiotic stress tolerance in plants

Aryadeep ROYCHOUDHURY*, Aditya BANERJEE, Vikramjit LAHIRI
Post-Graduate Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, West Bengal, India

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Abstract: Proline (Pro) accumulation is a common response of several plant species to combat abiotic stresses. Under stress conditions, Pro acts as an excellent compatible solute in the plant system, participating in the alleviation of stress sensitivity. Though the metabolic pathways associated with Pro are well studied, parts of its regulatory cascades are still not properly known. It has also been conjectured that epigenetic modifications regulate Pro metabolism during abiotic stress. Apart from Pro, the plant abiotic stress responses are essentially mediated by multiple effectors. Hence, proper analysis of the cross-talks of Pro with the other components of the abiotic stress response has turned out to be mandatory in order to design multistress-tolerant transgenic lines. Highlighting the relation between Pro and seed germination is also essential to understand the notion behind plant susceptibility and survival during stress. Generally, Pro has a universal mechanism to generate abiotic stress tolerance through stabilization of structural components, enzyme structures, and regulation of osmotic adjustments. The success achieved through recent transgenic approaches leading to more accumulation of Pro in the sink has also been focused on in the present review.

Key words: Proline, compatible solute, epigenetic modification, cross-talk, seed germination, abiotic stress, stress tolerance

1. Introduction

Considering the percentage of land area affected and loss of crop productivity, study of abiotic or environmental stress biology and its management continues to be a significant area of research in plant biotechnology. A report by the Food and Agricultural Organization in 2007 stated that only 3.5% of the global land area is free from any environmental constraints (http://www.fao.org/docrep/010/a1075e/a1075e00.htm). Thus, knowledge for combating abiotic stresses is essential to genetically design major stress-tolerant food crops. This is because their production is expected to decline in the future due to dearth of arable land, depletion of water resources, global warming, and drastic climatic alterations (Cramer et al., 2011). Globally, high salinity affects the largest count of crop production on at least 20% of irrigated land. The middle of the 21st century is forecasted to have around 50% loss in cultivable land due to increased salinity. Dehydration stress studies have also been diverted to salt stress because the response patterns in both are almost the same. Temperature in the subzero range results in the formation of ice crystals within the tissue intercellular spaces (Roychoudhury et al., 2013). Heavy metal toxicity, leaf wilting, electrolytic leakage, leaf abscission, changes in leaf area, generation of reactive oxygen species (ROS), accumulation of free radicals disrupting cellular homeostasis by membrane lipid peroxidation, etc. are among the various adverse effects of abiotic stresses on plants. Thus, the viability of each cell is challenged under such circumstances. Abiotic stresses are multigenic in nature, being governed by multiple loci and also occurring at multiple stages. Often, the plant is simultaneously affected by multiple stresses. Therefore, designing a genetic model that is stress-tolerant is extremely difficult and challenging (Yamaguchi-Shinozaki and Shinozaki, 2006). Stress-tolerant plants like *Craterostigma plantagineum*, *Mesembryanthemum crystallinum*, and *Thellungiella halophila* can be used as valuable models in which the systems biology can be studied and integrated in crop plants to enhance stress tolerance (Bartels and Sunkar, 2005).

Unlike animals, plants are sessile and do not have the advantage of fleeing away from adverse sites; they must stand and resist the stress conditions. Vivid molecular responses at the biochemical and molecular level are exhibited by plants to overcome stressful conditions (Roychoudhury and Nayek, 2014; Banerjee and Roychoudhury, 2015a, 2015b). The molecular responses involve interactions and cross-talks among several related

* Correspondence: aryadeep.rc@gmail.com

pathways. The universal stress hormone abscisic acid (ABA) acts as the chief regulator of abiotic stress responses, including osmotic stresses. The main physiological function of ABA during stress is to promote stomatal closure, minimize transpiration, and hence conserve water in the cells. ROS and reactive nitrogen species (RNS) are almost always the earliest signals in abiotic stresses (Molassiotis and Fotopoulos, 2011). ROS and RNS aid in the activation of a coordinated network of responses, among which those for the nitrosative effects of RNS are less documented (Cramer et al., 2011). A network of long-distance signaling is triggered by plants at the molecular level for perception of stress cues. This is accompanied by transduction of signals to activate the adaptive responses and finally cellular responses. Switching-on of a broad class of stress-specific genes, generally called the *osmotic stress responsive* (*OR*) genes, activates the cellular responses. The differences in signal perception and transduction mechanisms determine the differences in the level of stress tolerance among various genotypes of a plant species (Roychoudhury et al., 2013). The root growth and osmotic adjustment (OA) aid in maximizing water uptake during dehydration stress. This, along with minimizing stomatal and cuticular water loss, results in lower osmotic stress in the plants under such conditions. The osmotic homeostasis maintenance during salt stress and prevention of ice-nuclei formation in cold stress is done by OA. Plants mediate stress signaling via mainly three approaches: 1) reestablishing cellular homeostasis, via ionic and osmotic stress signaling; 2) controlling the extent of damages and inducing repair through a detoxification mechanism; 3) signaling to overcome the stressful period and aid the plant to regenerate with new potential through coordination of cell division and tissue expansion (Roychoudhury et al., 2013).

2. Compatible solutes

Compatible solutes help the plant cells to maintain optimum turgor and resume growth during ionic stress through OA. This is done by efficient water uptake by the reduction of the cytosolic osmotic potential. Such compatible solutes or osmolytes are extremely crucial for water retention during dehydration stresses, as they can maintain the hydration sphere of proteins, utilizing their high polarity and hydrophilicity (Roychoudhury and Chakraborty, 2013). Two theories, the 'preferential exclusion model' and 'preferential interaction model', have been proposed regarding the role of compatible solutes in water retention and stabilizing the protein structures. The preferential exclusion model proposes the exclusion of compatible solutes from hydration shell of proteins. This enhances protein stability and mutual interactions among proteins. The preferential interaction model basically depicts the interactions occurring between proteins and compatible solutes (Bohnert and Shen, 1999). Compatible solutes are potential low-molecular-weight chaperones stabilizing macromolecular assemblies and protein folding. The reduction of the inhibitory effects of ions on stress-induced enzyme activities is regulated by such compatible solutes to increase their thermal stability. Compatible solutes also prevent the dissociation of enzyme complexes and scavenge the toxic hydroxyl radicals to preserve the membrane and hence the cellular integrity. It can be suggested that compatible solutes also stabilize the photosystem II complex (Fatemeh et al., 2012). Osmolytes have been reported to function as cryoprotectants, thereby implicating a role in protection from cold stress and freeze–thaw cycles (Wangxia et al., 2003), an indication of overlap between cold stress and salinity stress responses. Common osmolytes accumulated during stresses are major carbohydrates like sucrose, fructose, and glucose; sugar alcohols like pinitol, ononitol, and cyclitol; polyols like adonitol, sorbitol, mannitol (all straight chain compounds), and myo-inositol (cyclic polyols); complex sugars like trehalose, raffinose, and fructans; free amino acids like proline (Pro) and glycine betaine (GB); organic acids like lactate, malate, citrate, succinate, fumarate, benzoate, salicylate, malonate, and γ-amino butyric acid (GABA); free ammonia and quaternary ammonium compounds like β-alanine-betaine, Pro-betaine, and hydroxyproline-betaine; and tertiary sulfonium salts like dimethylsulfoniopropionate and choline-o-sulfate (Hayat et al., 2012; Roychoudhury and Chakraborty, 2013). Salinity induces hyperaccumulation of Na^+ and Cl^-, but these do not act as osmolytes as they interfere with cellular functions at high concentrations and need to be sequestered to the vacuole. Compatible solutes, however, are not sequestered to vacuoles; they remain mostly in the cytosolic and chloroplastic compartments (Shinozaki and Yamaguchi-Shinozaki, 1999) where they perform their protective functions.

The low-molecular-weight aliphatic amines or polycations playing protective roles during stress are the polyamines (PAs), which constitute another major group of compatible solutes. At physiological pH, the positive charge residing on them helps in the interaction with the cell membrane. Thus, disintegration of the membrane during osmotic stress is prevented by the PAs. The potency of this action is maximum in tetramine spermine (Spm), followed by triamine spermidine (Spd) and then diamine putrescine (Put) (Roychoudhury and Das, 2014). The rate limiting step of PA biosynthesis is catalyzed by S-adenosylmethionine decarboxylase (SAMDC). The diamine oxidase (DAO) catalyzes Put catabolism, while polyamine oxidase (PAO) catalyzes Spd and Spm catabolism. Wimalasekera et al. (2011) showed

that *Arabidopsis* copper amino oxidase 1 (CuAO1), responsible for the degradation of Put, was a potential contributor of ABA-induced NO production and that this activity plays a central role in most stress responses. NO is responsible for posttranslational S-nitrosylation of proteins. Under stress conditions in *Citrus*, 271 proteins were found to be regulated by Put, Spm, and Spd by S-nitrosylation. The first plant PA-transporter, called the PA uptake transporter 1 (OsPUT1), was characterized in all tissues of rice, except seeds and roots (Mulangi et al., 2012). Cross-talk between the PA signaling pathway and ABA signaling has been reported (Roychoudhury and Das, 2014). The maximum accumulation of Put and Spd was recorded in rice seedlings treated with exogenous ABA. Total soluble PA content in ABA-treated aromatic rice variety Gobindobhog also increased, showing its salt-susceptible nature (Roychoudhury et al., 2009). It has been hypothesized that PAs stimulate the DNA binding activity of some TFs to the recognizing abscisic acid responsive elements (ABREs) of target genes (Roychoudhury and Das, 2014). The PAs control the activity of several ion channels indirectly by regulating the plasma membrane potential. This is mediated by the activation of H^+/ATPase and interaction of PAs with the 14-3-3 proteins (Garufi et al., 2007). During abiotic stresses, Spm inhibits stomatal opening and promotes closure by regulating the KAT1-like voltage-dependent inward K^+ channel in the guard cells of *Vicia faba* (Kusano et al., 2007a, 2007b).

Salt tolerance involves ion homeostasis and the inhibition of ROS by certain plasma membrane ion channels like the nonspecific cation channel (NSCC), while some other cation channels are activated by ROS-induced conductance (ROSIC). Such changes often promote efflux of K^+ and influx of Na^+. PA regulates ion homeostasis and also balances the Ca^{2+} level in cells. A recent model of Ca^{2+} homeostasis in plants has been put forward to be as follows: 1) ROS formation, degradation, and transport; 2) PA catabolism and transport; 3) feedback loops of NSCC, ROSIC, and NADPH oxidase; and 4) PA- and ROS-dependent Ca^{2+} pump activation (Pottosin et al., 2012).

All the compatible solutes, about which we have made a brief mention above, individually hold immense potential in helping the plant system to battle and win against the environmental challenges. Each compatible solute has a unique mode of action and downstream effects. However, inappropriate and incorrect gene expression of osmolytes is often accompanied by pleiotropic effects like growth retardation and necrotic lesions due to interference with the normal pathways of primary metabolism. To avoid such a scenario, metabolic engineering must be made stress-inducible and/or tissue-specific (Garg et al., 2002). In the following sections of this review, we broadly discuss the free amino acid Pro as a representative osmolyte, with a detailed focus on its interaction with other stress regulators and its role as a potent mediator of abiotic stress responses. In spite of more than 40 years of study on the role of Pro in combating abiotic stress, bits and pieces of information remain astray. Several observations on Pro by different groups, as knit together in this review, will probably aid in opening future prospects of research in this field.

3. Pro: a general account

Pro is often regarded as an imino acid due to the presence of a secondary amino group. The cyclic structure of Pro dictates restricted flexibility in conformation, for which it decides on the stability of secondary protein structures. Pro is a necessary and most common compatible solute found in diverse families of plants and bacteria experiencing abiotic stress. These properties make Pro unique among the proteinogenic amino acids (Lehmann et al., 2010). As mentioned earlier, Pro serves as a potent compatible solute during stress. Due to low molecular weight and highly soluble organic nature, Pro is nontoxic for the cell at high concentrations. High levels of Pro have been detected in plant species under stresses like high soil salinity, dehydration and water scarcity, chilling temperatures, ROS-mediated oxidative stresses, heavy metal toxicity, and ultraviolet (UV) ray exposure. Pro accumulation under abiotic stresses depends on the plant species and can be up to 80% of the cellular amino acid pool. This is mainly due to the increased synthesis of Pro, accompanied with its decreased degradation under such unfavorable conditions (Kavi Kishor et al., 2005). Salt stress in *Arabidopsis* triggers Pro accumulation to about 20% of the total amino acid pool (Liu and Zhu, 1997). Pro is also a stabilizer of the plasma membrane and the subcellular structures, which are necessary for the viability of the stressed cell. In such cases, Pro often acts as a protein-compatible hydrotope, alleviating cytoplasmic acidosis. The metabolic $NADP^+$/NADPH ratio, cellular pH, and cellular redox status are also maintained by Pro under stress conditions (Hayat et al., 2012). The members of Solanaceae can increase their Pro pool by more than two orders of magnitude when exposed to abiotic stress (Djilianov et al., 2005). The modern world is likely to face a tremendous shortage of food in coming times. Thus, it becomes the onus of agricultural biologists to carve out plans in order to solve such food scarcity problems in the future. It has been identified that several crop plants like rice that are obviously glycophytes mostly die of abiotic stresses, about which we have mentioned above. Pro as a compatible solute has shown a ray of hope in combating such stresses in crop plants through its overexpression in the appropriate tissue cells via genetic engineering. Exogenous application of Pro has also gained tremendous impetus in developing stress tolerance in

some plant species. Since the first report of the role of Pro in defeating stresses in wilting perennial rye grass (*Lolium perenne*), the quest to utilize this potentiality in crops has been on the rise (Szabados and Savoure, 2009).

4. Pro metabolism and transport

4.1. Pro biosynthesis

4.1.1. From glutamate

Pro biosynthesis was first characterized in bacteria (Kavi Kishor et al., 2005). Here, Pro is formed from glutamate via three steps. The pathway is initiated by the conversion of phosphorylated glutamate to γ-glutamyl phosphate and next to glutamate-γ-semialdehyde (GSA) by γ-glutamyl kinase and glutamate-γ-semialdehyde dehydrogenase respectively. The Δ^1 pyrroline-5-carboxylate (P5C) is subsequently formed from GSA via spontaneous cyclization. The direct catalysis of glutamate by the P5C synthetase (P5CS) yields GSA in plants and other eukaryotes. This reaction requires both reduced nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine trinucleotide phosphate (ATP). The P5C reductase (P5CR) then reduces P5C to Pro, utilizing NADPH in both prokaryotes and eukaryotes (Kavi Kishor et al., 2005; Lehmann et al., 2010). The γ-glutamyl kinase catalyzes the rate-limiting step of Pro biosynthesis in bacteria and yeast. The rate-limiting step in the case of plants is P5CS, which is regulated via the allosteric inhibitory effect of the end product of the pathway, i.e. Pro (Sekine et al., 2007).

The genomes of plants like *Arabidopsis*, *Medicago sativa*, *Medicago truncatula*, and *Oryza sativa* exhibit two homologous genes encoding P5CS (Armengaud et al., 2004). With specific roles varying among plant species, *P5CS* paralogs were reported to have different functions during plant life and development (Kavi Kishor et al., 2005). Blockage of Pro accumulation occurred in *Arabidopsis p5cs1* and *p5cs2* knockout mutants, due to the nonredundant activities of the mutated enzymes (Székely et al., 2008). The observed functional specialization of the *P5CS* genes was accredited to the duplication of the genes after monocot and dicot divergence, as revealed through phylogenetic analysis (Turchetto-Zolet et al., 2009). The normal site of P5CS1 localization is in the cytosol of leaf mesophyll cells as seen through green fluorescent protein (GFP) fusion tracing. However, in the embryonic cells and roots, its localization occurs within organelles similar to fusiform bodies (Szabados and Savoure, 2009). Experimental evidence also shows that P5CS1-GFP gets localized in the chloroplasts when the plants are exposed to salt stress. The P5CS2-GFP in *Arabidopsis* was found to accumulate in the cytosol (Székely et al., 2008). Thus, it can be inferred that the housekeeping Pro biosynthesis is mediated by the *P5CS2* gene. In response to abiotic stresses, Pro biosynthesis gets shifted to the chloroplast under the control of the stress-induced *P5CS1* gene (Szabados and Savoure, 2009). Copper-induced Pro accumulation has been reported in detached leaves. Such accumulation is mediated by abscisic acid (ABA) (Chen et al., 2001), and it was also associated with nitric oxide (NO) generation in *Chlamydomonas reinhardtii* (Zhang et al., 2008). This intracellular NO plays a potent role in Cu-induced Pro synthesis and downstream stress signaling. Further investigations disclosed that this effect was mainly due to the application of sodium nitroprusside, which potentially acts as a NO donor. This NO enhanced the activity of P5CS in the Cu-treated algae. This observation of Pro accumulation was not recorded when a NO scavenger instead of a NO donor was used (Zhang et al., 2008).

Biochemical identification of two isoforms of P5CR protein was done in pea and spinach. It remains unclear whether the origin of the two isoforms was from one or two genes (Murahama et al., 2001; Lehmann et al., 2010). The case is more clear for *Arabidopsis*, as it has been determined that a single gene encodes P5CR. The P5CR accumulates in plastids in response to unfavorable conditions, while the housekeeping concentration of Pro under normal conditions is maintained by P5CR in the cytosol (Szabados and Savoure, 2009). The P5CR activity maintains the redox potential of the cell by affecting the reduction of NADPH. The localization of P5CR in the plastids also indicates the function of this enzyme in counteracting the photoinhibitory damage of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) enzyme under adverse abiotic stress conditions (Kavi Kishor et al., 2005).

4.1.2. From arginine

Arginase converts arginine to ornithine, from which α-keto-δ-aminovalerate is generated by the enzyme ornithine-α-aminotransferase (αOAT). The α-keto-δ-aminovalerate is spontaneously cyclized to pyrroline-2-carboxylate (P2C) by the catalysis of P2C reductase. This pathway has not yet been reported in plant systems (Kavi Kishor et al., 2005). However, in plants, ornithine-δ-aminotransferase (δOAT) catalyzes the formation of P5C and glutamate from ornithine and α-ketoglutarate, via transamination reactions (Stranska et al., 2008). The normal Pro accumulation was observed in *δoat* mutants of *Arabidopsis*. The plants could not mobilize nitrogen from arginine or ornithine. This indicates that rather than its role in Pro biosynthesis, δOAT enzyme mainly regulates arginine degradation (Funck et al., 2008). The localization of δOAT is mainly in the mitochondria. This prevents the direct utilization of the δOAT-generated P5C by P5CR, due to the localization of the latter in the cytosol or plastids (Funck et al., 2008). More δOAT activity was observed in younger plants of *Arabidopsis* than older ones. In such young seedlings, the Pro content, *P5CS* mRNA, and δOAT activity increased under salt stress. Electroporation of *Vigna aconitifolia* cDNA expression library in Pro

auxotroph mutants of *Escherichia coli* restored the Pro prototrophy. The isolation of the cDNA clones encoding δOAT was successful through utilization of this novel '*trans*-complementation' (Kavi Kishor et al., 2005).

4.2. Pro catabolism and degradation

In plants and all higher eukaryotes, the site of Pro catabolism is the mitochondria. FAD as a cofactor is required for the oxidation of Pro to P5C by proline dehydrogenase (PDH), bound to the inner mitochondrial membrane. This step generates NADP/NADPH cycling or redox balance. The conversion of P5C to glutamate is carried out by pyrroline-5-carboxylate dehydrogenase (P5CDH), using NAD^+ as the cofactor (Tanner, 2008). Analyses in *Arabidopsis* and tobacco indicate the clear presence of two homologous *PDH* genes (Ribarits et al., 2007; Verbruggen and Hermans, 2008). Such instances have not been firmly reported for P5CDH, since this enzyme is encoded by a single copy of the corresponding gene in all plant species, though there is evidence that two copies of *P5CDH* are present in *Nicotiana plumbaginifoila* and *Zea mays* (Mitchell et al., 2006; Lehmann et al., 2010). A substantial level of identity was found between the cDNA clone of *Arabidopsis ERD5* (early responsive to dehydration stress), localizing in the mitochondria and yeast *PUT1* (proline utilization) (Kavi Kishor et al., 2005). Redox homeostasis is ultimately maintained through the cycling of Pro via its catabolic and anabolic pathways through glutamate. This is because the mitochondrial fraction had enhanced levels of ERD5 in response to Pro, added to *Arabidopsis* cultured cells, and this high accumulation was not detected when the cultured cells were undergoing dehydration. *ERD5* was, however, upregulated when the cells were rehydrated (Kavi Kishor et al., 2005).

4.3. Transport of Pro

The biosynthetic and catabolic compartmentalizations of Pro give a clear indication about the importance and intensity of Pro transport between the cytosol, chloroplasts, and mitochondria. Specific Pro transporters have been recruited by the cell for this purpose. The mitochondria of *Triticum durum* contains a Pro uniporter, which aids in the passage of Pro into the mitochondrial matrix. A Pro-glutamate shuttle between the cytosol and the mitochondrial matrix has also been identified in a Pro/glutamate antiporter in the mitochondrial membrane of *T. durum* (Di Martino et al., 2006). The delivery of arginine and ornithine through the mitochondrial membrane is sustained by the basic amino acid (BAC) transporters (Palmieri et al., 2006). The de novo biosynthesis, along with Pro transport, is essential. This was supported by the fact that the halophyte *Limonium folium* accumulated Pro in the vacuoles under normal conditions. When exposed to salt stress, high amounts of Pro remained in the cytoplasm (Gagneul et al., 2007). Some transporters have also been identified in *Arabidopsis* and tomato pollens. Under stresses, three transporters (Pro T1, Pro T2, and AAP_6) of the amino acid permease (AAP) family were expressed in *Arabidopsis*. The plants experiencing salt stress showed ubiquitous expression of Pro T1 in roots, stems, and flowers. Under dehydration stresses, Pro T2 was expressed with AAP_6 expression restricted to the sink tissues of roots and cauline leaves (Hayat et al., 2012).

4.4. Regulatory mechanism in Pro metabolism

CONSTANS (CO) is a transcriptional activator in the flowering pathway in *Arabidopsis*. It promotes flowering in response to long day length. *P5CS2* is also another target of CONSTANS (Samach et al., 2000). *P5CS2* expression is also upregulated during hypersensitive responses triggered by avirulent bacteria, salicylic acid (SA), and ROS (Fabro et al., 2004). The osmotic and salinity stresses induce higher expression of *P5CS1* through ABA-dependent and ABA-insensitive 1 (ABI1)-mediated pathways and also through H_2O_2-mediated signaling (Verslues et al., 2007). Light promotes *P5CS1* expression, while brassinosteroids inhibit the same (Szabados and Savoure, 2009). Under normal conditions, phospholipase D (PLD) functions as a negative regulator, and under salt stress, phospholipase C (PLC) and calcium function as positive regulators of Pro accumulation (Roychoudhury et al., 2013). However, the reverse effects of PLD and PLC on Pro accumulation was found in the halophyte *Thellungiella halophila*. The CaM4 calmodulin mediates the calcium burst and interacts with MYB2 TF in its active conformation. Activated MYB2 upregulates the transcription of *P5CS1* (Ghars et al., 2008). Both ABA-dependent and ABA-independent signaling pathways can influence Pro accumulation. Calcium plays an important role in Pro accumulation through the ABA-dependent pathway (Roychoudhury et al., 2013). The TFs that elevate abiotic stress response through the ABA-dependent pathway belong to the MYC/MYB families. Transgenic lines of *Glycine max* overexpressing *GmMYB76* showed higher expression levels of *responsive to dehydration29B* (*rd29B*), *DREB2A*, *P5CS*, *rd1*, *early dehydration inducible10* (*erd10*), and *cold-regulated78/responsive to dehydration29A* (*cor78/rd29A*). On the contrary, the expression levels of *rd29B*, *cor6.6*, *cor15a*, and *cor78/rd29A* was lowered in *GmMYB92* transgenic plants, though the levels of *DREB2A*, *rd17*, and *P5CS* remained high. This shows the different levels of stimulated tolerance depending on the nature of the host plants (Roychoudhury et al., 2013). Several complex developmental and osmotic regulations also decide the activity level of P5CR.

Plant growth regulators like indole-3-butyric acid (IBA), ABA, and kinetin when added exogenously mimicked and initiated Pro accumulation, similar to that during salt and water stress, in *Guizotia abyssinica* (Sarvesh et al., 1966). The benzyl aminopurine (BAP) induced the

same in *M. crystallinum*, while gibberellic acid (GA) did not. When NaCl and ABA or NaCl and kinetin were added, the response was additive, unlike when a combination of inducing phytohormones was administered, leading to a higher level of Pro accumulation than either individually. This is indicative of the fact that NaCl- or phytohormone-induced Pro accumulations are the end results of different signaling pathways (Sarvesh et al., 1966). Thus, salt and growth regulators are the independent initiators of Pro accumulation during stress. The convergent effect of the hormones in upregulating Pro biosynthesis during periods of abiotic stress remains to be completely worked out, although NaCl-induced growth inhibition can be alleviated by exogenous addition of GA and ABA (Sarvesh et al., 1966). The kinetin and ABA when supplied did not show the same response. The balance between cytokinin and ABA is also of significant importance. During stress, the level of ABA rises and that of cytokinin falls. In *Arabidopsis*, cytokinin reduced the level of *AtP5CS1* mRNA, while BAP enhanced *AtP5CS2* mRNA in leaves (Hu et al., 1992). In either case, the level of expression in the root was unaffected (Figure 1).

In contrast to the biosynthetic branch, Pro catabolism is accelerated under dark and stress-free conditions through PDH and P5CDH. A Pro- and hypoosmolarity-responsive element (PRE) motif ACTCAT has been identified through promoter analysis of the *PDH* gene. The TFs that bind to these motifs to activate *PDH* transcription are AtbZIP-2, AtbZIP-11, AtbZIP-44, and AtbZIP-53, all belonging to the bZIP family of TFs. A network of group-S bZIPs regulates *PDH* expression. This has been analyzed mainly through chromatin immunoprecipitation (Weltmeier et al., 2006). Pro can up regulate *P5CDH* expression, which under normal conditions is transcribed at low basal levels. The promoters of *P5CDH* in *Arabidopsis* and cereals also contain a short sequence similar to the PRE motif. The wounding and pathogen attack activates the *FIS1* gene, which encodes P5CDH in flax (*Linum usitatissimum*) (Mitchell et al., 2006). Small interfering RNA (24 nt and 21 nt siRNA) in *Arabidopsis* is generated through the natural antisense overlapping of the 3' untranslated region (UTR) of *P5CDH* and the salt-induced *SIMILAR TO RCD ONE 5* (*SRO5*) genes. The transcript levels of *P5CDH* are reduced by the cleavage of *P5CDH* by the generated siRNAs during stress, thus aiding in the accumulation of Pro (Szabados and Savoure, 2009).

4.5. Changes in DNA methylation pattern governs Pro accumulation

The modulation in DNA methylation induced by abiotic stress may play a functional role in plant stress tolerance (Karan et al., 2012). The metabolic processes may be subjected to regulation at the level of gene expression. DNA

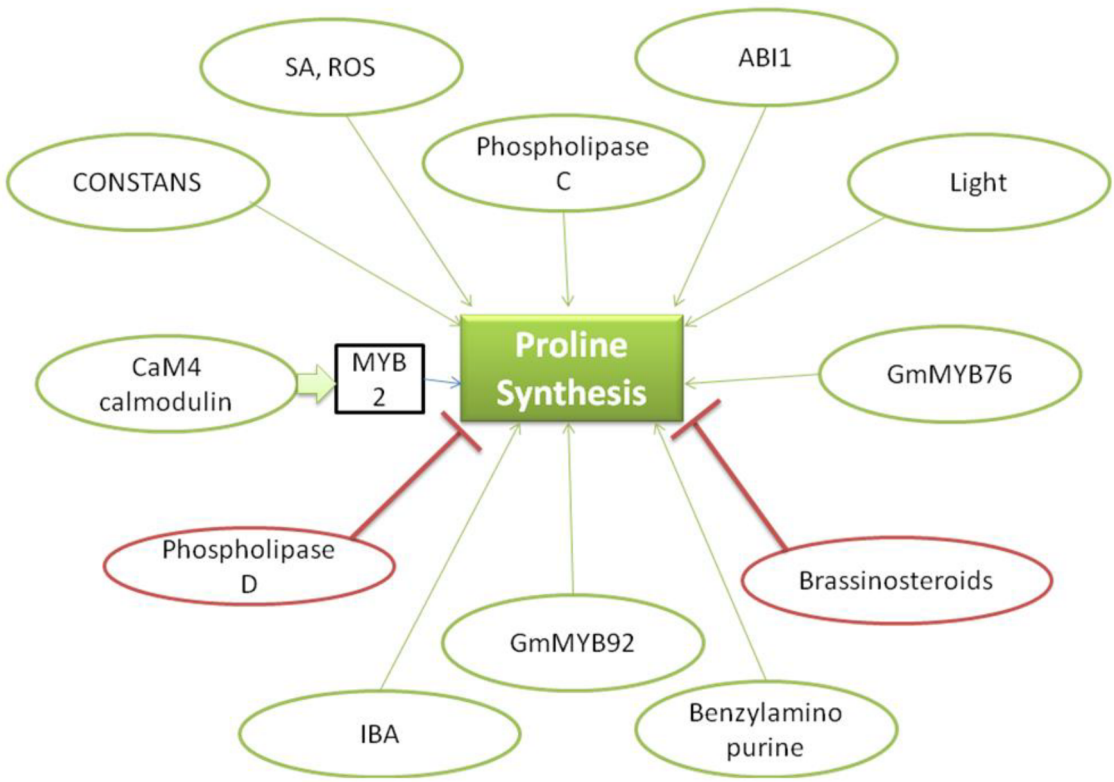

Figure 1. The biosynthesis of Pro under stressed conditions is responsible for the essential triggering of multiple factors. Inhibitory regulation is also achieved through phospholipase D and brassinosteroids.

methylation patterns and, more importantly, changes in the same are integral components of the epigenetic code, which is instrumental in marking transcriptional status of genes, with methylation generally being an inhibitory signal for transcription. Pro metabolism is also regulated by DNA methylation (Chan et al., 2005). The DNA methylation pattern of the three key genes involved in Pro biosynthesis was analyzed using methylation-sensitive amplification polymorphism and methylation-sensitive Southern blotting, where enhanced Pro accumulation in plants exposed to 15% (w/v) polyethylene glycol (PEG) was recorded, as opposed to control plants (Zhang et al., 2013). Many studies have demonstrated that osmotic stress induces increases in *δ-OAT* and *P5CS* abundance and activity. Thus, upregulation of *P5CS* and *δ-OAT* expression may contribute to the accumulation of Pro in response to osmotic and salinity stress (Verslues and Sharma, 2010). The selfed progenies (S1 and S2) of osmotically stressed plants (S0) accumulated higher concentrations of Pro in leaves under both normal and osmotic stress conditions than the unstressed control plants. In these S1 plants, *P5CS* and *δ-OAT* showed DNA demethylation in response to osmotic stress. The methylation state of the other gene, *P5CR*, was however unaffected. The nonrandomness of the change was validated by a housekeeping gene used as a control. The demethylation of *P5CS* and *δ-OAT* and their consequent upregulation contributed to the enhanced Pro synthesis and accumulation (Zhang et al., 2013). More significantly, most of these stress-induced epigenetic modifications are reset to the basal level once the stress is relieved, but some of the modifications may be stable, i.e. they may be carried forward as "stress memory" (Chinnusamy and Zhu, 2009) (Figure 2). This causes heritable changes in methylation pattern and consequently the phenotype in subsequent generations, thus helping the plants to cope with osmotic stress (Zhang et al., 2013). However, it cannot be confirmed whether the overexpression of the alleles undergoing stable epigenetic modifications (e.g., demethylation of *P5CS*) (Figure 2) to develop "stress memory" is suppressed via

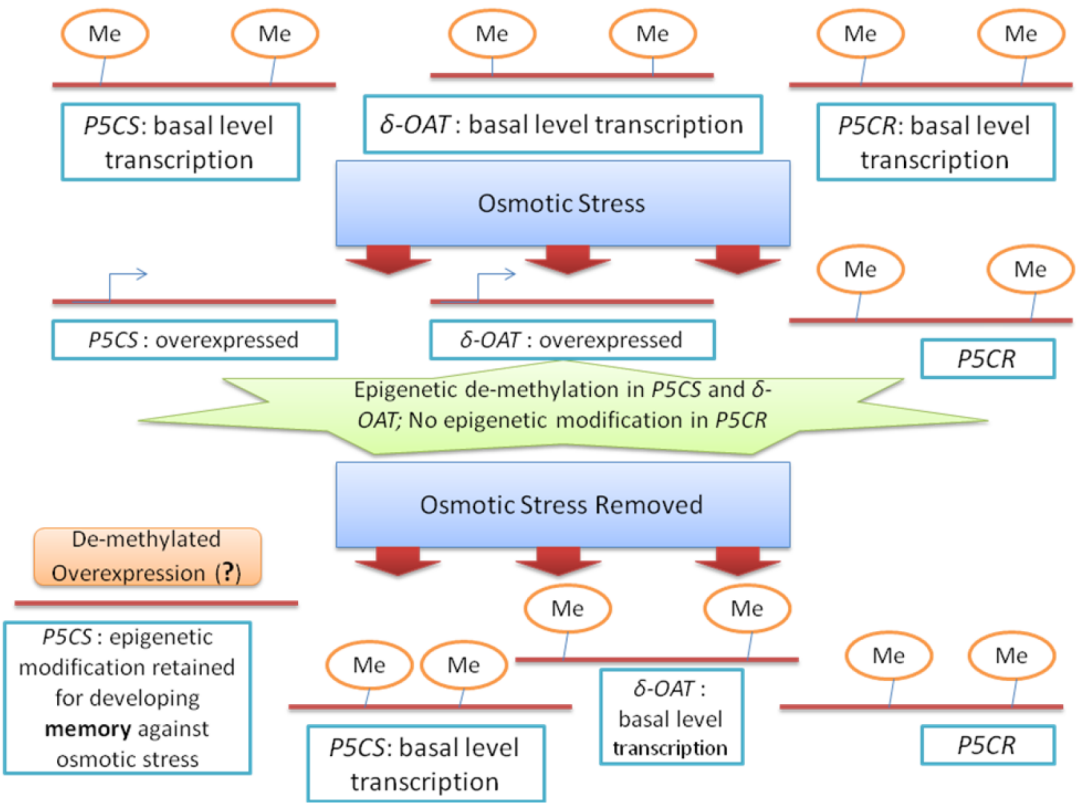

Figure 2. Epigenetic modifications are mainly responsible for overexpression of *P5CS* and *δ-OAT* in response to osmotic stress. *P5CR* expression is also responsible for overaccumulation of Pro. However, the overexpression pattern of this gene has not been found to be manipulated by epigenetic changes. On application of osmotic stress, demethylation occurs in *P5CS* and *δ-OAT*, probably leading to high promoter accessibility to the transcriptional complex and hence higher transcription rates. Upon removal of stress, basal levels of transcription are restored. However, some instances have shown that the modifications remain stable in order to develop a memory against osmotic stress. Such memory may trigger heightened responses against osmotic stress once the plant system faces it again. It is unknown how the overexpression of the alleles that undergo stable epigenetic modifications is suppressed after removal of stress.

other chromatin modifications after the stress is removed. Further studies are required for a definitive answer to this pertinent question.

5. Protective roles of Pro: cross-talks with other signaling pathways

5.1. Pro: plant–water relations, photosynthesis, and growth

Stress directly affects plant–water relations, indirectly affecting photosynthesis. This is because water uptake, ascent of sap, stomatal functioning, and chlorophyll biosynthesis are altogether hampered during stress, ultimately leading to reduced leaf water potential (Roychoudhury and Chakraborty, 2013). Exogenous Pro treatment resulted in a significant increase in the leaf water potential in *Vicia faba* during salinity stress. Restoration of photosynthesis occurred in *Olea europaea* 'Chemlali' facing salt stress. The pattern of restoration maintained a linear relation with increasing concentration of the exogenous Pro added (Ben Ahmed et al., 2010). Exogenous Pro in comparison to other compatible solutes like GB was more efficient in alleviating NaCl-generated stress in tobacco cells. If compared in terms of distribution, the resistance due to the effect of exogenous Pro was higher in the case of stomata on the abaxial surfaces than those on the adaxial surfaces. The even more striking fact is that lower concentrations of exogenous Pro have been found to be more effective than ABA spray in increasing stomatal resistance and also in maintaining turgidity in the leaves of stressed barley and wheat (Hayat et al., 2012). Pro also plays an important role in stabilizing the mitochondrial electron transport system (ETS) and other proteins including the CO_2-fixing RUBISCO, thus providing a direct link between Pro application and increased photosynthetic yield. Another means by which Pro regulates plant growth and development is by being the constituent of what are called Pro-rich proteins (PRP). A study showed that a hydroxyproline-rich protein called Sickle (Sic) is important in development and stress tolerance in *Arabidopsis*. The downregulation of the *sic* gene by miRNA-mediated posttranscriptional gene silencing produced plants with loss of function and undesirable characteristics such as dwarfism, delayed maturity and flowering, abnormal inflorescence or phyllotaxy, and serrated, sickle-shaped leaf margins. This demonstrated the importance of the Pro-regulated Sic protein for normal development in *Arabidopsis*. Pro also acts to promote hypersensitive response (HR) followed by programmed cell death (PCD) in incompatible plant–pathogen interactions. In such a scenario, there is an increase in ROS and Pro concentration in the infected tissue (Ayliffe et al., 2002). Interestingly, Pro plays a role in the modulation of *Agrobacterium* infection (Haudecoeur et al., 2009). Pro acts as the antagonist of the GABA-mediated quorum-quenching mechanism that protects the plants from colonization by *Agrobacterium*, thus allowing successful infection and transfer of the Ti plasmid.

5.2. Cross-talk between Pro and ABA

One of the major structural proteins in the plant cell wall is hydroxyproline-rich glycoproteins (HRGP). Based on domain characteristics, HRGPs can be classified into: 1) Pro-rich proteins with $(Pro)_3XYLys$ repeats; 2) extensin-type proteins with $Ser(Pro)_{3-5}$ repeats; and 3) arabinogalactan proteins (AGPs) with central domains rich in (Ser/Ala/Thr) Pro repeats. The nonbranched arabinose (Ara) oligosaccharides on hydroxyproline (Hyp) primarily contain the Pro repeats. The AGPs play important roles in plant growth and development, as both membrane-bound and secreted AGPs play roles in cell division, cell expansion, apoptosis, floral abscission, pollen tube guidance, pollen incompatibility, and plant–microbe interactions (Seifert and Roberts, 2007; Tseng et al., 2013).

Under abiotic stress, ABA affects the development and functions of the roots. The repetitive proline-rich proteins (RePRPs) have come up as chief examples of the cross-talk existing between ABA and structural Pro. The RePRPs in rice are ABA-responsive and root-specific proteins, with unusual PX_1PX_2 sequence motifs (Tseng et al., 2013). The first Pro-rich glycoprotein identified in rice was the shoot-specific OsPRP1 (Akiyama and Pillai, 2003). *OsPRP1* was suppressed by ABA and treatment with methyl jasmonate. The Pro content in specific rice RePRPs was found to be around 40%, with the X_1 and X_2 in the unique motif PX_1PX_2, containing polar residues like Lys, Asn, Glu, or Gln. These RePRPs are not classical AGPs as they do not bind to β-Yariv reagent and are heavily glycosylated with Ara and glucose, instead of Ara and galactose. The *ZmPRP* in *Zea mays* was found in the xylem of the root maturation region and are thought to be involved in secondary cell wall formation. *WPRP1* from wheat has been found in rapidly dividing tissues in shoots. Such PRPs with the unique PX_1PX_2 motif have not been identified in dicots (Tseng et al., 2013). The RePRP accumulation is inhibited by stress or ABA treatment, resulting in decreased growth rate in the elongation zone. However, in order to maintain the root length, the normal functioning of the cell division zone is essential (Yamaguchi et al., 2010). Pro in this case is not free and does not play the roles of an osmolyte. Still, we have introduced this significant fact to illuminate the effects of ABA on Pro associated with structural proteins.

Exogenous application of ABA triggered Pro content in the seedlings of three rice varieties, M-1-48, Nonabokra, and Gobindobhog (aromatic); however, the effect was most dramatic in the most salt-sensitive variety, Gobindobhog,

clearly pointing to the cross-talk between ABA and Pro in determining stress tolerance (Roychoudhury et al., 2009). A possible clue of cross-talk also exists with the fact that ABA and Pro sprayed together in stressed cotton plants significantly enhanced chlorophyll content, chlorophyll stability index, leaf relative water content, and dry matter accumulation at low water potential (Gadallah, 1995). Induction of Pro synthesis by ABA and salt stress correlates with a striking activation of *P5CS1* expression, whereas *P5CS2* is weakly stimulated. ABA and salt stress suppresses the *PDH* expression in shoots and roots of light-grown plants. The reason is that the maintenance of high Pro concentration in a stressed cell is essential for its viability. However, the light-dependent induction of *P5CS1* by ABA and salt stress is downregulated in dark-adapted plants. As a result, *PDH* activity significantly increases in such plants. The steroid hormone brassinolide regulates the inhibition of *P5CS1* during dark adaptation. This indicates the presence of a cross-talk between the transduction pathway associated with the reception of light and Pro content in plants. In ABA-hypersensitive *prl1* and brassinosteroid-deficient *det2* mutants, enhanced Pro accumulation and *P5CS1* induction have been recorded. The *prl1* mutation reduces the basal level of *PDH* expression, whereas the *det2* mutation increases the inhibitory effect of ABA over *PDH* (Abraham et al., 2003; Ibragimova et al., 2012). Application of 50 µM ABA to *Arabidopsis* wild-type, ABA-deficient *aba1-1* mutant, and ABA-insensitive *abi1-1* and *abi1-2* mutant seedlings triggered the expression of *AtP5CS* and not *AtP5CR*. The similar transcript levels in the wild-type and ABA-deficient mutants indicated that the expression of either of the genes was mediated by endogenous ABA (Savoure et al., 1997). However, the ABA-treated *abi1-1* mutants accumulated less Pro than the ABA-treated wild type. On exposing the *aba1-1* and *abi1-1* mutants to salt stress, Pro accumulation further decreased in them in comparison to the wild-type. This typically indicates the indirect role of ABA over Pro accumulation during salt adaptation (Savoure et al., 1997). Thus, the Pro biosynthetic genes are ABA-independent during stress exposure, although previous instances do show that their expression can be triggered by exogenous application of ABA. It has been suggested that the endogenous ABA levels can affect the accumulation of Pro during salt stress, and this possibly indicates posttranscriptional regulation of the biosynthesis of Pro in response to NaCl toxicity in soil (Roychoudhury and Chakraborty, 2013). Experiments with canola leaf discs (CLDs) subjected to hyperosmotic stress showed that exogenously supplied ABA downregulated *PDH* during poststress recovery, though the expression levels of *P5CS* were not relatively heightened. The ABA content in ABA-treated turgid CLDs was insufficient to determine the extent of Pro accumulation, as ABA levels were maintained low during *P5CS* expression in the tissues (Trotel-Aziz et al., 2003). The ABA-induced genes during abiotic stresses mainly encode proteases, chaperonins, enzymes of sugar, Pro and other compatible solute metabolism, S-adenosylmethionine decarboxylase (SAMDC) that catalyzes the rate-limiting step in PA biosynthesis, ion and water channel proteins, antioxidants, and TFs (Basu and Roychoudhury, 2014).

5.3. Cross-talk between Pro and antioxidants

The ROS play important roles in protecting plants against harmful pathogens that cause biotic stress. They contribute to the formation of tracheary elements, lignifications, and other important developmental processes (Das and Roychoudhury, 2014). However, nucleic acid damage, oxidation of proteins, lipids, and degeneration of chlorophyll pigments, along with uncontrolled K^+ efflux from cells, occur due to excess ROS accumulation in the plant system (Chen and Dickman, 2005). The ROS cause modifications in covalent bond, accompanied with direct oxidation of amino acids like Cys (to form disulfide bonds), Met (to form Met sulfoxide), Arg, Lys, Thr, and even Pro residues of crucial proteins (Anjum et al., 2014). Thus, it is crucial to regulate ROS generation within the compatible and optimum limits of the plant. Pro is a potent ROS scavenger (Chen and Dickman, 2005). This view was further corroborated when reduced ROS levels were documented in the roots of *Arabidopsis* upon exogenous treatment of Pro. The ROS-induced K^+ efflux also decreased significantly in this case. The main antioxidant enzymes like catalase (CAT), peroxidase (POX), and superoxide dismutase (SOD) also had their activities enhanced in the presence of Pro, which was applied exogenously to tobacco suspension cultures facing salt stress (Hoque et al., 2007a). The ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR) are the main enzymes regulating the ASC-GSH cycle. Exogenous Pro treatment of tobacco cultures exposed to salt stress upregulated the activity of these enzymes, resulting in the more efficient operation of the ascorbate-glutathione cycle (Hoque et al., 2007a). Exogenously applied Pro thus acts as a ROS scavenger (Kaul et al., 2008). Tobacco cells exposed to cadmium stress and treated with Pro had enhanced activities of SOD and CAT and hence a low rate of membrane lipid peroxidation (Islam et al., 2009). Salt stress in *Nicotiana tabacum* 'Xanthi' showed ROS production in the plant system. Along with ROS, the production of intracellular ammonia, and expression of *gdh-NAD*, *A1* (encoding the α-subunit of glutamate dehydrogenase, GDH) was also induced. During stress treatment, an increase in the immunoreactive α-polypeptide and assembly of anionic

isoenzymes were also reported. The α-GDH subunit expression is mediated by the salt stress-generated ROS signaling. As a result, production of glutamate for Pro synthesis occurs due to the anionic iso-GDHs, which also play crucial roles as antistress enzymes in detoxifying ammonia (Skopelitis et al., 2006).

5.4. Cross-talk between Pro and PAs

As mentioned earlier, the stress-induced regulation of the qualitative composition and the quantitative content of low-molecular-weight organic osmolytes is one of the key strategies of plants to adapt to adverse stress conditions. The cross-talk between Pro accumulation and PA synthesis has been recently postulated to be mediated by ABA (Shevyakova et al., 2013). Such cross-talk was studied in the glycophyte *Phaseolus vulgaris* exposed to salt stress. In a phytotron chamber and on Jonson nutrient medium, 2-week-old seedlings were grown for 6 days, being exposed to high NaCl concentrations of 50 mM and 100 mM. For the first 3 days, the roots were daily treated with 1, 5, 10, or 50 μM ABA for 30 min. High salt stress resulted in higher endogenous ABA level and a drastic 14-fold increase in Pro concentration. The concentration of free PAs (Put, Spm, Spd, and cadaverine) was reduced with the accumulation of 1, 3-diaminopropane, which is a product formed due to the oxidation of high molecular weight PAs. The steady maintenance in plant growth, stabilization of the water and sodium balance, and induction of chlorophyll and carotenoid biosynthesis showed that the ABA treatments aided the plants to overcome 100 mM NaCl stress. The ABA treatment actually suppressed the NaCl-responsive Pro and endogenous ABA accumulations. The normal levels of Put and Spd were restored. However, in contrast to wild-type plants, the Spm and cadaverine levels increased by 4- to 5-fold with a reduction in the contents of 1,3-diaminopropane and malondialdehyde (MDA) and also the activity of SOD (Shevyakova et al., 2013; Roychoudhury and Das, 2014). Thus, it was hypothesized that since both the biosynthetic pathways of Pro and PAs utilize glutamate as the common precursor, it is this step that is regulated by ABA in mediating the cross-talk. More studies obviously need to be performed for further approval.

The transgenic soybean plants overexpressing P5CR exhibited enhanced levels of PAs. This shows that even the manipulation of the Pro biosynthetic pathway can affect the accumulation of PAs, further supporting the possibility of cross-talk (Simon-Sarkadi et al., 2006). The transgenic plants had Pro levels that were about 124-fold higher in comparison to the wild-type plants during exposure to stress. These plants also had low levels of Spd and Put. This was explained by the fact that, due to the much higher rate of channeling of Arg and Glu for Pro biosynthesis, less was available to be used for PA biosynthesis. The wild-type plants, however, showed increased levels of Put and Spd on exposure to stress. The Pro and PA levels were simultaneously elevated in wild-type plants exposed to drought stress because these plants had more proteins degraded and required more osmolytes to recover from the injury (Simon-Sarkadi et al., 2006). In the case of transgenic plants, the extremely high Pro content seemed to have compensated for the activity of normal levels of Pro and PAs together. The greater increase in Pro content in the transformed plants helped them to better tackle the stress conditions than the wild-type plants during stress. However, the increased PA levels also make significant contributions in combating dehydration stress by reducing the stress-induced damages. Exogenous application of Spd and Spm during salinity stress in three rice varieties, namely M-1-48, Nonabokra, and Gobindobhog, enhanced the level of Pro over salt-treated counterparts; however, the effect was the most pronounced with Spd application in the most salt-sensitive variety, Gobindobhog, probably as a measure to ward off excess damages (Roychoudhury et al., 2011). An exceptional observation showed that the increase in Put content by salicylic acid in maize did not improve its tolerance against dehydration stress (Nemeth et al., 2002; Roychoudhury and Das, 2014). For better stress responses, the gradual changes in Pro and PA concentrations in the due course of time is more crucial than their level at the end of a long period of treatment.

6. Pro regulates seed germination during abiotic stress

Seed dormancy is a major factor in dictating plant fitness. This complex trait has been reported to be influenced by genetic components, tissues forming the seed, and also integrative environmental signals. Seed dormancy is absolutely essential as it delays germination unless the environment is not favorable to sustain the growth of the plant (Graeber et al., 2012). Apart from the phytohormones like ABA and gibberellins, nonenzymatic processes, chromatin factors, and dormancy-specific genes have also been found to regulate seed dormancy (Linkies and Leubner-Metzger, 2012). Heikal and Shaddad (1982) were among the first to link seed germination with Pro. They found that the 'interaction effect' between Pro and osmotic stress induced germination and growth in seeds under osmotic stress. Pro played roles as a major source of energy and nitrogen required during poststress metabolism and also as an osmotic regulator in seeds under stress. Exogenous application of Pro improved the growth rate and seed germination in *Arabidopsis* exposed to chilling temperatures (Hare et al., 2003). In a recently conducted study, Bhamburdekar and Chavan (2011) studied the pattern of Pro accumulation in germinating

pigeon pea seeds following NaCl, boron, and aluminum treatments. Pro level was promoted at different stages of seed germination after treatment with 10 and 50 ppm boron treatments. Pro biosynthesis was upregulated by aluminum during seed germination (Bhamburdekar and Chavan, 2011). The influence of different osmotic potentials (measured in MPa) on Pro content and percentage of seed germination of corn was tested. The reduced osmotic potential of the media increased the Pro content in the germinating seedlings. However, relation between the seeding Pro content and germination percentage was not detected (Shahriari et al., 2014). Recently, Ribeiro et al. (2014) reported on the activity of antioxidant enzymes and Pro accumulation in germinating seeds of *Erythrina velutina* Willd. exposed to abiotic stress. Antioxidant enzymes like APX showed high activities, along with increased accumulation of Pro in the cotyledons and embryonic axes of the seeds. Hence, it was concluded that the constant presence of Pro conferred stress tolerance to the seeds by regulating osmotic adjustment (Ribeiro et al., 2014).

Seed germination is negatively regulated by salinity stress. Such a finding was reported in *Pennisetum glaucum*. Seed dormancy was promoted in most seeds subjected to increased salt concentrations with drastic decrease in the germination percentage (Sneha et al., 2013). Pro and free amino acid contents, however, increased in the 14-day-old seedlings subjected to 50, 100, 150, and 200 mM NaCl. The measurements were recorded after 0, 12, 24, 48, 72, and 96 h (Sneha et al., 2013). The evaluation of the changes in Pro, CAT, and germination characteristics in barley seeds under salt stress showed that the Pro and CAT levels increased, while the germination percentage and germination index reduced with the increase in salt concentrations (Tabatabaei, 2013). The increase in Pro in the stressed seedlings obviously suggests its role as a crucial compatible solute in protecting macromolecular structures and as a free radical scavenger during the postgermination period under stress. Pro homeostasis is crucial to plant systems since it is involved in maintaining proper physiological development under long-term stress (Kavi Kishor and Sreenivasulu, 2014). This also indicates the role of the Pro sink in breaking seed dormancy and promoting germination. Thus, modern studies have adopted a holistic approach of developing transgenic stress-tolerant plants with an increased Pro sink in the reproductive tissues.

7. Pro: regulation under various forms of abiotic stress

7.1. Salinity stress

Salinity drastically affects the global agricultural productivity (Bartels and Dinakar, 2013). Salinity stress enhanced the Pro level almost 8-fold and 6-fold over the control in the salt-susceptible varieties M-1-48 and Gobindobhog, while the level remained unaltered in the salt-tolerant variety Nonabokra even after NaCl stress (Roychoudhury et al., 2008). The enhancement in Pro content was noted in the roots and leaves of NaCl-treated *Vigna radiata* seedlings (Roychoudhury and Ghosh, 2013). Both these observations suggest the protective role of Pro during salt stress. However, excess Pro in the growth medium does not always facilitate tolerance against salt stress. This was seen when the growth of tomato seedlings under salt stress was inhibited with Pro supplied in the growth medium (Heuer, 2003). The reason for such exacerbation may be due to the overaccumulation of P5C via Pro catabolism, thus accounting for Pro toxicity. Recently, in another study, two osmoprotectants (Pro and trehalose) were exogenously supplied to Pokkali (salt-tolerant) and Khao Dawk Mali 105 (KDML105 salt-sensitive) rice seedlings. The effects of Pro and trehalose were investigated for about 6 days on seedlings exposed to 200 mM NaCl. The growth of both the rice cultivars at this high concentration of salt was inhibited even after exogenous Pro treatment. Their growth patterns resembled that of the stress-exposed wild-type rice seedlings (Nounjan and Theerakulpisut, 2012). A negative relationship was also reported in the case of Pro accumulation and salt tolerance in *Vigna mungo*. Salt-resistant rice cultivars like Nonabokra and IR-4630 had less Pro accumulation than the salt-sensitive cultivars I Kong Pao and IR-31785. In tomato, no correlation between plant tolerance index and Pro accumulation was observed. It was concluded that Pro accumulation is not the driving force for salt tolerance in tomato (Almeida et al., 2014). Pro accumulation has been considered to be a minor route of combating stress in some plants, since some major alternative defense routes are present in them. In such plants, Pro behaves as an unchanged parameter and hence is an unsuitable marker for salt tolerance. In contrast, several plants have been identified in which the Pro content increases in response to salt tolerance, and it is considered as a major marker for detecting salt stress in the plants (Roychoudhury and Chakraborty, 2013). However, both trehalose and Pro played vital roles in the growth of the seedlings in the poststress recovery phase. Exogenous Pro application to KDML105 seedlings under salt stress enhanced the activities of POX, CAT, and APX. This resulted in the low accumulation of hydrogen peroxide in the plant tissues. In mung bean seedlings and olive plants under salt stress, such reduction in the level of hydrogen peroxide was observed on exogenous application of Pro (Ahmed et al., 2010; Hossain and Fujita, 2010). The SOD and POX activities in the salt-stressed Pokkali and KDML105 seedlings,

however, were reduced on exogenous application of Pro (Nounjan and Theerakulpisut, 2012). Similar results of reduced SOD activity were found in common ice plant under paraquat-induced stress (Shevyakova et al., 2009). A salt-sensitive cultivar of cucumber when exposed to salt stress after exogenous treatment of Pro showed enhanced POX activity. Increased APX activity was detected in Pro-treated grapes (Huang et al., 2009; Ozden et al., 2009).

Over 40 mM Pro was accumulated in the leaves of many halophytes to contribute to an osmotic pressure of over 0.1 MPa in the cells. The concentration of Pro in glycophytes is much lower, around 10 mM. Even at this lower concentration, they can function as osmolytes if partitioned exclusively in the cytoplasm (Munns and Tester, 2008). Pro contents in the leaves have been reported to be higher in the salt-tolerant than in the salt-sensitive lines of *Eruca sativa*, safflower, *Lens culinaris*, and sunflower (Roychoudhury and Chakraborty, 2013). The formation of strong hydrogen-bonded water shells around the protein moiety is stimulated by Pro in order to provide stability. In the salt-sensitive species of rice, alfalfa, maize, pigeon pea, and potato, high Pro concentration was recorded when these plants were exposed to stress (Cha-Um and Kirdmanee, 2008; Roychoudhury and Chakraborty, 2013). The roots of the salt-tolerant varieties of alfalfa plants had their Pro content doubled, whereas in the roots of the salt-sensitive variety, the rise in Pro concentration was slower. In comparison to the salt-sensitive pea genotypes (Ambassidar and PF-400), the salt-tolerant genotypes (Climax and Samarinazard) had higher accumulated Pro and lower concentration of sodium in the leaves. This indicated the existence of a correlation between OA and salt tolerance (Shahid et al., 2011). Pro accumulation in two sorghum genotypes contrasting in salt tolerance established the fact that Pro accumulation was not due to the response associated with salt tolerance, but was rather a reaction to salt stress (de Lacerda et al., 2003). Salt-sensitive rice varieties like Pathumthani1 and Black Sticky had less accumulation of Pro than salt-tolerant rice varieties like KDML105 and Sangyod. Sarsabz, Lu-26, and KTDH-22 are salt-tolerant genotypes of wheat that had higher Pro accumulation in their leaves, and hence a higher grain yield when exposed to salinity stress (Khan et al., 2009). Higher Pro accumulation occurred in the salt-tolerant ecotypes of *Agrostis stolonifera* in response to high salinity in comparison to the salt-sensitive ecotypes. A high degree of OA was exhibited in the leaves of salt-tolerant varieties of *Brassica juncea* along with a high critical point concentration of NaCl. The endogenous levels of Pro rapidly increased beyond this critical point. Several of the Pro anabolic enzymes like P5CR and ornithine aminotransferase increased in the salt-tolerant varieties of *B. juncea*. Halophytes like *Thellungiella halophila*, unlike glycophytes, are adapted to grow in saline soils. A vast array of physiological, biochemical, and structural adaptations against salinity have been reported in such halophytes. These properties have converted *T. halophila* into a model plant in which the molecular mechanisms related to salt tolerance can be studied (Bartels and Dinakar, 2013). The salt tolerance of *Thellungiella halophila* was enhanced due to lower expression of PDH in comparison to its salt-sensitive relative, *Arabidopsis thaliana*. Repression in Pro catabolism in *T. halophila* seedlings under salt stress was predicted mainly due to the hypersensitivity of the seedlings to exogenous Pro (Kant et al., 2006). The PEHM3 salt-tolerant varieties in maize showed higher contents of Pro, K^+, and Ca^{2+} in comparison with the salt-sensitive Navjot variety. The salt-susceptible variety had higher Na^+, Na^+/K^+, and Na^+/Ca^{2+} ratios than PEHM3 (Roychoudhury and Nayek, 2014).

In a recent report, it was seen that the wheat cultivars exhibited reduced plant biomass on exposure to salt stress due to the excess accumulation of Na^+ and inhibition in the biosynthetic pathway of chlorophyll a and b photosynthetic pigments (Talat et al., 2013). Wheat cultivars exposed to high salinity had less efficient substomatal CO_2 concentration, net CO_2 assimilation rate, stomatal conductance, transpiration rate, and water use. Reduced stomatal opening and transcription rates were observed in wheat cultivars treated with exogenous Pro. It was concluded that exogenous application of 100 mM Pro significantly ameliorates the harmful effects of salt stress on the growth, morphology, and physiology of wheat varieties (Talat et al., 2013). In another experiment on pistachio plants, the Pro content sharply increased at all levels of salinity except at 100 mM salt concentration. MDA content was used as a marker for cell membrane lipid peroxidation in the plants treated with Pro. It was observed that at higher salt concentrations of 200 and 350 mM NaCl, there was lower accumulation of MDA (3.9 and 1.85 μmol g^{-1} fresh weight respectively) accompanied by higher accumulation of Pro (3.5 and 4.1 μmol g^{-1} fresh weight respectively) (Abbaspour, 2012; Banerjee and Roychoudhury, 2014). This indicated that the increased Pro content helps in counteracting the lipid peroxidation directly or indirectly during salinity stress. The glutathione reductase (GR) activity also increased rapidly along with Pro in the pistachio plants treated with Pro and exposed to very high concentration of salt. The maximum GR activity was recorded in the leaves of plants grown in the medium with 500 mM NaCl. It has also been hypothesized that, under salinity stress, Pro accumulates consequently to inhibit plant growth and subsequent protein denaturation. The activities of the enzymes APX and CAT responsible

for the detoxification of hydrogen peroxide also increased (Abbaspour, 2012). Appreciable GR activity was recorded in eggplants treated with Pro and exposed to high salt stress. Thus, Pro has been suggested to act as an inducer of antioxidant activity and also as an enzyme-stabilizer during salt stress. In another study, it was found that chlorosis of the Akhisar 97 and Ozbas tobacco varieties occurred at NaCl concentrations greater than 150 mM and 200 mM respectively both in vitro and in vivo. Higher Pro content was recorded in Akhisar 97 in comparison with Ozbas. Analysis of the in vitro and in vivo experimental results for low concentrations of NaCl highlighted a difference existing between them. For in vivo studies, no difference existed in the Pro content between the leaves of plants exposed to 50 mM and 100 mM NaCl and leaves of control plants. However, for the in vitro studies with the same stress conditions, there was significant difference in Pro content in the leaves of the salt-treated and control plants (Çelik and Atak, 2012). Palmarosa callus tissue grown from cultured nodal explants was subjected to a range of NaCl concentrations. A positive correlation was observed between Pro accumulation and salt stress, since high levels of Pro were found in the salt-resistant callus lines (Patnaik and Debata, 1997). The concentrations of soluble carbohydrates and Pro were higher in the salt-resistant lines of *Glycine max* Merr 'Acme' callus cultures (Liu and van Staden, 2000).

Though Pro in some plants can be used as a marker to detect salt stress, the exact relationship between Pro accumulation and salt stress still remains obscure (Ku et al., 2011). The Pro content in transgenic plants overexpressing *P5CS* and subjected to salt stress was much higher than in the control plants (Hmida-Sayari et al., 2005). Salt-tolerant soybean mutant seedlings belonging to M3 generations of Ataem-7/150-68, S04-05/150-2, and S04-05/150-114 were exposed to 90 mM salt stress. Mutant S04-05/150-2 plants exhibited the maximum levels of Pro accumulation by having a 2.4-fold increase in Pro concentration after 1 week of salt treatment. Since the Pro contents of S04-05/150-114 transgenic plants and S04-05/150-114 control plants were found to be similar, it was inferred that this line did not require Pro to combat the salt stress. However, interestingly, the mutant plants of this line had a high expression level of *GmP5CS* (Celik and Unsal, 2013). The S04-05/150-2 soybean mutant also had the maximum accumulation of *GmP5CS* transcripts. Such high accumulation of *P5CS* has also been reported in *Arabidopsis*, soybean, tobacco, and *Medicago truncatula* (Kim and Nam, 2012; Celik and Unsal, 2013). It was suggested that due to the overexpression of *P5CS*, the reduction of P5C to Pro might be facilitated, and this conversion reduces stress injury to membranes and supplies more energy during post-stress recovery (Ma et al., 2008). Among the salt-tolerant mutants mentioned above, the highest *GmP5CR* transcript level was recorded in Ataem-7/150-68 plants after application of salt stress (Celik and Unsal, 2013). It was also reported that increased accumulation of Pro did not occur in *P5CR*-overexpressing transgenic plants (Stein et al., 2011). Salt stress in soybean seedlings, *Arabidopsis*, pea plants, and wheat varieties enhanced the levels of *P5CR* mRNA (Ma et al., 2008). Under salinity conditions, the P5CR catalyzes the rate-limiting step in Pro biosynthesis via glutamate and this *P5CR* transcript level accumulated in rice seedlings after 4 weeks of salt exposure (Sripinyowanich et al., 2013). Positive correlation between *P5CS* and salt stress in rice was more pronounced than that of *P5CR* (Yooyongwech et al., 2012). Pro catabolism is essential for the recovery of the plants from stress. Increased Pro breakdown produces reducing agents. These are converted to ATP, which is crucial for the maintenance of mitochondrial oxidative phosphorylation and the costly process of poststress recovery (Cvikrová et al., 2012). Pro accumulation has also been recently considered as a signal that activates the salt stress responses on exposure of the plants to high salinity.

Exogenous Pro application on salt-stressed *Pancratium maritium* resulted in higher growth in comparison with the wild-type plants under stress. Salinity-induced reduction in ubiquitin conjugate content occurred, while CAT and POX activity was retained in *Pancratium* during exogenous Pro treatment under salt stress. The oxidative damages caused by salinity stress were also overcome by the exogenous addition of Pro to the plant nutrient medium (Hayat et al., 2012). Exogenous Pro helped *Vicia faba* to recover completely from salinity-induced injuries, along with alleviations from salinity-induced membrane disruptions. This was also accompanied by increased leaf chlorophyll content, increased leaf relative water content, and higher plant growth (Gadallah, 1999). Decreased lipid peroxidation rate and increased chlorophyll content in the leaves of *M. crystallinum* were achieved upon treating the salt-stressed plants with exogenous Pro.

So far as tissue localization is concerned, the leaves accumulate more Pro to maintain chlorophyll level and cell turgor, in order to protect photosynthetic activity against stress (Silva-Ortega et al., 2008; Cha-Um et al., 2010; Batool et al., 2012). The P5CS activity in Jerusalem artichoke (*Helianthus tuberosus*) plantlets under NaCl stress was stimulated, while the OAT activity in due course was suppressed. Higher accumulation of Pro was detected in tissues with higher P5CS activity, while the enzymatic activity of OAT was low in the tissues due to salt treatment (Huang et al., 2013). Pro synthesis under stress is more species-specific; in contrast to *Helianthus tuberosus*, the δ-OAT activity in salt-stressed halophyte *Saussurea*

amara was high. It was also suggested that Pro synthesis in *H. tuberosus* predominantly occurs via the glutamate pathway. The pathway synthesizing Pro through ornithine has been regarded as the minor one (Wang et al., 2011). As in rice, the PDH activity in salt-stressed *H. tuberosus* was repressed more in the leaves than in the roots. This is in accordance with the high P5CS activity during salt stress that accounts for the high accumulation of Pro in the leaves (Huang et al., 2013). Under NaCl stress, *HtP5CS2* expression was induced within 12 h in different tissues and a similar trend was found when the plantlets faced varying NaCl dosages. The change of gene expression level of *HtP5CS1* was meager in comparison to *HtP5CS2*, but the relative expression of *HtP5CS1* remained unchanged (Huang et al., 2013). The *P5CS* expression peaked in *Opuntia streptacantha* on the ninth day of salinity treatment (Silva-Ortega et al., 2008). The role of *AtP5CS2* in the development of the seedlings of *Arabidopsis* has been found to be crucial. The hypersensitivity to salinity was observed in *Arabidopsis p5cs1* mutant plants with no functional *P5CS1* (Székely et al., 2008). It was seen that salinity stress reduced the expression pattern of *PDH1* in the stems of *H. tuberosus*. High Pro accumulation has also been reported in the halophytes *Thellungiella halophila* and *Lepidium crassifolium* without any exposure to stress. Due to increased *P5CS* and reduced *PDH* expression levels, the concentration of Pro in these unstressed halophytes was surprisingly much higher than in *Arabidopsis* plants subjected to salinity stress (Szabados and Savoure, 2009). Another halophyte, *Pancratium maritimum*, developed better tolerance of salinity stress upon high accumulation of Pro, which effectively stabilized the antioxidant enzymes and induced the expression of the stress-protective proteins, along with maintaining the protein turnover (Khedr et al., 2003). Thus, it has been inferred that Pro is a species-specific bioindicator of salinity stress and confers stress resistance to an extent in several halophytes through the different mechanisms as described above. However, it is also essential to note that Pro accumulation is not an absolutely essential criterion to adapt to extreme salinity, as we have also referred to plants that do not exploit the usual roles of Pro as an osmolyte during salt stress. A very recent improvement demonstrated that genome duplication increases salt tolerance in rice roots (Tu et al., 2014). The culture of diploid rice (HN2026-2x and Nipponbare-2x) and the corresponding tetraploid rice (HN2026-4x and Nippobare-4x) in 150 mM NaCl exhibited accumulation of Pro, soluble sugar, and MDA. Ultrastructural studies showed enhanced membrane, organelle, and nuclei stability in the tetraploids, along with low Na^+ content and high Na^+ efflux rate in their roots (Tu et al., 2014). Another study on reducing the detrimental effects of salinity in the roots of Pokkali and IR-28 was conducted by exogenously treating the plants with gallic acid. The stress caused high accumulation of Pro and hydrogen peroxide. This was the first study regarding the role of gallic acid in combating salt stress (Yildiztugay et al., 2014). The results showed significantly high activities of SOD, APX, and POX along with mitigation of much of the injuries (Yildiztugay et al., 2014).

7.2. Dehydration stress

The roles of Pro during water deficit have been thought to be important, like those under salinity stress, but the information available is quite scanty. Thus, further investigations are required to better characterize the positive correlation between Pro and dehydration stress. PEG 6000-mediated water stress induced Pro level 4-fold over the control in the two sensitive varieties IR-29 and Pusa Basmati, while the tolerant variety Pokkali showed no significant alteration with PEG treatment, suggesting that Pro is involved in stress tolerance in the two sensitive varieties (Basu et al., 2010). In another experiment, 4-week-old maize cultivars EV-1098 and AGAITI 2002 were subjected to dehydration stress after being exogenously treated with Pro. As expected, the stress-borne injuries in the plant were reduced in the Pro-treated cultivars in comparison to the wild-type (Ali et al., 2007). The plants under dehydration stress have almost the same effects as those under salinity stress, as in both cases there is high water deficit. Under dehydration stress, Pro protects endogenous proteins from misfolding, protects membranes of the cell organelles, and promotes poststress recovery of the plants (Ashraf and Foolad, 2007; Hoque et al., 2007b). The application of Pro is not always an absolute necessity for the plants to tackle stress. As discussed earlier, the effects of Pro applied as a foliar spray are highly dependent on the plant species, stage of development, time of application, and concentration of Pro in the spray (Ashraf and Foolad, 2007). In the case of maize subjected to dehydration stress, the application of 30 mM Pro was reported to be beneficial (Ali et al., 2007). Exogenous application of 30 mM Pro to rice seedlings proved to be crucial in combating dehydration stress (Roy et al., 1993). On the contrary, 20–30 mM Pro is required to ameliorate the adverse effects of dehydration in *Vigna radiata* cell cultures, while about 10 mM was found to be optimally effective in the case of tobacco suspension cells under stress (Ashraf and Foolad, 2007). These instances obviously indicate that the response to exogenous Pro treatment is highly concentration-dependent and species-specific.

In the absence of water in the conducting tissue cells, the photosynthetic capacity by regulating stomatal closure is reduced. This is accompanied by rapid loss of homeostasis

due to a surge in the number of damaging proteins associated with chlorophyll and photosystems PSII and PSI (Athar and Ashraf, 2005). Increased stomatal conductance and substomatal carbon dioxide, along with improved net carbon dioxide assimilation rate, was reported in the stressed maize cultivars after foliar application of Pro. This observation also indicated enhanced photosynthetic capacity following Pro treatment under dehydration stress (Ali et al., 2007). Adjustment between carbon uptake and water loss through transpiration is regulated by Pro applied exogenously to the stressed maize plants, since it was observed that Pro treatment did not appreciably improve the transpiration rate (Raven, 2002). This obviously proves that more effective utilization of water is promoted by Pro, and this is extremely vital for the viability of the plants during dehydration stress. Exogenous application of Pro simultaneously improved the biosynthesis of chlorophyll a and b and the photosynthetic rate in maize cultivars EV-1098 and AGAITI 2002. Such positive correlations between Pro and photosynthetic rate have not been limited to plants exposed to only dehydration stress. Such correlation has been reported in case of maize under water-logged conditions, in wheat under high salinity, in canola under dehydration, and even in trees under hypoxic conditions (Kauser et al., 2006; Raza et al., 2006; Ali et al., 2007). A positive correlation between photosynthetic rate and growth following application of Pro has also been observed in cotton, maize, and wheat (Arfan et al., 2007). In some experiments, PEG is used as a reliable inducer of drought stress in plants. PEG increases the solute potential and inhibits the roots to absorb water due to its nonpenetrating osmotic properties. Thus, in laboratory conditions, PEG can be used as a marker to identify the dehydration stress-tolerant plants. Pro, as discussed above, is often naturally accumulated in plants under dehydration stress as they regulate OA and safeguard the plants against subcellular damages (Chutia and Borah, 2012). Physiological drought was imposed in seven traditional rice varieties of Assam and their Pro accumulation levels were studied. The varieties Laodubi, Leserihali, Beriabhanga, and Borah portrayed the best drought resistance, with the plant height and seed number having a substantial genotypic coefficient of variability and heritability (Chutia and Borah, 2012). In another experiment on the drought-sensitive (S54) and drought-tolerant (S13) cultivars of *Morus alba*, the higher activities of P5CR, Pro oxidase, and PDH, as well as increased levels of quaternary ammonium compounds and chlorophyll stability, were reported when the aforesaid varieties were subjected to drought stress (Ramanjulu and Sudhakar, 2000). As expected, higher Pro levels were detected in S13 when compared to those of S54. Exposing the plants to 15 days of water stress resulted in 8-fold and 3.5-fold increase in Pro content in the leaves of S13 and S54, respectively. Under these conditions, the activities of Pro biosynthetic enzymes like P5CR were increased, while those involved in the degradation of Pro like Pro oxidase and PDH were suppressed. The higher degree of drought tolerance in S13 due to accumulation of Pro suggests an altered Pro metabolism in order to adapt to the adversities of stress. Apart from high Pro accumulation, the increase in the concentration of the quaternary ammonium compounds along with chlorophyll stability also accounted for higher drought tolerance in the S54 cultivar of mulberry (Ramanjulu and Sudhakar, 2000). *Agrobacterium*-mediated transformation was performed in *Helianthus annuus* under drought stress using a double-stranded RNA-suppressor against the *PDH* gene. The transformants exhibited a high accumulation of Pro during the early stages of in vitro cultivation under normal conditions. The enhanced drought tolerance of the transformants in vitro and in planta was also demonstrated (Tishchenko et al., 2014).

A set of 21 rehydration-specific genes were shown to be induced by Pro in *Arabidopsis*, most of which have a Pro-responsive element (PRE) in their promoter region and are the target of a set of bZIP TFs (Weltmeier et al., 2006). ABA-mediated Pro synthesis was also recorded in cowpea exposed to drought conditions. The accumulation of Pro helped in alleviating the damages caused by stress (da Costa et al., 2011).

7.3. Cold stress

Free Pro has significant roles in alleviating the detrimental effects of cold stress. The biosynthesis and stimulation of total phenolics as antioxidants are connected with the Pro-associated pentose phosphate pathway. In a research on three cool-season turf grasses, creeping bentgrass (*Agrostis stolonifera*), Kentucky bluegrass (*Poa pratensis*), and perennial rye grass (*Lolium perenne*), grown in chilling temperatures, the antioxidant response system and the accumulation of phenolics were mediated by the Pro-associated pentose phosphate pathway (Sarkar and Bhowmik, 2009). The positive link between high accumulation of phenolics and the Pro-associated pentose phosphate pathway was also reported in a turf grass species during cold acclimation. The cold-acclimated turf grass species exhibited decreased succinate dehydrogenase and increased glucose-6-phosphate dehydrogenase activities. This clearly leads to the fact that in order to develop higher cold tolerance and save energy during the stressed period, the carbon flux is channeled from the energy-costly tricarboxylic acid cycle to the energy-efficient Pro-associated pentose phosphate pathway (Sarkar and Bhowmik, 2009). Hydropriming *Vigna radiata* seeds with Pro resulted in better combating of cold

stress at 5 °C. If the applied spray consisted of increased concentrations of exogenous Pro, the seeds germinated at the said temperature, which was impossible for the control plants. The oxidative stress occurring during the low-temperature stress was also ameliorated by Pro, whose role as a typical ROS scavenger has been previously discussed (Das and Roychoudhury, 2014). Improved frost tolerance was observed in the leaves of *Solanum* when treated with exogenous Pro, which helped in tackling the cold stress. Another important aspect of Pro during chilling stress is that it can also be channeled into alternative catabolic pathways to act as a source of nitrogen and carbon for the stressed plant, thus favoring its growth. This function of Pro has been authenticated in *Vigna radiata* (Hare et al., 2003).

7.4. Heavy metal toxicity

Recently, a novel *dehydration-responsive element-binding protein 2B* (*DREB2B*) gene was isolated from the desert leguminous plant *Eremosparton songoricum*. It was characterized that *EsDREB2B* produces a truncated DREB2 polypeptide under the induction of multiple abiotic stresses including heavy metal stress and even exogenous ABA treatment (Li et al., 2014). The MDA, Pro, and chlorophyll contents did not differ among the wild-type and transgenic plants overexpressing *DREB2B*. However, within 6 h of heavy metal stress, *DREB2B* transcription levels peaked, accompanied by high accumulation of Pro in the transgenic lines. Upon measuring the MDA levels, it was confirmed that the transgenic lines experienced significantly less damage when compared to the wild-type plants (Li et al., 2014). In another novel study, *zinc-induced facilitator 2* (*ZIF2*) was overexpressed, as a result of which the zinc tolerance in *Arabidopsis* was enhanced (Remy et al., 2014). Among the two spice variants of ZIF2, in the one with the longer 5' UTR, i.e. *ZIF2.2*, transcripts were produced during high Zn concentrations in the soil. This is because the long 5' UTR helped in the formation of a stem-loop structure, which conferred higher stability, turnover, and translational efficiency to *ZIF2.2*. The transgenic plants overexpressing ZIF2 had higher chlorophyll content, shoot fresh weight, and longer primary root formation than the wild-type plants even at 750 μM Zn (Remy et al., 2014). We have discussed that the chlorophyll content and biosynthesis of chlorophyll pigments are linked to Pro accumulation during stress. Since the formation of ROS is bound to occur at such a high toxic metal concentration, the Pro content in such stressed plants should also increase, as Pro is the most abundant osmolyte and ROS scavenger found in plants.

Pro acts as a heavy metal chelator and specifically sequesters Cd and Zn ions by forming a Pro-metal complex, thereby preventing enzymes such as glucose-6-phosphate dehydrogenase and nitrate reductase from being inhibited by these metals (Sharma et al., 1998). It is also involved in the upregulation of phytochelatin synthesis, thereby providing a second level of heavy-metal detoxification, especially for Cd. In *Solanum nigrum*, Pro application helps to combat Se-induced stress (Aggarwal et al., 2011). However, Pro does not always act as an indicator of heavy metal toxicity. In an experiment on cowpea seedlings, it was seen that even at a concentration of 1 mM of Au^{3+} (auric ion), the levels of stress markers like MDA and Pro remained unaltered when compared to control plants grown on normal medium (Shabnam et al., 2014). Thus, out in the field, in spite of substantially high Au^{3+} concentration of the soil, one would assess the Pro levels of cowpea only to derive false information. This unchanged level of Pro in cowpea seedlings under high concentrations of Au^{3+} occurs because the Au^{3+} is made ineffective in the medium itself by phenolics released by the seed coat. The phenolics released by the seed coat of cowpea convert the Au^{3+} into blue crystalline nanoparticles, which are rather harmless to the plant. The presence of the gold particles was detected by using transmission electron microscope and X-ray diffraction (Shabnam et al., 2014). Experiments with cauliflower (*Brassica oleracea*) cultured under varying concentrations of heavy metals showed marked increase in Pro content (Theriappan et al., 2011). In seedlings stressed with 250 μM $CdCl_2$ for 15 h, the Pro content was reported to be twice as much as in the control plants. When treated with 500 μM $ZnCl_2$, the Pro content in the stressed plants became twice that in the control plants. The accumulation of Pro in the plants under $CdCl_2$ and $ZnCl_2$ stress was dependent on the concentration of the heavy metals in the medium. Incubation with 500 μM $HgCl_2$ for 15 h also resulted in 2-fold increase in Pro content in the stressed plants when compared with the control plants (Theriappan et al., 2011). The Pro content increased progressively in the salt-sensitive IR-29 and salt-tolerant Nonabokra rice varieties with gradual increase in $CdCl_2$ concentration (Roychoudhury et al., 2012). Roychoudhury and Ghosh (2013) also observed increment in Pro level in both roots and leaves of $CdCl_2$-treated seedlings of *Vigna radiata*. The role of Pro as an ion-chelator during heavy metal stress is documented. The biosynthesis of phytochelatins is stimulated by the accumulation of Pro, which chelates out heavy metals like Cd. The regeneration of shoots in *Solanum nigrum* exposed to Cd stress was facilitated during pretreatment with Pro. The endogenous Pro levels in *S. nigrum* treated with exogenous Pro aided in overcoming the detrimental effects caused by Se stress (Xu et al., 2009). In comparison to the nontolerant varieties, the heavy metal-tolerant varieties of *Deschampsia* and *Silene* constitutively maintained a higher Pro content. The Pro–metal complex

formation was reported in metal-tolerant *Armaria*, where Pro was shown to complex with Cu. In another instance, such complex formation with Cd and Zn rendered protection to the glucose-6-phosphate dehydrogenase and nitrate reductase, which exhibited normal activities during salt stress (Hayat et al., 2012). A recent experiment in *Cannabis sativa* showed the positive correlation of Pro and phenolics during Cd toxicity (Ahmad et al., 2015). The application of NPK fertilizers on the *C. sativa* plants grown in Cd contaminated soil increased the phytoaccumulation of Cd. Thus, the possibility of phytoextraction of Cd with increasing concentration of total phenolics and free Pro has been suggested (Ahmad et al., 2015). Pro accumulation has also been reported in *Cajanus cajan*, *Vigna mungo*, *Helianthus annuus*, and *Triticum aestivum* exposed to different heavy metals. In algae like *Anacystis nidulans*, *Chlorella* sp., and *Chlorella vulgaris* subjected to Cu stress, Pro accumulation occurred, and in *C. vulgaris*, exogenous Pro treatment reduced lipid peroxidation and checked the potassium efflux (Banerjee and Roychoudhury, 2014). Pro accumulation was surprisingly lowered in *Zea mays* inoculated with novel Zn-tolerant bacterial strain ZK1 of *Proteus mirabilis* (Islam et al., 2014). Though ZK1 improved the CAT, SOD, guaiacol peroxidase, and ascorbic acid activities, it lowered the Pro content in plant tissues, thus creating a negative correlation between Pro and heavy metal stress (Islam et al., 2014). In another experiment, pea plants were exposed to NaCl and/or $NiCl_2$ stress to investigate whether pure Pro or Pro enriched-leaf extract of *Lolium perenne* L. could effectively protect against phytotoxicity. Natural Pro (leaf extract) containing other essential nutrients proved to be a better protecting agent than pure Pro by altering the polyamine metabolism (Shahid et al., 2014).

7.5. UV stress

UV-B (280–320 nm) stands out as a crucial source of abiotic stress at high intensity in plants. This is due to the damaging effects of these rays on the chlorophyll pigments and the efficiency of photosynthesis. Thus, the common defenses adopted by plants during such radiation stress are the production of flavonoids and Pro (Saradhi et al., 1995). High Pro levels were recorded in the shoots of *Sisymbrium erysimoides* and the roots of *Plantago major* exposed to UV rays (254 nm) (Salama et al., 2011). The roots and shoots of desert annual plants like *Malva parviflora* and *Rumex vesicarius* also exhibited higher constitutive levels of Pro in comparison to the control plants when exposed to UV stress (Salama et al., 2011). During such radiation stress, the accumulated Pro mainly plays a role in scavenging the ROS and RNS generated. Exogenous Pro treatment in barley seedlings under UV-B stress resulted in decreased chlorophyll/carotenoid ratio, oxygen evolution rate, and photochemical efficiency of PSII. The chlorophyll/carotenoid ratio decreased due to the formation of the photosynthetic pigments, which rendered protection to the viable cells against UV-B (Fedina et al., 2003).

8. Adverse effects of Pro

In a biological system, overaccumulation of any metabolite is considered to be detrimental. The negative side effects of Pro present at high levels in the cells have been reported in tomato (Heuer, 2003). It has been noted that 30 mM exogenous Pro treatment is sufficient to aid plants in combating abiotic stress. However, treatment with 40–50 mM or higher concentrations of Pro proved to be toxic for rice seedlings, hampering their physiological processes (Roy et al., 1993). Pro-induced toxicity causes marked changes in the ultrastructure of mitochondria and chloroplasts and also initiates the early steps of programmed cell death (Deuschle et al., 2004). These may be attributed to a disruption in the redox balance of the organelles. The hyperaccumulation of Pro indirectly destabilizes the DNA double helix and increases its susceptibility to nucleases as well as lowering the melting temperature. Pro at lower levels activates cytosolic Pro biosynthesis from glutamate and also induces Pro catabolism in mitochondria. The catabolism generated $NADP^+$, which is crucial for cytosolic purine biosynthesis and reducing equivalents for phosphorylation of ADP in the mitochondria (Roychoudhury and Chakraborty, 2013). This cytosolic biosynthetic pathway gets blocked at very high concentrations of Pro due to the feedback inhibition of P5CS. This was reported in *Arabidopsis* and *Distichlis spicata*, due to which the organogenesis and growth in the respective plants were inhibited. Excess Pro has also been accounted for destabilizing the DNA helix, lowering the DNA melting point, making the DNA more susceptible to S1 nuclease, and increasing DNA insensitivity to DNase I (Hayat et al., 2012). In *Arabidopsis* mutants with nonfunctional *PDH* gene, plant growth was inhibited upon treatment with even less than 10 mM Pro, whereas the wild-type plants showed better growth at such concentrations of Pro (Nanjo et al., 2003).

Finally, to conclude, we have mainly concentrated in the present review on the response of Pro under various changing environmental conditions and its vivid roles in regulating the physiomorphological developments in the plant systems under stress conditions. It can be thus summarized that Pro can be used as a marker for abiotic stress in plants. This has been successful in several models subjected to salinity, dehydration, cold, heavy metal toxicity, and radiation stress. Thus, Pro can be regarded as a 'multipotent antistress component' in plant systems (Figure 3). However, Pro cannot be guaranteed as a sole reliable biomarker for detecting stress, as it is species-specific and dependent on other factors discussed earlier.

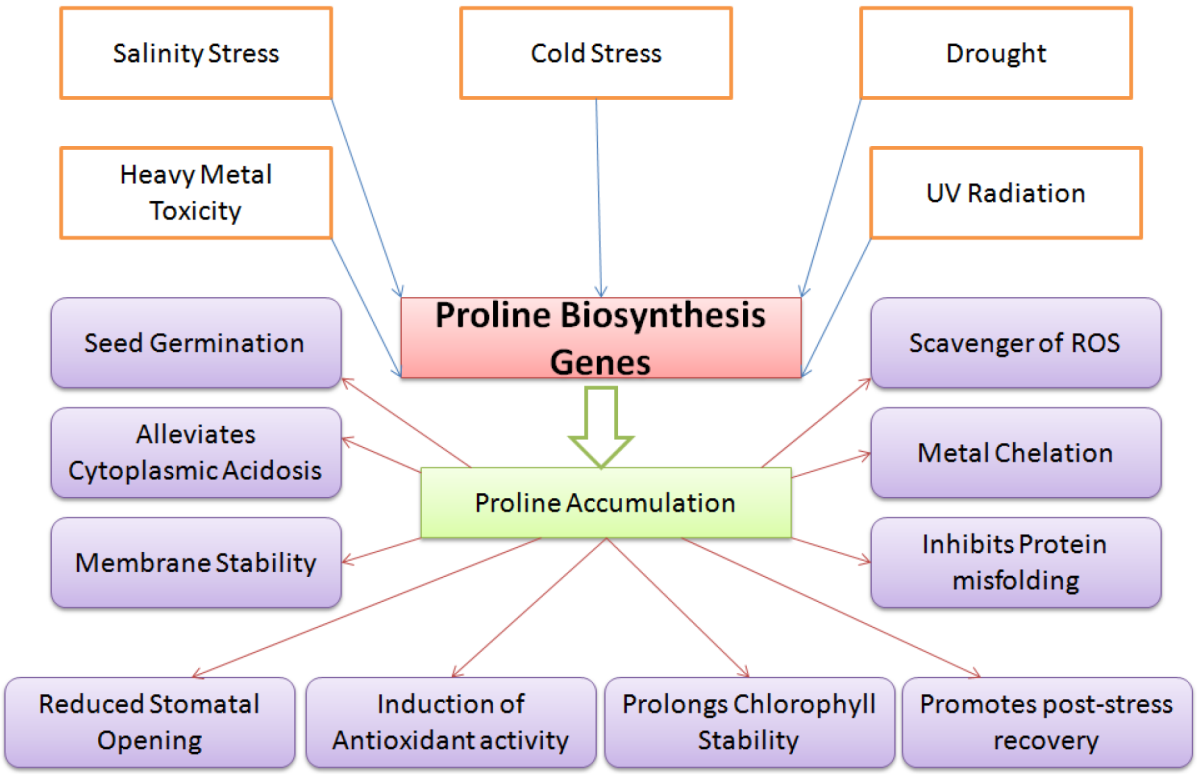

Figure 3. Pro biosynthesis is upregulated in response to multiple abiotic stresses like salinity, drought, cold, heavy metal toxicity, and radiation. This shows the importance of Pro in regulating plant responses against abiotic stress. The mechanism of Pro in mediating such stress tolerance is generally through stabilization of the cell membrane, reduction in stomatal opening under stress conditions, increase in the activity of antioxidants, reduced degradation of chlorophyll pigments, protection of protein structures, promotion of poststress recovery, etc.

The potential of Pro to tackle various abiotic stresses needs to be further exploited in order to design stress-tolerant viable crop cultivars with no deleterious effect on their productivity and growth. In spite of several years of stringent research on Pro, much remains to be known. The reason for the negative correlation of Pro and stress in some species is still not known perfectly, though some hypotheses have been put forward. This review presents detailed data on the developments in the field of Pro in counteracting abiotic stress. Exhaustive studies to elucidate the detailed signaling pathway of Pro and the downstream TFs that it activates (if any) can further promote the significance of Pro in developing tolerance against multiple abiotic stresses.

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