Chapter 11

Exogenous proline-mediated stress tolerance in plants

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11.1 Introduction

Since plants are sessile living, they are exposed to various stressors due to environmental factors in their natural environment, and this seriously affects plants' vital functions. These environmental factors damage the cell physiology, biochemistry, and genetic structures of plants, and these situations are defined as stress (Mansour & Salama, 2020). Environmental factors have been known as biotic and abiotic stresses. Biotic ones are defined as damage to a plant by other living things such as viruses, parasites, bacteria, and harmful and/or beneficial insects. On the other hand, abiotic stress gives rise to nonliving stressors such as drought, temperature stress, salinity, wind, mineral deficiency, and heavy metal stress that can harm the plant (Mansour & Salama, 2020).

It has been indicated that abiotic stresses can lead to various cellular dysfunctions in plants, including membrane damage, increased production of reactive oxygen species (ROS), protein denaturation, accumulation of toxic products, and altered gene expression (Kaur & Asthir, 2015). Mechanisms such as protein modulations, solute accumulation appropriate to the stress situation, antioxidant defense, and membrane lipids have been reported to be essential for enhancing plant tolerance to abiotic stresses (Mansour & Ali, 2017; Salama & Mansour, 2015). Plants activate the accumulation of proline, one of the most effective soluble agents to combat abiotic stress. Proline has been reported to play an important role in plants both during specific developmental processes and in the reaction to abiotic stress (Trovato et al., 2019; Unal et al., 2013). Plant cells could regulate the influx, synthesis, and accumulation of proline in response to abiotic stress conditions to maintain cellular turgidity and ensure homeostasis for growth and development. In addition, the amino acid proline can enhance the stability of cellular proteins and lipids by binding to hydrogen bonds, thus ensuring membrane integrity and increasing the cell's water uptake potential, serving as an osmolyte (Burritt, 2012; Hossain et al., 2019). Abundant evidence suggests that proline also has various roles as a metal chelator, an antioxidant molecule, a ROS scavenger, a protein stabilizer, and an inhibitor of programmed cell death (Butt et al., 2016; Orsini et al., 2018; Priya et al., 2019). With its broad range of active mechanisms, proline can enhance the stress tolerance of plants. In recent years, particularly through the exogenous proline treatment of plants, it has been proposed that it triggers stress tolerance mechanisms and leads to an improved response during subsequent stressful conditions (Aktas et al., 2007; Ashraf & Foolad, 2007; Priya et al., 2019). In this chapter, we will try to give information about exogenous proline treatment and the establishment of the response mechanism under abiotic stress conditions.

11.2 Proline biosynthesis and catabolism

The biosynthesis of proline, initially characterized in bacteria, occurs in three steps from glutamate (Kavi Kishor & Sreenivasulu, 2014). In plants, proline biosynthesis occurs through two main pathways dependent on glutamate or ornithine (Singh et al., 2022). Although the glutamate pathway is considered the main pathway for proline biosynthesis, the ornithine pathway has been reported to be active during seedling development and under supra-optimal nitrogen conditions (Armengaud et al., 2004; da Rocha et al., 2012). The glutamate-derived biosynthetic pathway is dominated by two

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consecutive steps. The bifunctional enzyme Δ1-pyrroline-5-carboxylate synthetase (P5CS) initiates this pathway by reducing glutamate to glutamate semialdehyde (GSA). Subsequently, GSA spontaneously converts to pyrroline-5-carboxylate (P5C), which serves as a common intermediate in both proline and ornithine metabolism. P5C is then further reduced to proline by the action of P5C reductase (P5CR). The electrons required for this process are provided by two NADPH molecules, and the biosynthesis of proline consumes one ATP molecule.

In plants, the biosynthesis and catabolism of proline occur in different cellular compartments (Szepesi & Szőllősi, 2018). Proline biosynthesis occurs in the cytoplasm and chloroplasts, and its degradation takes place in the mitochondria. Proline degradation in mitochondria is mediated by proline dehydrogenase (PDH or ProDH) and P5C dehydrogenase (P5CDH) enzymes (Szabados & Savoure, 2010). Therefore these enzymes involved in both the biosynthesis and catabolism of proline play a crucial role in the control of proline accumulation and tolerance to abiotic stress conditions (Dai et al., 2018; De Ronde et al., 2000; Rai & Penna, 2013; Wang et al., 2015; Zhang et al., 2014).

The molecular characterization of a gene or gene family is important not only for understanding evolutionary processes or interspecies relationships but also for elucidating its function within an organism or cell. In this regard, the *P5CS* genes and their orthologs have been isolated from various plant species, and studies have been done to investigate their biochemical and physiological aspects, shedding light on the molecular evolution of this gene (Kumari et al., 2008; Strizhov et al., 1997; Székely et al., 2008; Turchetto-Zolet et al., 2009). In a previous study, *P5CS* orthologs were identified in various organism groups, such as green algae, monocots, eudicots, and primates (Kumari et al., 2008). The results of the gene structure analysis in this study indicated that multicellular eukaryotes possess introns in the *P5CS* gene, whereas single-celled eukaryotes and prokaryotes lack introns. Additionally, it was reported that although plants have a higher number of introns compared to animals, the introns in plants are shorter in length (Kumari et al., 2008). Rai and Penna (2013) suggested that the observed differences in the sequence alignment of exons 6 and 7 and intron 6 between the *Arabidopsis thaliana* and *Oryza sativa P5CS1 and P5CS2* genes were primarily due to variations in intron length. During the evolution process, functional redundancy through gene duplications is a common phenomenon in many biological systems, particularly in metabolic pathways (Zhang, 2003). Phylogenetic analyses conducted in the plant kingdom reveal that the *P5CS* gene separates into two distinct groups (Ginzberg et al., 1998; Strizhov et al., 1997). In many plants, P5CS is encoded by two genes: *P5CS1* and *P5CS2*, which exhibit distinct transcriptional regulation. In vascular plants and animals, the P5CS enzyme exhibits dual functionality as γ-glutamyl kinases (GK) and γ-glutamyl phosphate reductases (GPR). In *A. thaliana*, there are two P5CS isoenzymes: AtP5CS1 (NP_001189715) and AtP5CS2 (NP_191120.2.). The amino acid residues 7−281 of the AtP5CS1 gene form the kinase domain, while the residues 296−692 contain the phosphate reductase domain (Fichman et al., 2015). Moreover, in silico analysis of the promoter regions of proline metabolism-related genes, including P5CS1, has revealed the presence of cis-regulatory elements (CREs) that contain binding sites for different transcription factors, including AP2/EREBP, MYB, WRKY, bZIP, and HD-HOX (Fichman et al., 2015).

11.3 Proline metabolism and functions in abiotic stress tolerance

In addition to being one of the 21 amino acids involved in protein synthesis, a more important role of proline as an active player in plant development has been identified in studies carried out by different scientists at the end of the last century (Chiang & Dandekar, 1995; Fujita et al., 1998; Schwacke et al., 1999). These studies have found large quantities of proline in the reproductive organs of some plants under nonstressful conditions. In another study, it was reported that proline biosynthesis genes were upregulated in seeds, fruits, and flowers of plants not exposed to abiotic stress (Schmidt et al., 2007). It has also been reported that proline, one of the osmotic regulators, protects the plant cells against environmental stress (Zouari et al., 2019). Proline accumulation is known to occur under conditions of salinity, drought, heavy metals, and extreme temperatures. Proline also acts as a protein-compatible hydrotrope, alleviating the acidic environment in the cytoplasm and keeping proper $NADP^+$/NADPH ratios in concert with metabolism (Gholami Zali & Ehsanzadeh, 2018).

Exogenous proline treatment(s) in plant species exposed to abiotic factor(s) have been reported to make a significant contribution to alleviating the effects of stress (Zouari et al., 2019) (Fig. 11.1). Depending on the variety of abiotic stress factor(s) and the plant species, the concentration of proline to be treated may vary (Ashraf & Foolad, 2007; Zouari et al., 2019).

Salinity stress is an essential abiotic stress factor(s) that drastically influences plant growth, development, and yield starting from the germination of seeds to crop yield (El Moukhtari et al., 2020). Due to increasing salinization in semiarid and arid lands in different regions of the world, the productivity of crops is gradually declining, and this poses a threat to global food security (Farooq et al., 2017). Numerous harmful effects of salinity related to osmosis and ion toxicity are known (Kolomeichuk et al., 2020). With osmotic stress (induced by salinity), water intake is restricted, and it

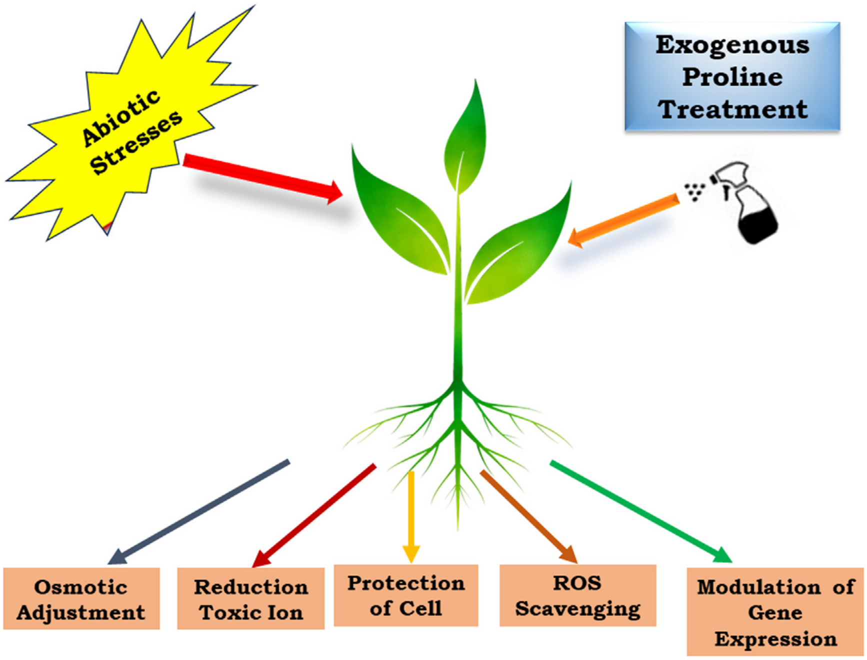

FIGURE 11.1 Exogenous proline treatments in alleviating abiotic stress. Exogenously applied proline alleviates the effect of abiotic stressors through osmotic adjustment, reduction of ion toxicity, cell protection, ROS scavenging, and modulation of gene expression. *ROS*, Reactive oxygen species.

is stated that this will cause decreases in plant growth and development parameters (Farooq et al., 2015). To overcome salinity, plants can promote various physiological and biochemical mechanisms related to water relations, photosynthesis, respiration, ion distribution, hormones, and antioxidant defense (Turkyilmaz Unal et al., 2014).

Salama and Mansour (2015) reported that proline is the most widespread internal osmolyte that accumulates when plants are exposed to various abiotic stress factor(s). It was determined that external proline application had a positive effect on some growth, development, and yield parameters in maize (*Zea mays* L.) plants in the presence of salt stress (Alam et al., 2017). Ashraf and Foolad (2007) discussed the effects of exogenous proline application on seed germination, development of seedling, and ion balance. It has been determined that proline treatments in various plants exposed to salinity are effective in promoting the antioxidant defense system (Abdelhamid et al., 2013; Butt et al., 2016; Qirat et al., 2018). Mahboob et al. (2016) reported that exogenous proline improves physiological and biochemical processes and alleviates the effects of stress by increasing the chlorophyll a and b, glycine-betaine, proline, total phenolic, and total soluble sugar content and regulating the $K+:Na+$ ratio in wheat exposed to salt stress.

Studies have revealed that these genes have distinct functions in response to abiotic stresses. In *Arabidopsis*, the *P5CS1* gene has been reported to play a role in proline accumulation and osmotic stress resistance, while its involvement in salt stress is associated with transcriptional memory (Feng et al., 2016; Kesari et al., 2012).

11.3.1 Drought stress

Drought stress, which is often correlated with another important abiotic stress factor(s), is one of the leading abiotic stress factor(s) that threaten the vital functions of the plant by reducing plant yield. Drought stress is becoming a formidable challenge because of global climate change, declining water supplies, and growing water demand (Hosseinifard et al., 2022).

Alkahtani et al. (2021) found that by applying exogenous proline to sugar beet under drought stress, stress symptoms were reduced by increasing proline amount, total phenolic, and antioxidant enzymes in the plant. Similarly, Farooq et al. (2017) stated that applied proline caused an increase in osmolytes such as glycine betaine, proline, chlorophyll, and total soluble phenolics in wheat plants.

11.3.2 Heavy metal stress

Lead, copper, nickel, etc. heavy metal amounts are increasing rapidly in water and soil due to mines, industrial wastes, chemical fertilizers, and pesticides. Especially cadmium, cobalt, and lead are highly toxic and nonbiodegradable, and they can accumulate and harm living things (Tangahu et al., 2011). They can disrupt cell membranes and DNA structure and cause protein and enzyme denaturation.

It is known that proline plays a role as a signal molecule, metal chelator, and antioxidant defense molecule in plants exposed to heavy metals (Ghosh et al., 2022; Hayat et al., 2012). Zouari et al. (2016) applied different concentrations of proline to olive trees exposed to cadmium stress and found that photosynthetic activity and antioxidant enzyme activity increased, thus increasing growth and yield.

11.3.3 Temperature stress

Both high and low temperatures pose a major threat to the life of plants, and the degree of impact varies with exposure time, severity, plant species, and age (Imran et al., 2021). Exogenous proline application has a healing effect especially in high-temperature stress, vegetative, and generative plant parts. Priya et al. (2019) reported that when they applied exogenous proline to mung beans exposed to high temperatures, it had a positive effect on yield as well as plant growth. Mohammadrezakhani et al. (2019) exogenous proline applications in three citrus species exposed to cold stress increased the amount of ascorbic acid, oxalic acid, citric acid, flavonoids, and some antioxidant enzymes. In *Vigna radiata* seedlings under cold stress, the applied proline also served as a nitrogen and carbon source and improved growth and development (Posmyk & Janas, 2007).

11.3.4 Flooding/waterlogging

Floods cause hypoxic conditions at the roots of plants. It causes damage to plants by affecting mineral uptake, photosynthesis, and ROS production (Arbona et al., 2016; Pucciariello et al., 2014). To cope with this, morphological, anatomical, physiological, and biochemical changes occur in plants. An increase in proline content was determined in rice exposed to flooding (Chanu & Sarangthem, 2015). It was found that proline accumulation in the leaves of peach plants increased when inoculated with arbuscular mycorrhizal fungi (AMF), and this application increased the tolerance to water accumulation (Tuo et al., 2015).

11.4 Proline metabolisms and epigenetic regulation

The P5CS gene and its transcript levels are of great importance in the transcriptional regulation of proline accumulation under stress conditions. Studies conducted in potatoes and chickpeas suggest that the expression of P5CS can be post-transcriptionally regulated by micro-RNAs (Shui et al., 2013; Yang et al., 2013). However, although these micro-RNAs have been shown to exhibit a negative correlation with P5CS transcript levels, their precise roles in the regulation of this gene remain not fully understood. Furthermore, several studies propose that changes in the methylation pattern play a significant role in the regulation of the P5CS gene.

It is suggested that modulation of gene expression at the level of DNA methylation plays a significant role in plant stress tolerance (Karan et al., 2012). Epigenetic regulation occurs through DNA methylation patterns and their associated changes. Chan et al. (2005) reported that proline metabolism is regulated by DNA methylation. The DNA methylation patterns of three key genes involved in proline biosynthesis were found to be altered in response to PEG treatment, resulting in increased proline accumulation (Zhang et al., 2013). In a study conducted on drought stress, it was reported that the P5CS1 gene possesses transcriptional memory (Feng et al., 2016). Similarly, it has been revealed that the expression of the P5CS1 gene is swiftly induced in response to the priming drought stress and contributes to the drought memory response in rice (Li et al., 2019).

11.5 Future aspect

As highlighted in this review, proline metabolism plays a significant role in plant adaptation to abiotic stresses. Genetic manipulation is an important tool to enhance the ability of plant species to adapt to adverse environmental conditions. In the last decade, the identification of several gene families involved in proline biosynthesis and particularly the characterization of the functions of the P5CS1 gene in stress tolerance have paved the way for new strategies to improve plant performance. Particularly, the promising CRISPR/Cas9 system has emerged as a convenient tool for effectively introducing mutations in both regulatory and coding sequences. In this context, it is crucial to elucidate the genomic changes associated with external proline application. Furthermore, the modification of genes involved in the regulation of internal proline metabolism through various biotechnological tools is also important for enhancing crop productivity under different stress conditions.

In conclusion, gaining a thorough understanding of the adaptive and regulatory mechanisms in plants that contribute to growth performance under the influence of multiple abiotic stress factors is essential for the development of modern varieties with enhanced adaptability to dynamic field conditions.

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