








REVIEW ARTICLE

Assessment of proline function in higher plants under extreme temperatures

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INTRODUCTION

The unprecedented risks associated with environmental factors like temperature, rainfall, and radiation, which are linked to projected climate change, can have pernicious impact on agriculture (Dhankher & Foyer 2018; Varshney *et al.* 2021; Farooq *et al.* 2022). Plants are subject to various environmental stresses throughout their life, which restrict growth and development

ABSTRACT

Climate change and abiotic stress factors are key players in crop losses worldwide. Among which, extreme temperatures (heat and cold) disturb plant growth and development, reduce productivity and, in severe cases, lead to plant death. Plants have developed numerous strategies to mitigate the detrimental impact of temperature stress. Exposure to stress leads to the accumulation of various metabolites, *e.g.* sugars, sugar alcohols, organic acids and amino acids. Plants accumulate the amino acid 'proline' in response to several abiotic stresses, including temperature stress. Proline abundance may result from *de novo* synthesis, hydrolysis of proteins, reduced utilization or degradation. Proline also leads to stress tolerance by maintaining the osmotic balance (still controversial), cell turgidity and indirectly modulating metabolism of reactive oxygen species. Furthermore, the crosstalk of proline with other osmoprotectants and signalling molecules, *e.g.* glycine betaine, abscisic acid, nitric oxide, hydrogen sulfide, soluble sugars, helps to strengthen protective mechanisms in stressful environments. Development of less temperature-responsive cultivars can be achieved by manipulating the biosynthesis of proline through genetic engineering. This review presents an overview of plant responses to extreme temperatures and an outline of proline metabolism under such temperatures. The exogenous application of proline as a protective molecule under extreme temperatures is also presented. Proline crosstalk and interaction with other molecules is also discussed. Finally, the potential of genetic engineering of proline-related genes is explained to develop 'temperature-smart' plants. In short, exogenous application of proline and genetic engineering of proline genes promise ways forward for developing 'temperature-smart' future crop plants.

and impose significant threats to food security worldwide (Dhankher & Foyer 2018; Farooq *et al.* 2022). Recent predictions on climate change suggest that the sustainability of major crops could be severely affected by both high and low temperatures during current and future extreme climate events (Fig. 1) (Lesk *et al.* 2016; Abd-Elmabod *et al.* 2020; Giordano *et al.* 2021; Raza *et al.* 2021; Haider *et al.* 2022; Raza 2022; Raza *et al.* 2022a). The yield deficit for major field crops is 6–7% per

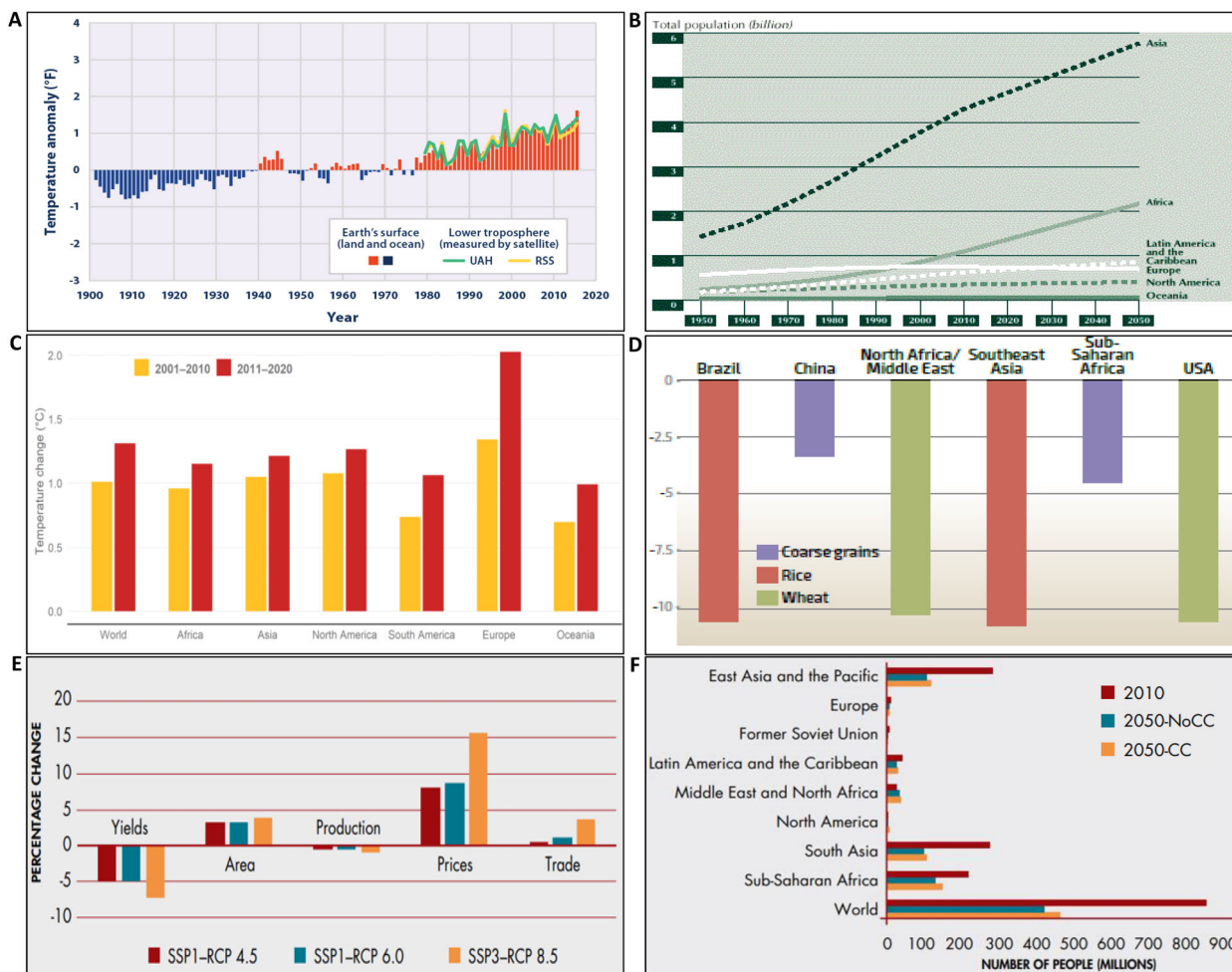


Fig. 1. Impact of climate change on temperature variation and crop yield. (A) Temperature variations worldwide from 1900–2020; (B) Continent-wise population (1950 to 1990) and projections for 1995 to 2050; (C) Global trend in mean annual temperature in recent decades; (D) Projected decline in cereal yields (without adaptation) through temperature and climate change in 2050; (E) Global impact of climate change on crop yield, area, production, price, and trade by 2050; (F) Regional risk of hunger due to climate change in the population by 2050. Sources: (A) https://19january2021snapshot.epa.gov/climate-indicators/climate-change-indicators-us-and-global-temperature_.html; (B) <https://www.fao.org/3/w2612e/w2612e04b.htm>; (C) <https://www.fao.org/3/cb4410en/cb4410en.pdf>; (D) <https://www.fao.org/ag/save-and-grow/MRW/en/1/index.html>; (E, F) <https://www.fao.org/3/6030e/6030e.pdf>.

1 °C rise in seasonal mean meteorological conditions (Fig. 1) (Lesk *et al.* 2016). Figure 1 shows the historical and predicted influence of climate change on temperature deviation and crop yields that increase the risk of hunger in growing population. This population growth around the world and extreme climate change events significantly impacts crop production and food supply (Fig. 1). Like most stresses, extreme temperatures (cold stress, CS; heat stress, HS) influence plant growth and development individually or in combination with other stresses. Ultimately, they can cause oxidative damage, cellular injury, and even cell death after long-term exposure, imposing significant threats to food security worldwide (Basavaraj & Rane 2020; Giordano *et al.* 2021; Raza *et al.* 2021; Raza *et al.* 2022a; Sharma *et al.* 2022a; Saeed *et al.* 2023).

The phenological stage of a species has an optimal temperature range for normal growth and development. Any deviation (low or high) from this range either activates tolerance mechanisms or damages the plant (Ji *et al.* 2021). At the morphological level, damage as a result of temperature stress is manifested

as inhibition of root and shoot growth, leaf scorching/abscission, stem burning, and fruit discoloration, ultimately decreasing crop yield (Chaudhary *et al.* 2020). At the cellular level, temperature-induced overproduction of reactive oxygen species (ROS), *viz* hydrogen peroxide (H₂O₂), singlet oxygen (¹O₂), hydroxyl ([•]OH), and superoxide (O₂^{•-}) radicals, lead to oxidative damage affecting metabolic functions and thereby limiting plant growth and development under temperature stress (Bita & Gerats 2013; Mittler 2017; Kohli *et al.* 2019; Hasanuzzaman *et al.* 2020a; Mittler *et al.* 2022; Raza *et al.* 2022c).

To offset the adverse effect of extreme temperatures, plants have evolved mechanisms that can accumulate heat shock proteins, osmoprotectants, and secondary metabolites; thus modifying phytohormone signalling, and upregulating antioxidant defence systems (Raza *et al.* 2021; Haider *et al.* 2022; Raza 2022; Raza *et al.* 2022a; Bhardwaj *et al.* 2023). Among these responses, the accumulation of proline, a proteinogenic amino acid, affects survival of plants under diverse stress conditions,

such as salinity, drought, heavy metal, or temperature stress (Wang *et al.* 2007; Liang *et al.* 2013; Signorelli *et al.* 2013a; Bouthour *et al.* 2015; Rodríguez-Ruiz *et al.* 2019). Proline has diverse roles in stress conditions, such as maintaining cell turgor, as a source of carbon and nitrogen, chelation of metals, and as a signalling molecule (Kavi Kishor & Sreenivasulu 2014; Bouthour *et al.* 2015; de Freitas *et al.* 2018; Aghdam *et al.* 2019). In many cases, proline accumulation acts as a general ROS scavenger under diverse stresses. Some data suggest that Pro interacts with the hydroxyl radical ($\cdot\text{OH}$) (Signorelli *et al.* 2014), others consider that it is not directly involved in protection against $\text{O}_2^{\cdot-}$, singlet oxygen ($^1\text{O}_2$), nitric oxide ($\cdot\text{NO}$), nitrogen dioxide ($\cdot\text{NO}_2$) or peroxyxynitrite (ONOO^-) (Signorelli *et al.* 2013b, 2016). Therefore, caution should be taken in maintaining proline as a generic antioxidant. Proline is also synthesized in unstressed environments, where it modulates the floral transition (Mattioli *et al.* 2008, 2022) and development of the male gametophyte and seeds (Mattioli *et al.* 2012, 2018). Studies have reported the involvement of proline in essential pathways, such as the pentose phosphate, tricarboxylic acid, urea cycle, and phenylpropanoid pathways (Hare & Cress 1997; Shetty 2004; Priya *et al.* 2019).

On the other hand, exogenous application of proline has been shown to mitigate some stresses, including heat and cold stress (Liang *et al.* 2013; Shin *et al.* 2018; Priya *et al.* 2019; Mattioli *et al.* 2020). Proline application can decrease oxidative stress damage and membrane injury, increase antioxidant enzymes, leaf water content, and photosynthesis under stress (Priya *et al.* 2019; Shakeri *et al.* 2019; Ghosh *et al.* 2022). The mechanisms of proline-mediated stress management and growth enhancement are poorly understood. Proline metabolism under conditions of salinity and heavy metal stress is well documented; nevertheless, molecular and physiological functions of proline accumulation under stress are yet to be resolved (Ghosh *et al.* 2022). Notably, the dynamic role of proline in plant tolerance to heat stress (HS) and cold stress (CS) has not been well documented, with special emphasis on its interaction with other metabolites, hormones, and signalling molecules. This review focuses on proline metabolism under CS and HS and how its exogenous application improves plant tolerance to such extreme temperatures. We also present an overview of effects and responses to extreme temperatures on plants, highlight crosstalk of proline with other signalling molecules, and examine genetic engineering of proline enzyme-encoding genes that have great potential in mitigating the adverse effect of temperature stress through development of 'temperature-smart' transgenic plants.

EFFECTS AND RESPONSES OF TEMPERATURE STRESS ON PLANTS: AN OVERVIEW

Plants are exposed to diverse abiotic stresses that have devastating impacts on growth and development (Kapoor *et al.* 2022; Sharma *et al.* 2022a, 2022b; Raza *et al.* 2022b, 2022e). Heat stress (HS) is a serious abiotic stress, and its intensity is increasing, posing a serious threat to crop productivity and food security (Lesk *et al.* 2016; Abd-Elmabod *et al.* 2020; Giordano *et al.* 2021; Raza *et al.* 2021, 2022a; Kapoor *et al.* 2022; Raza 2022). Germination is essential for plant life, and HS exposure substantially reduces germination by disrupting enzyme activity as well as uptake and availability of water and

nutrients (Essemine *et al.* 2010; Giordano *et al.* 2021; Malabarba *et al.* 2021; Raza *et al.* 2022a). Heat stress also reduces root growth, seedling growth and triggers significant yield losses (Kumar *et al.* 2011; Saeed *et al.* 2023). Moreover, HS also reduces cell size, growth, divisions and, ultimately, death, thereby causing significant reductions in growth and biomass (Srivastava *et al.* 2012). Additionally, HS often induces morphological changes to the plant body, including scorching of leaves and stems, leaf rolling, necrosis and chlorosis (Omae *et al.* 2012).

High temperature negatively affects plant osmotic adjustment by increasing evapotranspiration, which irreparably affects solute production that is essential for stress tolerance (Hassan *et al.* 2021; Raza 2022; Raza *et al.* 2022a; Saeed *et al.* 2023). Increasing temperatures also reduce photosynthesis and increase respiration, which in turn reduces assimilation and causes substantial yield loss (Hassan *et al.* 2021). HS disturbs cell metabolism by downregulating water uptake, leading to turgor loss (Machado & Paulsen 2001). Moreover, HS also induces ROS, which damage cellular membranes and increase leakage of important osmolytes (Wahid & Close 2007; Mittler 2017; Mittler *et al.* 2022; Saeed *et al.* 2023). Additionally, HS reduces carbohydrate metabolism, which disrupts reproductive events, such as flower development, fruit set, seed development that lead to yield loss in a wide variety of crops (Huang *et al.* 2012; Kapoor *et al.* 2022).

The source–sink relationship is important in plant growth and development (Huang *et al.* 2008); HS disturbs this relationship and causes a substantial reduction in assimilate production. HS also disturbs and reduces nutrient uptake and thus assimilation in plants (Hungria & Kaschuk 2014). Furthermore, HS reduces the acquisition of nutrients by reducing root growth, biomass productivity and nutrient uptake (Hassan *et al.* 2021). Increasing temperatures also deplete liable carbon and reduce transport of carbohydrates from roots to shoots, which in turn causes a significant reduction in nutrient uptake (Huang *et al.* 2008; Yang *et al.* 2011). HS also alters chloroplast structure and disorganizes the thylakoids, leading to a major reduction in photosynthesis (Wang *et al.* 2009). HS also critically damages activity of photosystem II (PS-II), stomatal conductance and intercellular CO_2 concentration, all of which reduce photosynthesis and result in a substantial decrease in plant growth (Mohammed & Tarpley 2010; Greer & Weedon 2012; Sabagh *et al.* 2020; Sharma *et al.* 2022c).

Heat stress disturbs C and N distribution and metabolism in plants, which negatively affect leaf protein and starch metabolism, leading to a considerable reduction in yield and quality (Calderini *et al.* 2006; Yang *et al.* 2011). Plant reproduction is sensitive to HS, which can severely damage overall yield (Lobell *et al.* 2011), decreasing the formation of floral buds and fruit set and causing flower abortion, seed sterility and extensive reductions in productivity (Maheswari *et al.* 2012). HS also increases respiration, ATP production and generation of ROS that damage cellular structures (Bryla *et al.* 2001). Plants activate enzymatic and non-enzymatic antioxidant defence systems to cope with heat-induced oxidative stress (Hassan *et al.* 2021); they accumulate osmolytes, including sugars, proline and glycine betaine (GB) to mitigate adverse effects of HS (Sabagh *et al.* 2021). Different phytohormones have important roles in plant responses to HS. Nonetheless, HS clearly affects the synthesis and degradation of hormones which influence plant

growth and survival (Hussain *et al.* 2018; Sabagh *et al.* 2021; Raza *et al.* 2022c).

Cold stress (CS) also induces morphological changes and significantly reduces plant growth and development. Common symptoms of CS are stunted growth, chlorosis, necrosis and wilting (Hussain *et al.* 2018; Manasa *et al.* 2021; Zhang *et al.* 2022). Cold stress also reduces seed germination, internode and coleoptile elongation and causes substantial decrease in growth (Jame & Cutforth 2004). CS also limits growth and surface area of roots, which reduces nutrient and water uptake and substantially reduces growth (Kul *et al.* 2020). Moreover, CS also deforms pollen tubes and induces flower loss, resulting in reduced grain development (Ji *et al.* 2017; Hussain *et al.* 2018).

Plants need stable membranes to survive stress conditions, but CS reduces membrane stability leading to loss of important osmolytes (Chen *et al.* 2018; Barajas-Lopez *et al.* 2021). The formation of ice crystals under CS reduces water potential in the apoplast, causing water outflow from the cell (Puhakainen *et al.* 2004). CS often leads to drought stress, a major reason for reduced plant growth (Hassan *et al.* 2021). The onset of drought under CS reduces root growth, turgidity and hydraulic conduction, leading to reduced water and nutrient uptake and significant yield losses (Hassan *et al.* 2021). Moreover, CS also disturbs soil microbial activity that affects plant nutrient relationships and uptake. Ice crystal formation increases electrolyte leakage, punctures cells, and leads to increased cytosolic outflow (Sun *et al.* 2019).

Cold stress negatively affects photosynthesis by reducing efficiency of PS-II, and reducing stomatal conductivity, chlorophyll biosynthesis, and activity of RuBisCO (Cvetkovic *et al.* 2017; Khan *et al.* 2017). Further, CS impairs respiratory activity and the cytochrome pathway of electron transport, which damages mitochondria and decreases the flow of kinetic energy, thus affecting normal functioning in plants (Banerjee & Roychoudhury 2019; Ikkonen *et al.* 2020). ROS substantially increase under CS, causing significant damage to proteins, lipids and cellular structures (Hassan *et al.* 2021; Mittler *et al.* 2022). Plants activate antioxidant defence systems to cope with the deleterious impacts of CS (Sun *et al.* 2019; Ritonga & Chen 2020; Lei *et al.* 2022). Nonetheless, plant response depends on various factors, including signal perception and transduction (Ganeshan *et al.* 2008). Plants also activate stress-responsive genes to cope with CS and many genes have been identified that play important roles against it (Kosová *et al.* 2008; Knight & Knight 2012; Park *et al.* 2015; Mittler *et al.* 2022; Zhang *et al.* 2022; Raza *et al.* 2022d). Plants also accumulate osmolytes, which protect them from ROS, to maintain functioning and counter CS (Thakur & Nayyar 2013; Yokota *et al.* 2015; Raza *et al.* 2021, 2022a). Additionally, plants accumulate cryoprotective polypeptides (*e.g.* COR15a) that protect them from CS, ensuring their survival and better growth under CS (Ritonga & Chen 2020).

In chickpea plants, CS leads to electrolyte leakage that damages cellular function and structure, accompanied by ROS production, leading to decreased growth after 6 days under CS (Amini *et al.* 2021). In another study, CS initiated oxidative bursts through accumulation of ROS and expression of antioxidant genes related to defence in rice, which markedly suppressed growth (Song *et al.* 2021). Repkina *et al.* (2021) found that a temperature drop below 4 °C triggers ice nucleation and

electrolyte seepage, preventing normal functioning of photosynthetic pigments and reducing development in sensitive wheat cultivars. Jha & Mohamed (2022), found that 4 °C diminished seed germination, growth indices and membrane stability, concurrent with hormone loss through oxidative stress in maize.

PROLINE METABOLISM IN HIGHER PLANTS

The cellular metabolism of proline is compartmentalized, while its synthesis takes place mainly in the cytosol, and its catabolism occurs in the chloroplast and mitochondrion. Biosynthesis can be either *via* the glutamate or the ornithine pathway (Fichman *et al.* 2015). However, the functional significance of the ornithine pathway is complex. Some authors claim that the ornithine pathway can contribute to proline synthesis (Shin *et al.* 2016). It is widely accepted that glutamate is the major pathway for proline synthesis, especially under stress conditions (Fichman *et al.* 2015). However, Funck *et al.* (2008) demonstrated that ornithine aminotransferase (OAT) is used for glutamate production but not for proline synthesis, and that the functional relevance of OAT for proline synthesis is questionable.

In the chloroplast, glutamate is reduced to glutamate semialdehyde (GSA) in a reaction catalysed by Δ -1-pyrroline-5-carboxylate synthetase (P5CS), followed by the spontaneous conversion of GSA to an intermediate, pyrroline-5-carboxylate (P5C). P5CS is a rate-limiting enzyme, and its transcriptional regulation and feedback inhibition monitor its activity (Peng *et al.* 1996; Hong *et al.* 2000; Székely 2008; Zhang *et al.* 2016; Meena *et al.* 2017). Several factors regulate P5CDH, *e.g.* abscisic acid (ABA), natural antisense short interfering RNA (nat-siRNAs), epigenetic regulators or alternative splicing (Borsani *et al.* 2005; Zhang *et al.* 2014). The enzyme Δ -1-pyrroline-5-carboxylate reductase (P5CR) catalyses the final step of proline synthesis. Loss in function of the proline responding-1 (*Pro-1*) gene that encodes P5CS inhibits proline biosynthesis and accumulation in maize (Wang *et al.* 2014). Lack of proline increases the level of uncharged tRNA^{Pro} AGG codon, resulting in a significant reduction in protein synthesis, indicating that proline plays a crucial role in the synthesis of proteins (Szepesi & Szöllösi 2018).

On the other hand, in the ornithine pathway, which takes place in the mitochondrion, arginine is first reduced to ornithine by the action of arginase, which is further transaminated by OAT, leading to formation of GSA and P5C. The last reaction is similar to the glutamate pathway, where P5CR converts P5C to proline by P5C dehydrogenase. In the P5CR cycle, P5C either goes toward glutamate production or exits the mitochondrion and returns to the cytosol to be reduced to proline again. The ProDH and P5CR cycles occur in both plant and animal cells (Meena *et al.* 2017; Szepesi & Szöllösi 2018). Information on proline transport within the cell is limited and the existence of glutamate/proline transporters has been suggested (Funck *et al.* 2010; Szepesi & Szöllösi 2018). Future studies are required to identify the transporters of proline across the boundaries of chloroplasts and mitochondria.

The catabolism of proline to glutamic acid (Glu) is initiated through catalytic activity of proline dehydrogenase (ProDH) in mitochondria (Fig. 2). This enzyme is bound to the inner membrane of mitochondria and is involved in transfer of

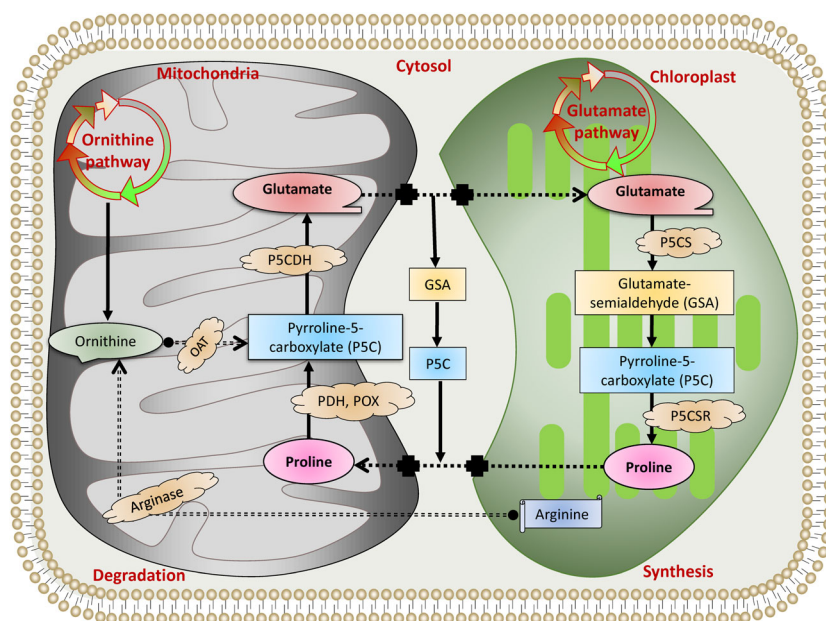


Fig. 2. Proline biosynthesis and degradation in plants. Mainly, proline synthesis occurs in the chloroplast and cytosol and takes place in two ways: *i.e.* the glutamate and ornithine pathways. In the glutamate pathway, glutamate is reduced to GSA by the P5CS enzyme and spontaneously converted to P5C. The P5CR enzyme further reduces the P5C intermediate to proline. Similarly, in the ornithine pathway, arginine is first reduced into ornithine by the action of the arginase enzyme. Ornithine is next transaminated by the OAT enzyme, generating GSA and P5C, which is converted to proline. Proline degradation occurs in mitochondria *via* the sequential action of PDH or POX enzyme, resulting in P5C from proline, the P5CDH enzyme then converts P5C to glutamate. GSA, glutamate-semialdehyde; P5CS, pyrroline-5-carboxylate synthetase; P5C, pyrroline-5-carboxylate; P5CR, P5C reductase; OAT, ornithine-delta-aminotransferase; PDH, proline dehydrogenase; POX, proline oxidase; P5CDH, P5C dehydrogenase.

electrons to ubiquinone in the mitochondrial electron chain (Kiyosue *et al.* 1996; Liang *et al.* 2013). The first reaction of ProDH involves oxidation of proline into P5C, followed by oxidation of P5C by P5CDH to glutamate (Funck *et al.* 2010; Szepesi & Szöllősi 2018). Onset of stress downregulates the catabolic pathway, thereby allowing the build-up of proline (Szepesi & Szöllősi 2018). In addition to stress-induced downregulation of the catabolic pathway, proline build-up is also related to the strong upregulation of anabolic genes (Szepesi & Szöllősi 2018). Downregulation of the catabolic pathway also depends on the action of *DFR1* (Ren *et al.* 2018). Reduced activity of P5CDH can induce a significant increase in ROS under stress conditions; hence, homeotic control of ROS levels is achieved through enhanced proline oxidation by P5CDH, which prevents intense P5C–proline cycling and ROS production from electron run-off in mitochondria (Szepesi & Szöllősi 2018). Figure 2 provides a model of proline metabolism and the enzymes involved in the different subcellular compartments.

EXOGENOUS APPLICATION OF PROLINE MITIGATES EXTREME TEMPERATURE STRESS IN PLANTS

Proline plays a role in plant metabolism, particularly in the mechanism of plant responses to multiple abiotic and biotic stresses. Proline forms complexes with heavy metals or cross-talk with other signalling molecules (Sharma *et al.* 1998; Liang *et al.* 2013; Ghosh *et al.* 2021). Foliar application of proline can successfully alleviate the adverse effects of CS and HS in various crops. Table 1 summarizes representative studies on exogenous

proline-induced physiological response under temperature stress. Plants have evolved proline-induced physiological, biochemical, and molecular processes to respond, adapt and achieve tolerance to CS and HS. Figure 3 provides a comprehensive representation of all the metabolic processes that are impacted by the exogenous application of proline to induce temperature tolerance in plants.

Cold stress management by proline application

Cold stress (CS) can be classified into (i) chilling stress, where plants experience temperatures between 0–15 °C, and (ii) freezing stress at temperatures below 0 °C. CS affects plant physiology and biochemistry by targeting the photosynthetic machinery, cytosolic viscosity, and overproduction of ROS, which cause chlorosis, necrosis, and even cell death, under severe stress (Zbierzak *et al.* 2013; Manasa *et al.* 2021; Raza *et al.* 2021, 2022a). Disorganization of chloroplasts and mitochondria under CS limits basic metabolic reactions responsible for growth in tropical and subtropical plants (Heidarvand & Maali Amiri 2010; Karami-Moalem *et al.* 2018; Ngoc *et al.* 2021). The positive role of proline in improving stress tolerance has been elucidated (Table 1).

Quinoa (*Chenopodium quinoa*) exposed to CS (4 °C) has decreased chlorophyll (Chl), ascorbic acid, and phenolic content, together with increased lipid peroxidation that is ameliorated by exogenous application of proline (Yaqoob *et al.* 2019). Cold stress enhances levels of methylglyoxal and lipid peroxidation in tea buds. Application of 20 mM Pro and 20 mM betaine relieve CS by downregulating lipid peroxidation and upregulating

Table 1. Representative examples of the beneficial role of proline-induced temperature stress tolerance in different plant species. Abbreviations are explained in the main text.

Plant species	Stress condition	Growth stage	Proline concentration	Protective role	References
Cold stress					
<i>Saccharum officinarum</i>	15 °C, 8 h	Bud sprouting	10 mM	CS prevented bud sprouting and biomass increase, while proline treatment stimulated growth through decreased H ₂ O ₂ generation, increased endogenous proline, K ⁺ , and Ca ²⁺ levels	Rasheed <i>et al.</i> (2010)
<i>Brassica napus</i> L.	4 °C, 14 days	Seedling	5, 10, 15 and 20 mM	Cold stress-induced membrane injury <i>via</i> electrolyte leakage, while proline relieved CS by increasing the endogenous proline pool, total soluble sugars, and down-regulating electrolyte leakage	Jonytiene <i>et al.</i> (2012)
<i>Capsicum annuum</i>	4, 8 °C,	Callus sample	12 and 24 mM	Exogenous proline strengthened plant defence system through increased accumulation of endogenous proline and apoplastic protein under CS	Koç (2013)
<i>Spinacia oleracea</i>	2.5/–1/0 °C, 14 h	Seedling	10 mM	Exogenous proline increased freezing tolerance through elevated amino acid production, <i>i.e.</i> arginase, lysine, and proline under CS	Shin <i>et al.</i> (2018)
<i>Chenopodium quinoa</i>	4 °C, 20 h	Seedling	25 and 50 mM	Prolonged CS disrupted Chl and decreased SOD, POD and CAT, while proline application increased growth <i>via</i> elevated Chl content, fresh and dry biomass, SOD, POD, and CAT activity, and decreased MDA content	Yaqoob <i>et al.</i> (2019)
<i>Citrus reticulata</i>	1, –1, –3 °C, 3 days	At maturity	0, 15, 20 mM	CS inhibited antioxidant activity and growth, whereas high proline levels augmented the plant defence system and growth through increased CAT, APX, and endogenous proline levels	Mohammadrezakhani <i>et al.</i> (2021)
<i>Zea mays</i> L.	4 °C/4 °C, 5 days	Germination	15 mM	Proline treatment improved germination potential, increased activity of α -amylase, P5CS, and OAT, and enhanced proline synthesis pathways and accumulation, while alleviating reduced IAA, ZR, and GA	Zuo <i>et al.</i> (2022)
<i>Solanum lycopersicum</i> L.	5 ± 1 °C (dark) for 12 h and 10 ± 1 °C (light) for 12 h, continues for 7d	Seedling	0, 5, 10 and 15 mM	Proline application (mainly 10 mM) protects from negative effects of CS and enhances antioxidative enzyme activity like APX, CAT, and SOD	Uzal (2022)
Heat stress					
<i>Saccharum</i> sp.	25 °C, 5 h	Bud sprouting	20 mM	Heat stress diminished bud sprouting and mesophyll cell area, while proline application relieved stress on bud sprouting, increased plant biomass, K ⁺ , and Ca ²⁺ levels, and decreased H ₂ O ₂	Rasheed <i>et al.</i> (2010)
<i>Agrostis stolonifera</i>	35/30 °C, 35 days	Seedling	10 mM	Proline reduced leaf senescence induced by HS and increased HS tolerance by increasing activity of Chl synthesis enzymes, PSII efficiency, and suppressing CHLase enzyme activity	Rossi <i>et al.</i> (2020)
<i>Brassica napus</i> L.	38/28 °C, 24 and 48 h	Seedling	2 mM	Exogenous proline application stimulated regulatory interactions between methylglyoxal and ROS detoxification and improved thermotolerance through increased water content photosynthetic pigments and downregulation of H ₂ O ₂ , LOX, and MDA	Hasanuzzaman <i>et al.</i> (2020)
<i>Agrostis stolonifera</i>	35 °C, 28 days	Seedling	10 mM	Foliar spray with proline alleviated HS and improved turf quality through increased Chl and regulation of amino acids, <i>viz</i> glutamic acid, aspartic acid, leucine, valine, threonine, and tryptophan	Rossi <i>et al.</i> (2021)
<i>Abelmoschus esculentus</i> L.	45/35 °C day/night, 7 days	Seedling	2.5 mM	Exogenous proline decreased adverse effects of HS by improving turgor, Chl content, amino acid content, CO ₂ assimilation, and antioxidant concentration	Hussain <i>et al.</i> (2021)

Table 1. (Continued)

Plant species	Stress condition	Growth stage	Proline concentration	Protective role	References
<i>Oryza sativa</i> L.	35–36 °C, 10 days	Seedling	10, 20 and 30 mM	HS diminished Chl, antioxidants and osmoprotectants at reproductive and vegetative stages. Proline application alleviated the stress by increasing SOD, POD, CAT, total soluble sugars, inhibiting MDA and ROS formation in thylakoids	Hanif <i>et al.</i> (2021)

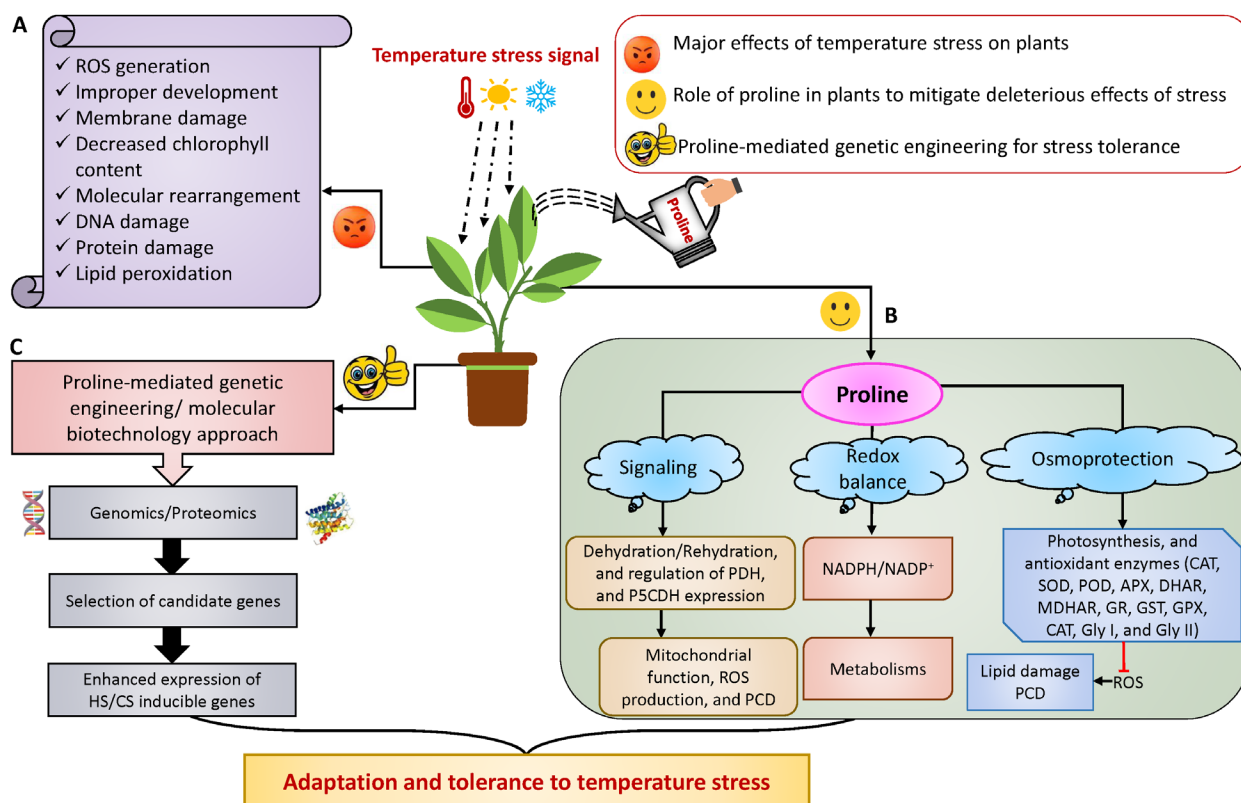


Fig. 3. An overview of proline-mediated temperature stress tolerance in plants. (A) Major effects of temperature stress on plants, which ultimately reduce the overall plant growth and production; (B) protective role of proline in mitigating the adverse effects of temperature stress. Proline directly takes part in signalling networks, helps maintain redox balance (as ROS scavenger), and serves as an osmoprotectant; (C) proline-mediated genetic engineering of proline-encoding and temperature stress-related genes. These candidate genes can be manipulated using various approaches, such as overexpression, knockout *via* CRISPR/Cas system, etc. Some related examples are documented in the main text and in Table 2. Abbreviations are explained in the main text.

glutathione-S-transferase activity, thereby protecting glyoxalase pathway enzymes in tea buds (Kumar & Yadav 2009). Cold stress-induced injury to biomembrane function, such as disruption of membrane-bound ion pumps in pepper callus, was relieved with 24 mM proline under different CS treatments (4, 8, 16, 24 °C). Proline increased tolerance by enhancing bioaccumulation of antifreeze protein, which inhibits ice crystal formation in pepper tissue culture (Koç 2013).

Cold stress (10 °C) triggered abortion of flowers and pod set in chickpea (*Cicer arietinum* L.) (Kaur *et al.* 2011). This loss was mitigated by spraying with 10 μ M proline at the flowering stage, which increased sucrose and trehalose content of leaves, thus favouring flower retention and pod set, leading to improved yield under CS (Kaur *et al.* 2011). Similarly, endogenous proline and total soluble sugar content were improved in

proline-treated rapeseed (*Brassica napus* L.) (Jonytiene *et al.* 2012). The beneficial effect of proline in 5-week-old spinach seedlings was evaluated by pre-treatment with proline (10 mM) before freezing stress (−1 °C). The pre-treated plants also showed a six-fold increase in endogenous proline content. Other amino acids, *e.g.* lysine, leucine, and arginine, also increased 2.8-fold and may contribute toward freezing tolerance in spinach (Shin *et al.* 2018).

A recent study examined the response of *Citrus* species to foliar application of proline and putrescine (0, 15, 20 mM) under CS treatment (1, −1, −3 °C) for 6 h (Mohammadrezakhani *et al.* 2021). Cold stress exposure altered physio-biochemical processes and negatively affected growth. Exogenous treatment with proline and putrescine resulted in lower ROS production, and improved osmotic balance and phenolic compound content

Table 2. Examples of proline-mediated genetic engineering to improve temperature stress tolerance in different plant species. Abbreviations are explained in the main text.

Gene	Stress	Plant species	Key mechanism	References
<i>P5CSF129A</i>	40 °C/60 min	<i>Nicotiana tabacum</i>	Proline promotes protection against oxidative stress, net photosynthesis, water use efficiency, Chl content and xanthophyll cycle pigments were higher in transgenics	Pospisilova <i>et al.</i> (2011)
<i>BrCIPK1</i>	10 °C	<i>Brassica rapa</i>	<i>BrCIPK1</i> is involved in stress adaptations through activation of pyrroline-5-carboxylate synthase in the proline biosynthetic pathway	Abdula <i>et al.</i> (2016)
<i>CcHyPRP</i>	48 °C, 2 h	<i>Oryza sativa</i>	Increased activity of catalase, SOD, and lower levels of MDA in <i>CcHyPRP</i> -transgenics as compared	Mellacheruvu <i>et al.</i> (2016)
<i>P5CS</i>	4 °C, 3 days	<i>Petunia violacea</i>	<i>P5CS</i> alleviates negative stress caused by CS and improves cold tolerance by enhancing overexpression of ROS scavenging genes like <i>APX</i> , <i>CAT</i> , and <i>SOD</i>	Jamshidnia <i>et al.</i> (2018)
<i>PhP5CR</i>	40 °C/35 °C, 7 days	<i>Petunia hybrida</i>	During HS, <i>APX</i> activity increases and enhances stress tolerance under abiotic stress by eliminating or reducing ROS	Yue <i>et al.</i> (2019)
<i>MtPDI</i>	26 °C, 16 h	<i>Oryza sativa</i>	Higher MDA and proline content, SOD and POD activity, and free thiols group in transgenic plants confer HS tolerance	Wang <i>et al.</i> (2019)
<i>AmP5CS</i>	40 ± 1 °C, 7 days	<i>Avicennia marina</i>	<i>AmP5CS</i> is involved in HS response by enhancing expression of ROS scavenging genes like <i>APX</i> , <i>CAT</i> , and <i>SOD</i>	Liu <i>et al.</i> (2020)

(Mohammadrezakhani *et al.* 2021). Similarly, exogenous proline decreased harmful effects of CS by upregulating activity of membrane transporters and acquisition of mineral nutrients in sugarcane (Rasheed *et al.* 2010). In proline-treated plants, the number and area of mesophyll cells and vascular bundles was significantly increased, thus leading to an increase in number of leaves in CS plants (Rasheed *et al.* 2010).

Addition of proline upregulated CS tolerance in rapeseed, while downregulating leaf membrane injury. In proline-treated plants, electrolyte leakage declined by 4–18% in shoots in contrast to untreated controls under CS conditions. Endogenous proline levels and total soluble sugar content improved tolerance to oxidative stress in proline-treated plants (Jonnytiene *et al.* 2012). Notably, proline treatment effectively lowered oxidative stress markers in mung bean (*Vigna radiata* L.) seedlings exposed to CS (5 °C). Exogenous application of L-proline resulted in increased C and N, leading to higher root and hypocotyl growth in mung bean seedlings subjected to CS. These findings suggest that proline acts as a compatible osmolyte that protects structures and functions of proteins under CS (Posmyk & Janas 2007). Proline has been effectively used as a seed priming agent, together with other compounds, improving germination-related indices under CS in crops like muskmelon (Kaur & Gupta 2017) and squash (Dimple *et al.* 2017).

Proline also collaborates with phytohormones in improving stress tolerance and crop productivity. For instance, use of 20 mM proline in conjunction with 0.1 mM salicylic acid in seed germination of muskmelon (*Cucumis melo* L.) under CS increased vigour indices and improved seed germination in the seeds relative to controls (Kaur & Gupta 2017). These authors also reported that CS inhibited seed germination and activity of antioxidant enzymes (peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD)) through the generation of ROS in cucumber seeds. Under CS, treatment of cucumber seeds with 10 mM proline and salicylic acid contributed to stabilizing cell structures and improving germination percentage and antioxidant enzyme activity (Kaur & Gupta 2015). Dimple *et al.* (2017) studied proline and salicylic acid seed priming in squash under CS. Seed priming with 10 and 20 mM proline and salicylic acid

increased germination percentage, root growth, fresh and dry biomass, and vigour under CS. Application of 20 mM proline was more effective in increasing total soluble sugar, protein content, and germination in squash, compared with control plants (Dimple *et al.* 2017).

Proline effectively overcomes chilling injury in fruit and vegetables. Chilling stress can lead to internal browning and limited post-harvest life of peach (*Prunus persica* L.) (Gohari *et al.* 2021). Exogenous treatment with proline (0, 5, 10, 15 mM) and L-cysteine diminished excess total soluble solids and decreased peach fruit firmness during storage. Further, exogenous proline significantly decreased malondialdehyde (MDA), H₂O₂, and electrolyte leakage, concurrent with increased antioxidant enzyme activity in treated fruits, improving the quality of flat peach fruits (Gohari *et al.* 2021). A study evaluated the role of proline in improving the shelf life of potato slices and prevent browning at 2–4 °C, where pretreatment with proline (90 mM) solution decreased browning, total phenolic content, polyphenol oxidase, and activity of several amino acids, while concurrently increasing accumulation of proline and antioxidative capacity of freshly cut potatoes (Liu *et al.* 2022).

All these examples indicate that the exogenous application of proline is beneficial to alleviate the negative effects of CS at different stages of plant development, from germination to fruit formation.

Heat stress management with proline application

Temperature fluxes, particularly HS, significantly impact plant development, resulting in decreased quality and yield loss. HS damages the plasma membrane, disrupts nucleic acid structure, protein synthesis and activities of mitochondria and chloroplasts, leading to ROS production and oxidative stress (Raza *et al.* 2021; Seth *et al.* 2021; Haider *et al.* 2022; Raza 2022; Sharma *et al.* 2022c). Similar to the CS response, exogenous proline application plays an important role in improving HS tolerance in crops (Table 1).

Chilli (*Capsicum annum* L.) seeds sown in plastic pots were subjected to HS (40/30 °C day/night) 30 days after

germination. HS decreased morphological and physiological attributes of chilli plants under HS (Akram *et al.* 2021). Foliar spray application of proline (0, 5, 10 mM) significantly mitigated HS in chilli and upregulated growth and biomass; subsequently increasing photosynthetic pigments, regulating stomatal conductance and transpiration, resulting in increased tolerance to HS. Exogenous proline was further associated with increased water flux and intercellular CO₂ concentration, coupled with increased photosynthesis to cope with HS (Akram *et al.* 2021). Recently, Hussain *et al.* (2021) subjected 4-week-old okra (*Abelmoschus esculentus* L.) seedlings to HS extending from 28/22 to 45/35 °C (day/night), where foliar spray application of 2.5 mM proline resulted in an optimum increase in root and shoot biomass, leaves per plant, and water use efficiency under HS, accompanied by an increase in activity of antioxidant enzymes. Similarly, sugar beet (*Beta vulgaris* L.) under HS (50 and 55 °C) given foliar proline (25, 50, 100 mg·L⁻¹) had improved height, root morphology, and biomass through increases in photosynthetic pigments, soluble sugars, and amino acids (Khalil *et al.* 2020).

Chickpea plants under HS (40/35 °C) had reduced growth and inhibition of photosynthesis related to C metabolism, while at 45/30 °C they also suffered membrane damage and had lower osmotic potential. However, application of proline (10 µM) significantly increased the chlorophyll (Chl) content, and plant height, associated with a subsequent decrease in oxidative damage in terms of MDA and H₂O₂ content in heat-stressed plants. Further, enzymes linked with C fixation, *i.e.* Rubisco activity, were upregulated (Kaushal *et al.* 2011). In another case, the simultaneous application of proline with GB in barley (*Hordeum vulgare* L.) leaves subjected to HS (45 °C) for 10 min enhanced tolerance by improving the connectivity of PSII antennae, thereby improving the stability and efficiency of PSII under HS (Oukarroum *et al.* 2012). Similarly, mungbean exposed to 45/33 °C had significant damage to cell membranes, osmotic content, and cellular respiration, as well as decreased PSII and Chl function, CO₂ fixation and assimilation, but proline treatment significantly increased its thermo-tolerance (Priya *et al.* 2019).

Foliar application of L-proline (0–1600 mg·L⁻¹) to tomato (*Solanum lycopersicum* L.) resulted in increased total soluble sugars and water content, with concomitant decreased oxidative markers, such as MDA and H₂O₂, thereby increasing total fruit yield per plant under HS (Tonhati *et al.* 2020). Moreover, plants treated with exogenous proline (100 mg·L⁻¹) had higher carboxylation efficiency, photosynthetic pigments, and activity of SOD, CAT, GR, and APX, which increased thermo-tolerance in tomato, in contrast to untreated controls (Tonhati *et al.* 2020). Recently, 10-day-old rapeseed plants were treated with 2 mM proline in combination with GB and exposed to HS (38/28 °C). Proline treatment mitigated HS fluctuations in the membrane lipid bilayer and upregulated osmolytes involved in the glyoxylase system. Exogenous proline positively impacted cellular redox balance and antioxidant activity of glutathione reductase, glutathione S-transferase, and glutathione peroxidase enzymes, along with stabilization of photosynthetic apparatus activity by lowering MDA and H₂O₂ content (Hasanuzzaman *et al.* 2020b). Plant subjected to drought stress and HS have severely inhibited productivity, since photosynthesis is greatly affected by these combined stresses. Heat stress mainly targets activity of PSII and disrupts Chl structure and function.

In rice (*Oryza sativa* L.), exogenous proline ameliorates HS by increasing potassium (K⁺) uptake and Chl efficacy and upregulating osmotic regulators, such as total soluble protein and endogenous proline that act as ROS scavengers to avoid oxidative damage under HS (Hanif *et al.* 2021).

Like other growth regulators, proline also interacts with other molecules in regulating HS responses and tolerance mechanisms. Rossi *et al.* (2020) evaluated the beneficial role of proline (10 mM) in combination with gamma-aminobutyric acid (GABA) and ammonium nitrate under HS in creeping bentgrass. They found that exogenous proline relieved heat-triggered leaf senescence through increased Chl synthase and suppressed activity of Chlase, which is involved in Chl degradation. Plant defence against HS requires adjustments and association with amino acid metabolism (Rossi *et al.* 2020). In another experiment, Rossi *et al.* (2021) treated creeping bentgrass with 10 mM proline in combination with GABA and ammonium nitrate under HS. Plants treated with proline showed increased plant defence through elevation of glutamic acid involved in Chl biosynthesis. These results suggest that proline application enhanced glycine formation, a precursor of GB that confers HS tolerance and reduces ROS production. HS inhibited bud sprouting activity and caused membrane damage through accumulation of MDA, indicating oxidative injury, in sugarcane (Rasheed *et al.* 2010); however, pretreatment with 20 mM proline and GB protected bud sprouting. These findings revealed that exogenous proline increased accumulation of K⁺ and calcium (Ca²⁺), which helped the sugarcane buds to withstand HS during sprouting. Moreover, proline treatment markedly reversed HS damage and led to significant expansion and number of mesophyll cells and vascular bundles, thus increasing sugarcane thermo-tolerance (Rasheed *et al.* 2010). All these examples indicate that proline can trigger defence mechanisms and prevent damage associated with high temperatures.

GENETIC ENGINEERING OF PLANTS FOR PROLINE-MEDIATED TEMPERATURE STRESS TOLERANCE

Biotechnology has great potential to improve conventional crop protection, quality management, and other crop traits. Genetic engineering comprises isolating the gene of interest (GOI), ligating the gene into the chosen vector to form recombinant DNA, and subsequently transferring it into the plant genome to develop a new function (Parmar *et al.* 2017). Creating transgenic plants for temperature (CS and HS) tolerance is an efficient and cost-effective approach to maximize crop production under changing climates (Raza *et al.* 2021, 2022a). Proline has a significant role in plant abiotic stress tolerance. Studies have revealed that proline accumulation is as an expected outcome of stress; its concentration varies with stress duration and ontogenetic stage of the plant (Kaur *et al.* 2011; Liang *et al.* 2013; Ibragimova *et al.* 2022). Moreover, studies have confirmed that engineering proline metabolism-related genes encoding key enzymes enhance tolerance to temperature stress (Table 2, Fig. 3). Moreover, genes that are not directly related to proline metabolism but somehow affect proline metabolism can be engineered to develop 'temperature-smart' future crops.

For instance, the transformation of rice with the *CIPK1* gene from *B. rapa* encoding a Ca²⁺-regulated protein kinase increased

rice tolerance to CS by activating pyrroline-5-carboxylate synthase in the proline biosynthetic pathway (Abdula *et al.* 2016). The *Ubi-1::BrCIPK1* rice lines have higher biomass, water content, and proline and free sugar content than the wild type (WT). Overexpression of the rice *OsSIZ1* gene in *Arabidopsis* improves HS tolerance. Plants overexpressing *OsSIZ1* had higher transcript levels of *P5CS*, a gene involved in proline biosynthesis; therefore, proline acts as an osmoprotectant to alleviate damage caused by HS. These results demonstrate that the rice gene *OsSIZ1* has great potential to improved crop tolerance to several abiotic stresses (Mishra *et al.* 2018). Transgenic tomato plants expressing *AtDREB1A* were assessed for tolerance to CS by exposing them to 4 °C for 5 days. CS increased ROS production, while the transgenic plants had an active antioxidant system due to enhanced activity of SOD, CAT, and ascorbate peroxidase (APX). Accumulation of osmoprotectants, like proline and soluble sugars, assists transgenic plants in retaining an osmotic balance under CS. Stress-responsive genes *pyrroline-5-carboxylate synthase*, *lipid peroxidase*, *SOD*, and *CAT* were more highly expressed under CS in transgenic tomato plants (Karkute *et al.* 2019).

Proline-rich proteins (PRP) are cell wall and plasma membrane-anchored factors involved in cell wall maintenance and stress-induced fortification (Stein *et al.* 2011). Thus, overexpression of *OsPRP3* (rice proline-rich protein), which triggers proline accumulation, resulted in CS tolerance (Gothandam *et al.* 2010). Furthermore, functional analysis of *PtrPRP*, a *HyPRP*-encoding gene of *Poncirus trifoliata*, showed that the *PtrPRP* protein contains 176 amino acids, among which 21% are proline residues, and has an 8-cysteine motif (8 CM) domain at the C-terminal, which is a signal peptide, and a proline-rich region at the N-terminal (Peng *et al.* 2015). Transgenic *P. trifoliata* with knock-down *PtrPRP* using RNA interference (*RNAi*) was created to examine the role of *PtrPRP* in CS tolerance. Under CS, *PtrPRP-RNAi* plants were more sensitive relative to the WT, as shown by higher MDA content and electrolyte leakage. These outcomes suggest that *PtrPRP* is positively involved in CS tolerance through ROS homeostasis and maintaining membrane integrity (Peng *et al.* 2015).

Park *et al.* (2021) studied overexpression of *OsERF115/AP2EREBP110* to improve thermotolerance at the vegetative growth stage of rice plants and seeds. Moreover, *OsERF115/AP2EREBP110*-overexpressing plants had higher proline levels and enhanced expression of the proline biosynthesis *P5CS1* gene. Water use dynamics of individual plants showed that *OsERF115/AP2EREBP110*-OE plants had better water-saving characteristics under combined heat and drought stress. These findings suggest the possible use of *OsERF115/AP2EREBP110* as a candidate for genetic engineering to develop heat and drought stress-tolerant crops (Park *et al.* 2021). *P5CS* is involved in multiple stress responses, and the molecular cloning of *AmP5CS* in *Avicennia marina* has been reported (Liu & Wang 2020). To determine whether *AmP5CS* could be induced by HS (40 °C) in *A. marina*, the *AmP5CS* transcript level was assessed over 168 h. Expression of *AmP5CS* was significantly upregulated within the first hour, indicating it is involved in the HS response.

The introduction of the CRISPR/Cas system for genome editing has revolutionized plant science as it allows targeted mutagenesis and site-directed substitutions/insertions/deletions at specific sites in an organism's genome (Chen *et al.* 2019; Saeed

et al. 2020; Sukegawa *et al.* 2021). Genomics-driven CRISPR/Cas-based genome editing of proline-encoding genes is crucial for high quality, high yielding, ecofriendly, and climate-resilient agriculture (Yaqoob *et al.* 2023). Proline-rich proteins have multiple biochemical and physiological roles in stress response and plant growth. Nawaz *et al.* (2019) created rice mutant plants using CRISPR/Cas9 technology to examine the role of *OsPRP1* in CS. They reported that the *OsPRP1* knockout caused CS sensitivity in rice – *OsPRP1* mutant plants were more sensitive to CS, with low survival rate and lower root biomass than the WT. Mutant lines had less antioxidant enzyme activity and lower proline, Chl, ABA, and ascorbic acid content than the WT under CS. The results suggest that knockout of *OsPRP1* enhanced cold sensitivity in rice at the seedling stage by modulating antioxidants and maintaining crosstalk through signalling pathways (Nawaz *et al.* 2019). *OsProDH* encodes a putative proline dehydrogenase, and qRT-PCR analysis showed that *OsProDH* transcripts were relatively higher in root tissues and leaf blades. HS treatment repressed expression of *OsProDH* (Guo *et al.* 2020). These authors developed *OsProDHOE* lines and knockout mutant lines through CRISPR/Cas9, which has reduced endogenous proline content, while the mutation of *OsProDH* improved proline content. The mutant and overexpressing lines were, respectively, more tolerant and more sensitive to HS than the WT. The data suggest that mutation of *OsProDH* led to significantly higher proline accumulation, which reduced H₂O₂ accumulation and oxidative stress, ultimately improving survival, despite HS (Guo *et al.* 2020).

In recent years, molecular biotechnology and genetic engineering have significantly advanced our understanding of nested loops in proline biosynthesis and metabolism. Although there has been substantial progress in understanding these processes, some aspects are still unknown and demand further research. Genes involved in proline biosynthesis and metabolism are suitable targets for genetic engineering to improve plant abiotic stress tolerance, including temperature stress. Genome-editing systems like CRISPR/Cas9 are one of the recent revolutionary technologies for modern agricultural biotechnology. There is a need to further explore proline biosynthesis genes to develop 'temperature-smart' future crops.

PROLINE INTERACTION AND CROSSTALK WITH OTHER MOLECULES UNDER TEMPERATURE STRESS

Plants accumulate various osmolytes as protection from the harmful impacts of abiotic stresses. Crosstalk between hormones and osmoprotectants generates positive and negative feedbacks, which affects hormones/osmolyte synthesis, transport, and signalling (Liang *et al.* 2013; Iqbal *et al.* 2014; Ghosh *et al.* 2021; Hassan *et al.* 2022; Mir *et al.* 2022; Raza *et al.* 2022f). Proline accretion has been reported in tomato leaves and floral buds under temperature stress; however, proline content was lower in leaves than in floral buds (Shin *et al.* 2016). Proline plays an essential role as an osmolyte, and its metabolism is regulated by phytohormones (Meena *et al.* 2019). Phytohormones are endogenous growth regulators which regulate plant growth, germination, and many physiological processes under stress conditions (Khan *et al.* 2013; Raza *et al.* 2022c). Recent findings shows that proline interacts and has crosstalk with other molecules under temperature stress (Fig. 4).

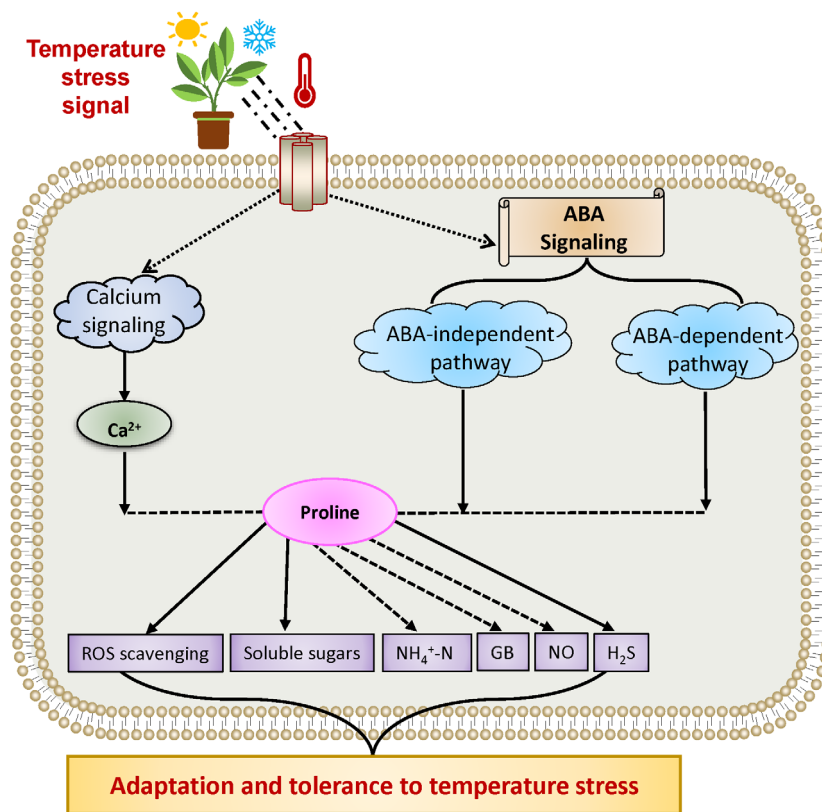


Fig. 4. An overview of interaction and crosstalk of proline with osmolytes (soluble sugars) and hormones under temperature stress. Proline accumulation has positive crosstalk with Ca^{2+} and ABA availability under temperature stress. Moreover, proline also increases accumulation of NO, GB, NH_4^+-N , and H_2S , resulting in increased tolerance to temperature stress. Abbreviations are explained in the main text.

Proline accumulation under stress occurs *via* ABA-independent or -dependent pathways (Verslues & Bray 2006). Accumulation of proline also depends on the availability of Ca^{2+} and chloride. An increase in Ca^{2+} availability increases accumulation of proline under stressful conditions (Meena *et al.* 2019). ABA has a regulatory impact on proline accretion and its subsequent mobilization in response to stress. Plant proline accumulation is positively linked with HS tolerance (Verslues & Bray 2006), and this has been shown by overexpression of the proline biosynthesis gene (*P5CS*) (Seki *et al.* 2007). However, some authors also found a negative correlation between proline and ABA under HS. For instance, An *et al.* (2014) found that ABA application decreased proline accumulation under HS, which suggests that ABA may not have a long-term positive impact in regulation of proline accumulation (An *et al.* 2014). Proline also has positive crosstalk with GB and soluble sugars under HS (Fig. 4). Application of proline substantially increased accumulation of GB, proline, and soluble sugars in sugarcane, indicating positive crosstalk between proline, GB, and soluble sugars under temperature stress (Rasheed *et al.* 2011).

Molinari *et al.* (2007) also reported a significant increase in proline accumulation in transgenic sugarcane under HS, which protected Chl content, PS-II and increased MDA, responsible for improved antioxidant activity. Nonetheless, these authors also noted that osmotic potential in transgenic sugarcane decreased with higher proline accretion, which

does not support its role in osmotic adjustment (Molinari *et al.* 2007). There is also a relationship between N and proline accumulation under stress conditions (Iqbal *et al.* 2015). Thus, Rivero *et al.* (2004) suggested a positive association between proline accumulation and NH_4^+-N under HS. These authors suggested that HS tolerance is related to higher accumulation of proline with NH_4^+-N than with NO_3^- . Moreover, proline is also positively associated with glutathione under stress conditions. An increase in proline accumulation in soybean under heat and drought stresses substantially increased antioxidant activity and glutathione content (Kocsy *et al.* 2005).

Abscisic acid (ABA) induces HS tolerance in chickpea by increasing accumulation of proline, GB, and trehalose, indicating a synergistic association between these important osmolytes in plants under different abiotic stresses (Kumar *et al.* 2012; Iqbal *et al.* 2019). Exogenously applied salicylic acid increased accumulation of proline in wheat under both CS and HS. This increase in proline content is linked with regulation of enzymes responsible for proline synthesis and its catabolism by salicylic acid (Ignatenko *et al.* 2019). Crosstalk of proline with essential hormones (IAA, GA, CK, and ethylene) has not yet been explored. Therefore, future research must determine the relationship of proline with these hormones at organ, tissue, and cellular levels. Such studies will increase knowledge of proline as an osmolyte and its crosstalk with different hormones in conferring stress tolerance.

In addition to phytohormones and osmoprotectants, proline also interacts with gaseous molecules under stress conditions (Fig. 4). Nitric oxide (NO) works as a signalling molecule and promoter of proline synthesis (Neill *et al.* 2002; He & He 2020; Iqbal *et al.* 2021); however, little is known about regulation of NO by proline and the interaction of these two molecules under HS (Alamri *et al.* 2019). Nitrate reductase (NR)-dependent NO production triggered expression of *P5CS1*, which substantially improved CS tolerance in *Arabidopsis* (Zhao *et al.* 2009). Also, proline accumulation leads to an increase in NO content in banana under cold storage (Wang *et al.* 2013). Exogenous NO application in combination with ABA triggers proline accumulation in wheat under HS, which is accompanied by an increase in antioxidant enzymes, providing higher heat tolerance (Iqbal *et al.* 2022). Similarly, exogenous NO enhances proline accumulation, providing higher cold tolerance in tea (*Camellia sinensis*) roots (Wang *et al.* 2020).

Recently, hydrogen sulfide (H₂S) has also been documented as a gastrotransmitter with various comparable functions to those of NO (Corpas *et al.* 2019; Raza *et al.* 2022f). Application of H₂S alleviated cold injury in banana plants by increasing proline accumulation through an increase in P5CS activity and reduction in ProDH activity (Luo *et al.* 2015). Under extreme temperature conditions (CS and HS), H₂S similarly increases proline accumulation by increasing P5CS activity and decreasing the activity of ProDH (Li *et al.* 2013; Luo *et al.* 2015). H₂S also modulates proline accumulation in cucumber, which, in turn, increases CS tolerance (Nasibi *et al.* 2020). These authors suggested that modulation of proline by H₂S might involve the NO pathway; however, this pathway needs further investigation (Nasibi *et al.* 2020; Raza *et al.* 2022f).

CONCLUSION AND FUTURE OUTLOOK

Climate change, temperature variations, and crop productivity are directly linked. The deleterious effects of temperature variations (CS and HS) hinder normal physiological, biochemical, and molecular mechanisms in plants. One such stress response is the stimulation of synthesis of ROS, which cause considerable damage to cellular membranes, proteins, lipids, and DNA. Plants have developed strategies to alleviate the adverse effects of environmental stresses. For instance, plants accumulate osmolytes, including amino acids (proline), to mitigate the adverse impacts of HS and CS. Among these molecules, proline protects plants from the toxic effects of extreme temperatures by promoting antioxidant systems to avoid uncontrolled ROS overproduction, thus allowing osmotic adjustment and maintaining membrane stability. Moreover, proline also increases Rubisco activity, PS-II efficiency, total soluble proteins, soluble sugars, and activity of antioxidant enzymes. It also collaborates with phytohormones and other signalling molecules, thereby improving plant performance under temperature stress (CS and HS). Proline also stabilizes subcellular structures, buffering cellular redox potential. Additionally, it behaves as a protein-compatible hydrotrope that maintains appropriate NADPH/NADP⁺ ratios, alleviates cytoplasmic acidosis, and ensures better photosynthetic activity and subsequent plant performance.

Proline-mediated genetic engineering could be a promising approach to resolving the molecular basis of stress tolerance by regulating proline biosynthesis. The state-of-the-art CRISPR/

Cas-based gene-editing helps in manipulating these genes, opening new doors for plant breeders to develop 'temperature-smart' cultivars with higher productivity. Previous studies confirmed that engineering proline metabolism-related genes enhances tolerance to CS and HS, which could help to feed growing population with 'temperature-smart' future crop plants.

Nevertheless, there are many unanswered questions. The roles of proline with different hormones and osmolytes is poorly studied. The role of proline in seed germination under extreme temperature conditions is also poorly studied; therefore, it would be fascinating to explore this in germination mechanisms under temperature stress (CS and HS). Similarly, the accumulation of proline in different plant organs under extreme temperature conditions must be investigated in future studies. The role of proline in nutrient uptake is also poorly studied; thus, more studies are needed to explore this effect on nutrient signalling, nutrient transport, and nutrient channels under CS and HS. The role of proline in photosynthesis is well explored; however, its role in stomatal signalling is still unknown and it is essential to explore this role under CS and HS. The role of proline osmolyte accumulation and its crosstalk with hormones is poorly studied. The complex relationship of proline with essential hormones (IAA, GA, CK, ethylene) and osmolytes (GB and soluble sugars) must be determined at organ, tissue, and cellular levels. Moreover, it would be fascinating to determine the role of proline on the different enzymes and genes linked with the synthesis of the aforementioned hormones and osmolytes. Such studies will increase knowledge of proline as an osmolyte and its crosstalk with different hormones and osmolytes in conferring stress tolerance. Moreover, advanced 'omics' techniques allow exploration of the proline-mediated CS and HS at plant metabolome, proteome and transcriptome levels. Last, the engineering of proline-mediated signalling and metabolic pathways will open a new window into current knowledge to explore proline-mediated CS and HS tolerance mechanisms in plants. In short, the current literature suggests that the adverse effects of HS and CS can be mitigated with exogenous proline application. Nevertheless, genetic engineering of proline biosynthesis genes should be further explored to regulated proline level/content in transgenic plants that can withstand extreme temperatures.

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AUTHOR CONTRIBUTIONS

AR conceived the idea. AR, CS, SA, MUH, FS, and SH wrote the manuscript. AR, SC, SA, and MUH designed the tables and figs. AR, RS, AA, FJC, WJ, and RKV reviewed and edited the manuscript. All authors have read and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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