REVIEW

The role of proline in the adaptation of eukaryotic microalgae to environmental stress: An underestimated tool for the optimization of algal growth

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Abstract

Microalgae are considered the most promising source of renewable fuels, high-value bio-products and nutraceuticals. Potentially, microalgae can satisfy many global demands, but in large-scale cultivation the average productivity of most industrial strains is lower than maximal theoretical estimations, mainly due to sub-optimal growth conditions. Although microalgae have developed complex strategies to cope with environmental stresses, cultivation in outdoor photobioreactors is limited to few species and it is not yet sufficiently remunerative. Indeed, most microalgal species are very sensitive to environmental conditions, and changes in solar irradiation, temperature, and medium composition can drastically decrease biomass yield. Developing new strategies for improving algal tolerance to stress conditions is thus greatly desirable. One of the first responses that occur in both higher plants and microorganisms following the exposure to abiotic stress conditions, is an increased synthesis and accumulation of the amino acid proline. While the role of proline accumulation in stress adaptation is well-recognized in higher plants, in microalgae the implication of proline in stress tolerance still awaits full elucidation. In this review we summarize available data on proline metabolism under environmental stress in eukaryotic microalgae. Possible implications toward optimization of algal growth for biotechnological purposes are also discussed.

Keywords Proline · Microalgae · Environmental stress · Biomass productivity · Heavy metals

Introduction

Microalgae are (mainly) unicellular photosynthetic organisms living mainly in aquatic habitats, and show high adaptability to a wide range of temperatures, salinities, pH values and different light intensities, allowing the colonization of oceans, lakes, rivers, ponds, and waste waters (Tirichine and Bowler 2011). Their high biodiversity makes them a rich source of interesting and useful metabolites. A large number of species are naturally lipid accumulator under stress, and they can be exploited for next generation biofuels production. Green microalgae include genera which are among the most widely used for industrial applications, such as

Haematococcus, *Nannochloropsis*, *Chlorella* and *Dunaliella* spp. Some species produce a wide range of bioproducts, including polysaccharides, pigments, vitamins, antioxidants and bioactive compounds. Moreover, microalgae can be employed in wastewater treatment and atmospheric CO_2 mitigation (Benedetti et al. 2018).

The interest in exploiting microalgae is increasing for several reasons. In contrast to higher plants, algae do not require arable lands and need far less freshwater for their growth. Moreover, the possibility to cultivate algae in indoor photobioreactors reduces a lot the effect of the seasonal cycle on the biomass yield (Chu and Majumdar 2012). Despite these potentialities, the biomass productivity in large-scale plants, both indoor and outdoor, is still too low to make the whole process profitable. Thus, an optimization of the cultivation methodologies and genetic engineering/selection for microalgae growth enhancement are required.

Microalgal growth enhancement could also be obtained through increased resistance to environmental stress conditions. Several microalgal species are naturally resistant to harsh environmental conditions, such as excess salt

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(*Dunaliella salina* (Bonnefond et al. 2017; Xu et al. 2018; Ahmed et al. 2017)), cold and drought (*Klebsormidium* sp. (Rippin et al. 2019)) and high irradiation (*Chlorella ohadii* (Levin et al. 2021)). The cultivation of most of these species is still limited to lab scale, yet the possibility to transfer some of their abilities to microalgal species of commercial interest could improve the productivity of the latter in large scale cultivation. However, a proper knowledge of the metabolic bases for increased stress tolerance is required for this aim.

Several factors are involved in the algal response to abiotic stress that could be exploited to increase algal biomass production under stress conditions, such as amino acid (Arora et al. 2022), osmolyte (Kaur et al. 2022) and antioxidant metabolism (Nowicka 2022). In higher plants, the synthesis and accumulation of the amino acid proline is believed to play a multifaceted role in stress tolerance, being involved in the cellular response to excess salt, dehydration, low temperature and oxidative stress (Forlani et al. 2019b). Much less is known about a possible protective role of proline in microalgae, which have to cope with somehow different environmental stress conditions. In this review, we describe the state-of-the-art about the involvement of proline metabolism in stress responses of eukaryotic microalgae.

Proline synthesis in higher plants and eukaryotic microalgae

Intracellular concentration of free proline is mainly determined by four metabolic processes: biosynthesis, degradation, use of proline for protein biosynthesis and release of proline during protein degradation (Hildebrandt 2018). In plants, free proline is detectable in the cytoplasm, where its biosynthesis proceeds by the sequential action of two enzymes: a δ^1-pyrroline-5-carboxylate (P5C) synthetase (P5CS) that reduces glutamic acid to γ-glutamic semialdehyde (GSA), which spontaneously cyclizes to P5C with loss of one water molecule; and a P5C reductase (P5CR) that reduces P5C to proline (Trovato et al. 2019). The former enzyme strictly requires NADPH as the electron donor (Sabbioni et al. 2021) whereas the latter may use either NADPH or NADH with various efficiency and affinities (Forlani et al. 2015) (Fig. 1A). In *Arabidopsis thaliana* and other angiosperms, two isoforms of P5CS are present, respectively encoded by *P5CS1* and *P5CS2* gene (Székely et al. 2008). P5CS1 has been identified as the major contributor to stress-induced proline accumulation, while P5CS2 has been shown important for embryo development and growth (Funck et al. 2020). Several papers reported increased expression of *P5CS* genes in plants exposed to environmental stress conditions such as drought, the presence of high salt or heavy metals concentrations, resulting in increased free proline levels (Wang et al. 2015). Besides

this main pathway, in some species proline biosynthesis from ornithine has also been described (da Rocha et al. 2012). Within the mitochondrion, GSA/P5C can be produced starting from ornithine, which is directly produced by arginase or imported from the chloroplast and used as substrate by an ornithine-δ-aminotransferase (OAT), which transfers an amino group to α-ketoglutarate yielding glutamate and GSA (Winter et al. 2015). OAT is not essential for proline production (Funck et al. 2008), but its overexpression seems to be correlated to the response to some environmental stress, such as excess salt (Roosens et al. 1998).

From the cytosol, proline also can be translocated to the mitochondrion (Di Martino et al. 2006), where its catabolism takes place by means of two enzymes. A proline dehydrogenase (ProDH) using FAD as the electron acceptor converts proline to P5C, which is reduced back to glutamic acid by a NAD-dependent P5C dehydrogenase (P5CDH) (Trovato et al. 2019) (Fig. 1A).

In microalgae, the information about proline metabolism is still largely incomplete, and only a few studies investigated in detail the metabolic routes involved. As in plants, also in the model organism *Chlamydomonas reinhardtii* proline biosynthesis takes place in the cytosol, but two separate enzymes are responsible for the initial conversion of glutamate to P5C (Merchant et al. 2007). Two isoforms of γ-glutamyl kinase (G5K and PROB, also known as GGK1 and GGK2) phosphorylate glutamic acid to γ-glutamylphosphate (G5P), which is subsequently reduced to P5C by a GSA dehydrogenase (GSD1) (Miyoshi et al. 2011; Zalutskaya et al. 2020) (Fig. 1B). The biosynthetic route is therefore more similar to that found in prokaryotes than in higher plants (Vallon and Spalding 2009). Recent studies carried out with *Auxenochlorella protothecoides* have identified two genes encoding P5CS (*P5CS1* and *P5CS2)*, two genes encoding P5CR (*P5CR1* and *P5CR2*) and one gene encoding PDH involved in proline degradation (Xing et al. 2022).

In *C. reinhardtii* proline biosynthesis can also start from ornithine in the mitochondrion through the enzyme OTA1, which converts ornithine into GSA. P5C produced within the mitochondrial matrix is oxidized by ALD1 into glutamate, which is translocated to the cytosol and possibly re-utilized for proline synthesis (Fig. 1B).

As in most eukaryotes, proline catabolism proceeds in the mitochondrion by means of two separate enzymes, POX1 and ALD1 (Fig. 1B). Recent studies demonstrated the involvement of nitric oxide (NO) in adaption of *C. reinhardtii* to various environmental stresses. NO treatment led to a significant accumulation of proline. In contrast, expression levels of the gene encoding OAT (OTA1) decreased after treatment with NO, suggesting the predominance of the glutamate pathway over the ornithine pathway under stress (Zalutskaya et al. 2020).
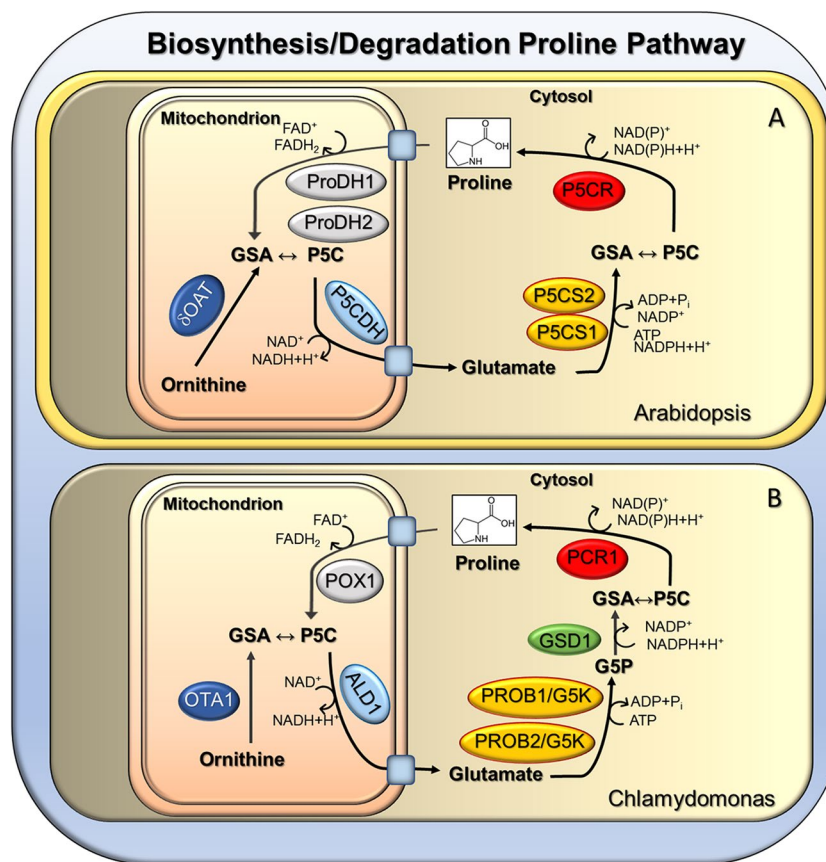
Fig. 1 Pathways for proline biosynthesis and degradation in *Arabidopsis thaliana* (A) and *Chlamydomonas reinhardtii* (B). (**A**) Proline biosynthesis proceeds in plants by the sequential action of two enzymes: an ATP- and NADPH-dependent P5CS that reduces glutamic acid to GSA, which spontaneously cyclizes to P5C with loss of one water molecule; a P5CR that reduces P5C to proline. P5CR may use either NADPH or NADH as cofactor with various efficiency and affinities. Proline degradation takes place in mitochondria, where

a FAD-dependent ProDH converts proline to P5C, and a P5CDH oxidizes P5C back to glutamic acid. In mitochondria GSA is also produced starting from ornithine by the action of OAT. (**B**) Within the cytosol, P5C biosynthesis in *C. reinhardtii* is accomplished by two separate enzymes: G5K/PROB, which phosphorylates glutamic acid into G5P, and GSD1, which reduces G5P to P5C. In the mitochondrion, POX1 oxidizes proline to P5C, which is in turn oxidized to glutamate by ALD1

Proline accumulation as an early response to various stress conditions

Microalgae and higher plants are constantly exposed to abiotic stress conditions, leading to frequent adjustment and remodelling of the cell defence machinery that involve metabolic reconfiguration. When exposed to harsh environment causing hyperosmotic stress, the cell often accumulates an array of metabolites, known as compatible osmolytes, such as sugars, polyols, quaternary ammonium compounds and amino acids, among which proline is the most common. An increased level of free proline represents also one of the earliest responses to a wider range of stressful conditions that do not require osmotic adjustment (Mattioli et al. 2009). Higher concentrations of free proline were found for instance after the exposure to heating and cooling treatments of *A. thaliana* plants (Hildebrandt 2018). Even if the pivotal role of proline accumulation in

stress adaptation is well-recognized, the molecular bases of its beneficial effects are still unclear, and several mechanisms have been hypothesized to explain them (Forlani et al. 2019b).

Proline may protect proteins from high ion concentrations by a direct interaction with water molecules present on their surface. High salt concentration affects the stability of protein hydration shell, destabilizing protein conformation. Proline acts as a kosmotropic molecule by lowering the entropy under hyperosmolarity and maintaining the water layer surrounding proteins intact. The pyrrolidine ring allows the interaction with hydrophobic surface residues of proteins, thereby increasing their hydrophilic area (Arakawa and Timasheff 1983, 1985) and counteracting the negative chaotropic effect of high salt concentration. On the other hand, due to its amphipathic nature, proline stabilizes membranes by intercalation between phospholipid head groups (Rudolph et al. 1986).

Interestingly, several studies reported that proline is involved also in stress defence mechanisms induced by presence of high concentration of heavy metals. It has been formerly proposed that proline is capable of reactive oxygen species (ROS) detoxification. However, it has been later demonstrated that its chemical properties do not allow scavenging of singlet oxygen, superoxide, NO and nitrogen dioxide (Signorelli et al. 2016). According to recent studies, it seems that proline can act exclusively as a hydroxyl radical scavenger (Signorelli et al. 2015). Notwithstanding this, the exogenous supply of proline to *A. thaliana* roots resulted in reduced ROS levels (Cuin and Shabala 2007). Moreover, the activity of antioxidative enzymes, such as peroxidase (POD) and superoxide dismutase (SOD), was enhanced in presence of high concentration of exogenous proline in tobacco cell suspensions exposed to high salts concentrations (Hoque et al. 2007). It seems therefore that proline may contribute indirectly to ROS scavenging, but this topic is still subject of debate. Notably, proline synthesis and degradation may play also a key role in redox balancing between cytosol and mitochondria, since these processes cause a significant fluctuations of NAD(P)$^+$/NAD(P)H pools (Giberti et al. 2014).

Microalgae occupy mostly aquatic habitats, and their life cycle is strictly relater to water. Aquatic ecosystems are constantly subjected to changing in temperature, due to sea currents or the presence of geothermal sources. Also light, which reaches the inner layers of rivers and seas, is strongly influenced in quality and quantity by rapid and constant movements of water masses. In open pond large-scale cultivation, in which water masses are constantly moved, the continuous light-shade alternation generates light stress and affects algal growth. In addition, also fluctuations in salt concentration and the presence of pollutants and heavy metals can act as stressors (Fig. 2). As a consequence, microalgae have developed complex defense systems against rapid changing of these environmental factors.

Proline involvement in stress defence towards harsh environmental conditions in microalgae

Salt stress

During recent years, the ongoing climate change and human interventions caused salinization of a significant part of freshwater resources on a global level. Large-scale microalgal cultivation needs massive amount of freshwater, therefore the availability of halotolerant strains is very attractive. High salinity is a challenging environmental stress for organisms to overcome, and most of green microalgae are extremely vulnerable to excess salt, not only due to ionic imbalance, but also to the generated ROS interfering with the photosynthetic machinery (Fal et al. 2022).

Fig. 2 Schematic representation of major abiotic stresses that affect plant and microalgal growth. The thickness of the arrows represents the relative importance of each stressor in the two cases
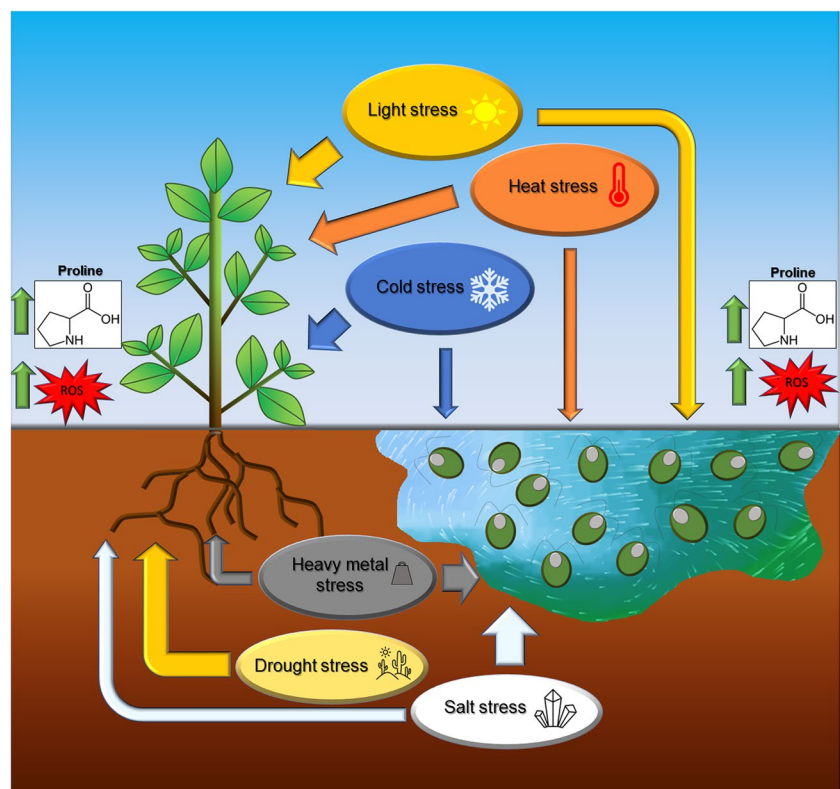
To face hyperosmotic stress, microalgae developed a wide range of physiological, metabolic and molecular responses (Wang et al. 2018), including the accumulation of carbohydrates and lipids as storage molecules to maintain microalgae survival (Anand et al. 2019). Microalgae exposed to high salinity accumulate ROS-detoxifying enzymes such as SOD, ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), and POD, as well as osmoprotectant molecules, such as glycine betaine, glycerol and proline (Pancha et al. 2015) (Ismaiel et al. 2018). Such osmolytes prevent water loss and contribute to maintain the osmotic balance (Anand et al. 2019). Contrary to higher plants, in microalgae mannitol and sorbitol are also used in salinity adaptation, especially in brown algae (Wegmann 1986). In addition to organic solutes, inorganic ions also play an important role in osmoregulation (Hellebust 1985).

Several halotolerant microalgal species have been isolated that can grow in saline environments. Among them, either freshwater or marine water species of the same genus, such as *C. reinhardtii* and *Ch. vulgaris* (freshwater species) and *Chlamydomonas pulsatilla* and *Chlorella salina* (marine species). Salt-resistant green microalgae are characterized by the capability to over-produce compatible solutes, and often by the presence of a cellulosic cell wall.

In *Chlamydomonas* spp., high salinity adaptation involves changes in cell morphology and aggregation state, termed as *palmelloid*. In palmelloid of *C. reinhardtii*, 4–16 cells are clustered, lose flagella and increase secretion of exopolysaccharides (Shetty et al. 2019). These strategies are necessary to face both temporary and prolonged osmotic shock. When *C. reinhardtii* cells were exposed to salt stress and subsequently treated with exogenous proline, the treatment ameliorated the negative effects of high salinity (Reynoso and de Gamboa 1982). *Chlamydomonas pulsatilla* can withstand hypersaline environments by increasing glycerol levels, and can survive at lethal salt concentrations by forming resting spores (Hellebust 1985). In *C. reinharditii* the endogenous content of free proline increased during 24 h of treatment at NaCl concentration above 100 mM (Mastrobuoni et al. 2012). Further evidences have shown increased expressions of proline synthesis metabolic pathway genes under osmotic stress in *Chlamydomonas* sp. ICE-L, which is periodically exposed to extreme salinity concentrations inside the brine channels in the Antarctic Sea ice (Zhang et al. 2020). Stressed cells of *C. reinhardtii* accumulated carotenoids and proline as osmoprotectant (Fal et al. 2022).

Chlorella and *Dunaliella* spp. do not usually form palmelloids after the exposure to high salinity stress, and show different defence mechanisms. *Chlorella* spp. are characterized by a rigid cellulosic cell wall that limits their ability to change cell volume. Therefore, osmoregulation is maintained through the production of organic solutes and accumulation of inorganic ions. On the contrary, *Dunaliella* lacks a rigid cell wall, allowing rapidly changes in cells volume during high salinity stress by adjusting ion and glycerol concentration within the cell (Chen et al. 2009). Although *Chlorella* and *Dunaliella* spp. show great differences in cell morphology, a number of studies found a similar increase of free proline content immediately after high salinity treatments. *Chlorella vulgaris* is considered a freshwater species, but it can tolerate up to 0.8 M NaCl, while *Ch. salina*, a marine species, can survive up to 2 M NaCl. *Chlorella autotrophica*, another marine species, is able to grow in environments characterized by fourfold seawater salt concentration, about 2.4 M (Ahmad and Hellebust 1984). It has been found that in *Ch. vulgaris* and *Ch. autotrophica* free proline content increases under salinity stress, whereas in *Ch. salina* it remains constant (Farghl et al. 2015). When *Ch. vulgaris* and *Chlorococcum humicola* (a halotolerant microalga) were compared, proline content in cells treated with different NaCl concentrations was found in both cases to increase with increased salinity. Maximal level (threefold that in untreated controls) was found at 500 mM NaCl for *Ch. vulgaris*, but only at above 1000 mM NaCl for *Chl. humicola* (Singh et al. 2019). In another study on *Ch. vulgaris*, cells were treated for 30 days with NaCl concentrations ranging between 0.1 and 0.4 M. In this case also, proline increased with the treatment. Remarkably, the content of glycine betaine was also found to increase, showing that the adaptive response of *Ch. vulgaris* can involve other compatible solutes (Hiremath and Mathad 2010). More recently, lipid accumulation was induced in *Ch. vulgaris* strain YH703 by means of NaCl treatments, which caused high H_2O_2 production and increased APX activity and proline contents. Since *Ch. vulgaris* YH703 strain can mitigate ROS generated under salinity stress, a possible involvement of proline in ROS scavenging has been hypothesized (Yun et al. 2019).

Several studies have revealed the implication of proline metabolism in high salinity stress response also in *Scenedesmus*, a genus that has been actively studied for bio-diesel production, and has one of the highest biomass productivity among green algae, mainly under heterotrophic growth (da Silva et al. 2009; Mandal and Mallick 2009; Yoo et al. 2010; El-Sheekh et al. 2013). Some *Scenedesmus* strains contain also a considerable amount of carbohydrates (about half of their dry weight), which makes them attractive candidates for bio-ethanol production (John et al. 2011). In *Scenedesmus* sp. CCNM 1077, a dose-dependent increase in proline content (up to 1.9-fold) was found under salinity stress in the midpoint of the treatment, but it decreased thereafter (Pancha et al. 2015). *Scenedesmus* sp. IITRIND2 exposed to high salt concentration exhibited an increase of proline and glycine betaine content that was paralleled by an increase of the activity levels of some antioxidant enzymes, in particular CAT and APX (Arora et al. 2017, 2019).

Proline is the major osmolyte detected in halotolerant *Picochlorum* sp. (Henley et al. 2004). It has been reported that *Picochlorum soloecismus* could be a new platform for the production of renewable fuels (Gonzalez-Esquer et al. 2019). In addition, *Picochlorum* strain SENEW3 accumulated proline under high salt stress conditions through upregulation of one gene involved in proline synthesis (Foflonker et al. 2016). An increase of free proline content was also detected in *Acutodesmus dimorphus*, a halotolerant microalga able to accumulate a large amount of lipids under heat stress (Chokshi et al. 2015, 2017), and in *Neochloris oleoabundans*, an oleaginous microalgal species that can be cultivated both in freshwater and salt water (Jaeger et al. 2018). Another microalgal species, *Micractinium* sp., following exposure to high NaCl concentrations showed increased free proline level, together with higher lipid accumulation (Yang and Hu 2020).

Dessication is often related to hypersaline environments, and it is doubtless one of major abiotic stressors that affect microalgal growth. Recently two microalgal clades, *Pseudostichoccus* and *Deuterosticoccus*, were found able to survive in total absence of water in both saline and non-saline conditions. *Pseudostichococcus*, which was able to recover fully after desiccation with or without salinity stress, showed a higher proline:sorbitol ratio compared to untreated controls. These results suggest the possible implication of proline in desiccation resistance, and open the way for further investigations on the possible applications of this genus in salinity bioremediation (Van and Glaser 2022).

Although much evidence of increased free proline content under osmotic stress has been obtained in a wide range of microalgal species, it is still unclear whether this amino acid acts mainly as osmoprotectant, or it is involved in the activation of ROS detoxification response. Hence, proline could act also as signal molecule involved in activation of multiple responses under osmotic stress. Unfortunately, lack of complete overview of how many and which genes of proline metabolism are upregulated under saline stress, and their interconnection with ROS detoxification pathways, hampered to date full understanding the role of proline in salt resistance.

Heavy metal stress

The presence in soil and water of an excess of heavy metals caused by anthropogenic pollution represents one of the harshest conditions to cope with. Heavy metals comprise both essential micronutrients (Cu, Zn, Co, Fe, Mn, Mo) and cations that do not play any function in living organisms (Ag, As, Cd, Cr, Hg, Ni, Pb, Sb, U, V, W). Accumulation of heavy metals in the cell induces oxidative damages by generating ROS, which lead to enzyme inactivation, protein degradation, pigment bleaching and lipid peroxidation (Nowicka 2022) One of the first defence mechanisms adopted by microalgae is a fast antioxidant response.

One of the first studies to correlate increased proline content with an excess of heavy metals ions in microalgae was published in the 90's, when high levels of free proline were detected in *Ch. vulgaris* cells exposed to high concentrations of Cu ions. If proline was exogenously supplied before the treatment, Cu uptake was drastically reduced (Wu et al. 1998). Further studies revealed that free proline content increases drastically within a few hours after the exposure of *Ch. vulgaris* cells to increasing concentrations of Cu, Cr, Ni and Zn cations. The authors suggested that rapid accumulation of free proline may confer resistance by inhibiting heavy metal-induced lipid peroxidation (Mehta and Gaur 1999).

Excess Cu affected also the growth of *C. reinhardtii* causing lipid peroxidation and generation of NO and induced a consistent accumulation of free proline. Higher expression levels of enzymes involved in proline synthesis were detected in presence of NO analogues, suggesting a regulatory function of NO in proline metabolism under heavy metal stress (Zhang et al. 2008). Exposure of *C. reinhardtii* cells to high concentrations of Hg ions induced accumulation of free proline and significantly increased the expression of ROS scavenging enzymes (Elbaz et al. 2010) (Wei et al. 2011). The importance of proline in heavy metal detoxification was strengthened by the results obtained overexpressing mothbean *P5CS* in *C. reinhardtii*. Recombinant cells showed 80% more free proline than the wild type, and were able to grow better in Cd-supplemented medium, most likely because of higher ROS scavenging, and higher GSH levels that facilitate phytochelatin synthesis and Cd sequestration (Siripornadulsil et al. 2002).

Free proline content alterations in response to heavy metal treatment were assessed in many other microalgal species. Studies conducted on *Scenedesmus obliquus* revealed significant increasing in both proline and polyphenol contents after Pb^{2+} exposure (Danouche et al. 2020). Treatment of *Scenedesmus* sp. with Cu or Zn ion concentrations below 10 μM induced hyperaccumulation of heavy metals and increased proline biosynthesis. Notably, concentrations above 10 μM of Cu ions had, on the contrary, an inhibitory effect on proline synthesis. Cu and Zn ions induced strong oxidative stress in *Scenedesmus* sp. by increasing membrane permeability and lipid peroxidation. A pre-treatment with 1 mM proline for 30 min increased GR activity levels and totally prevented lipid peroxidation, suggesting that it acts by scavenging ROS rather than by chelating metal ions (Tripathi and Gaur 2004). Further evidences of proline involvement in Cu ions detoxification in *Scenedesmus* sp. were reported in (Tripathi and Gaur 2004) and (Kováčik et al. 2010). The exposure of *Scenedesmus quadricauda* cells to high concentration of Cd^{2+} ions inhibited growth and pigmentation, and induced accumulation of proline and malondialdehyde, reflecting high level of lipid peroxidation (Çelekli et al. 2013). The uptake of other heavy metals such

as As enhanced proline accumulation in *Scenedesmus* sp. IITRIND2A (Arora et al. 2018).

Several microalgal species showed a high tolerance against a variety of heavy metals concomitant with the accumulation of proline, as in *Euglena gracilis* (Cervantes-García et al. 2011) and in *Desmodesmus* sp. (Buayam et al. 2019) exposed to excess Cu^{2+} (Liu et al. 2020). Increased proline content goes hand in hand with higher GSH level, yet it is not clear how proline acts in redox balancing. In the model proposed by Siripornadulsil and co-workers, Cd-induced hydroxyl radicals react directly with free proline (Siripornadulsil et al. 2002). This hypothesis is consistent with results obtained in *Brassica juncea* showing that proline was able to quench free radicals in thylakoids isolated from plants exposed to high light intensities (Alia and Mohanty 1997). Proline could react with hydroxyl radical with the formation of hydroxyproline, but no evidence supporting this scavenging mechanism has been reported (Smirnoff and Cumbes 1989; Matysik et al. 2002). Further studies demonstrated in plants that proline reacts with hydroxyl radicals to form P5C, which is converted back to proline by P5CR in the so called Pro-Pro cycle (Signorelli et al. 2014).

Stress generated by the presence of pollutants

Besides heavy metals, wastewater contains a wide range of compounds such as herbicides, insecticides, cleaning detergents, plastic processing wastes etc., which can be toxic for aquatic organisms. To cope with water contamination, during the last decades many works have exploited the capability of microalgae of sequestering and detoxifying chemical pollutants, the so-called *microalgae wastewater bioremediation*. In several cases, proline metabolism has been found involved in providing tolerance to such toxic chemical compounds. Studies carried out on a freshwater *Chlamydomonas mexicana* strain exposed to high doses of the insecticides acephate and imidacloprid showed increased SOD and CAT activity levels and high accumulation of proline (Kumar et al. 2016). Significant build up of proline and glycine betaine was found in *D. salina* following the treatment with polyethylene glycol, whose presence in wastewater limits the use of large water resources. Activity levels of some antioxidant enzymes, namely CAT and APX, were simultaneously enhanced (Tafvizi et al. 2020). Perfluorooctanoic acid (PFOA) has been found in various ecosystems and is receiving growing attention due to its biomagnification properties that increase its toxicity in aquatic environments. PFOA inhibited the growth of *C. reinhardtii* but only partially affected growth of *S. obliquus*. In both cases, the exposure to this pollutant enhanced proline content (Hu et al. 2014). All these studies clearly showed a correlation between the treatment with chemical pollutants and proline accumulation, yet the results do not allow to understand whether the increase of proline content is simply due to the need of scavenging ROS generated within the cell by toxic chemicals, or proline metabolism may play a more specific role in their detoxification.

Stress induced by temperature and light fluctuations

The major constraints in outdoor microalgae mass cultivation is the susceptibility of many strains of commercial interest to climate variations, as temperature and light intensity fluctuations drastically affect microalgal productivity. Under open ponds conditions, microalgae are subjected to night-day and season cycles in which temperature and sunlight irradiation may vary sharply. Heat stress affects pigments metabolism, damages mitochondrial function, and causes lipid peroxidation. Cold stress causes membrane rigidification, affects the stability of proteins or protein complexes and induces intracellular H_2O_2 accumulation (Choudhury et al. 2017; Ding et al. 2019; Chokshi et al. 2020). The intracellular responses induced by temperature fluctuations reflect an imbalance in ROS homeostasis (Muhlemann et al. 2018; Wang et al. 2019; Chokshi et al. 2020). As a consequence, microalgal yield is strongly reduced. In *Ch. vulgaris* a variation of 5 and 10 ^oC above the temperature optimum decreased productivity by 50% and 100%, respectively (Converti et al. 2009). In *S. obliquus* grown under a range of temperatures lower than the optimum, an up to 80% yield reduction was found (Chalifour and Juneau 2011).

Studies on *A. protothecoides* pointed out different effects of heat and cold stress. High temperature treatments caused a drastic increase of ROS levels. Consequently, the expression of key enzymes involved in ROS detoxification was enhanced, but no significative changing in proline levels was found. On the contrary, under cold stress free proline content raised up to 3.8 fold respect to untreated controls (Xing et al. 2022). When the expression levels of the two isoforms of *P5CS* and *P5CR* genes and *PDH* gene were determined under cold stress, *P5CS* and *P5CR* were found up-regulated, while *PDH* was down-regulated, showing that proline accumulation relies upon both increased biosynthesis and reduced catabolism. Under heat stress, only an increase of *P5CR* expression was found (Xing et al. 2022). Conversely, *C. reinhardtii* did not show any fluctuation in free proline content under cold stress conditions (Valledor et al. 2013; Cvetkovska et al. 2022). The relationship between proline metabolism and heat stress was investigated also in *Acutodesmus dimorphus*, a potential species for biofuel production. In cells exposed to high temperature (45 and 50 °C), free proline content initially showed a rapid increase up to 12 h, but decreased drastically thereafter. Once again, SOD and CAT were also found to increase (Chokshi et al. 2020).

Concerning the effect of light fluctuations, the maximal theoretical efficiency of photosynthetically active radiation (400–700 nm) solar energy conversion into biomass under low light conditions at the lab scale is about 27%, but the efficiency drops to about 6% in outdoor cultivations under high light (Weyer et al. 2010; Zhu et al. 2010). Under low-irradiance, the photosynthetic rate increases linearly with light intensity, while at high irradiance the increase is not linear and reaches a plateau at light saturation (P_{max}) (Li et al. 2009). Above P_{max}, photoinhibition and ROS generation occur, and excess energy is dissipated into heat, with a consequent drop in productivity.

Although proline metabolism, as emphasized above, is often involved in ROS detoxification, it is still unclear whether proline may play a role in microalgae resistance to the oxidative stress generated by excess light. In plants, a possible correlation has been hypothesized based on the high rate of NADPH and ATP consumption required for proline synthesis from glutamate (Hare and Cress 1997). Proline production dissipating excess reducing power was proposed as a compensatory strategy to sustain photosynthesis and prevent photoinhibition under excess light in *A. thaliana* mutants lacking a chloroplast NADP-dependent malate dehydrogenase (Hebbelmann et al. 2012). Using reducing equivalents for enhanced proline biosynthesis could limit the generation of ROS through pseudocyclic photophosphorylation, and avoid the consequent cell damage (Ben Rejeb et al. 2014). Tissue-specific differences in proline metabolism, where proline synthesis in photosynthetic tissues regenerates NADP$^+$, while its catabolism in meristematic and expanding cells sustains growth by increased energy availability, further strengthened this hypothesis (Sharma et al. 2011).

A similar correlation has been suggested in *C. reinhardtii* the use of excess reducing equivalents for proline accumulation ameliorates the redox imbalance caused by high light irradiance in the chloroplast. Moreover, proline can be subsequently translocated into the mitochondrion and used to sustain the increased growth rate observed in high light acclimation (Davis et al. 2013). Consistently, a four–fivefold increase of free proline content was found in *Asteracys* sp. cells grown mixotrophically under high irradiances respect to the cells grown under low light (Agarwal et al. 2019). Increased proline concentrations were recently found also in *Ch. humicola* exposed of UV-B light, but in this case a direct role in scavenging the ROS produced under these conditions seems more likely (Singh et al. 2019).

Proline metabolic engineering, state of the art and perspectives

During the last decades the interest in microalgae for biotechnological applications has increased, yet the large-scale cultivation of most microalgal species is still not sustainable. Currently, only the green algae *Ch. vulgaris*, *D. salina* and *Haematococcus pluvialis* are cultivated on a large scale (Borowitzka 2018), but for the production of single products and with several limitations. Most of the industrial scale microalgal growth for food/feed production is performed in closed systems in mixotrophy or heterotrophy under axenic conditions. This implies a consequent high energy consumption for the maintenance of optimal temperature, light irradiation, gas fluxes, stirring and sterilization cycles. The possibility to find new approaches for lowering costs of large-scale cultivations would open new perspectives and widen microalgal applications. Several solutions have been already proposed, including recycling media, using nutrients derived from wastewater, phototrophic growth in sunlight, and ameliorating of photobioreactor architecture. Another possible approach to maximise productivity is represented by the identification of microalgal strains resistant to abiotic stress conditions. This result could be obtained by conventional selection of spontaneous or chemically-induced mutants. However, the newest genetic engineering techniques are opening the way to engineer strains with highly desirable traits, such as hyper salinity tolerance, resistance to excess light, heat and cold stress, capability to growth under non-axenic conditions and in presence of pollutants. Even more interestingly, these techniques could allow the transfer of these traits from donor strains to commercial strains with high productivity.

Genetic manipulation through CRISPR/Cas9 genome editing has demonstrated the high potential and plasticity of microalgae. For instance, several *C. reinhardtii* strains with characteristics of commercial interest have been obtained by transforming nucleus or chloroplast (Benedetti et al. 2018), and the introduction of *PtxD* gene allowed increased control of pest contamination (Loera-Quezada et al. 2016; Changko et al. 2020; Cutolo et al. 2020). Engineering of *C. reinhardtii* opened the possibility to utilize microalgae also as cell bio-factories to produce high-value compounds at low cost, with application in pharma, cosmetics, human nutrition (García et al. 2017) and treatment of lignocellulosic biomass waste (Benedetti et al. 2021). Although results demonstrated the robustness of these techniques, it will be necessary to apply them to microalgal species with higher productivity.

Genetic transformation of microalgal strains of commercial interest as *Chlorella* spp. is extremely challenging, one of the major constraints being represented by the presence of a cellulosic cell wall (absent in *Chlamydomonas*), which hinders the insertion of exogenous DNA. The first successes in chloroplast biolistic transformation of non-model microalgae have been described only recently. Efficient transformation of *Ch. vulgaris* chloroplast for the production of two foreign peptides (Wang et al. 2021),

and transformation of *Nannochloropsis gaditana* and *Tetraselmis* sp. with reporter genes (Cui et al. 2014, 2021) have been reported. Another group succeeded in transforming *Picochlorum renovo*, a green microalga characterized by high-productivity, halo- and thermotolerance (Dahlin et al. 2019). These studies represent an important breakthrough in microalgae genetic manipulation, but they are still far from the production of strains with characteristics exploitable on industrial scale. The attainment of improved growth rates can be pursuit by alternative strategies, such as random mutagenesis and subsequent selection of strains characterized by desirable phenotypic traits. With this approach, *Chlorella* sp. and *N. gaditana* strains characterized by high productivity and low sensitivity to oxidative stress under high light irradiance have been obtained (Cazzaniga et al. 2014; Perin et al. 2015; Guardini et al. 2021).

Under this perspective, proline metabolism represents an attractive target. As discussed above, proline metabolism seems strictly involved in the cell response to a wide range of environmental stresses, and in many instances a higher proline content, or a more active proline synthesis, was found to correlate with a higher tolerance to abiotic stress (Forlani et al. 2019a). In plants, successful strategies in proline metabolic engineering were developed. Overexpression of a *Vigna aconitifolia* P5CS in tobacco led to a higher proline accumulation under stress (Kishor et al. 1995), and the removal of feed-back inhibition allowed to obtain plants showing water stress tolerance (Zhang et al. 1995). Similar results were obtained in chickpea and rice (Karthikeyan et al. 2011), protecting plants from drought (Surekha et al. 2014). Proline metabolism tuning, obtained by expressing P5CR under the control of a heat-inducible promoter, conferred drought tolerance in soybean (De Ronde et al. 2004). *Arabidopsis thaliana* antisense mutants defective in *ProDH* expression showed increased free proline content and enhanced tolerance to excess salt and low temperature (Nanjo et al. 1999), without any detrimental effect on plant development (Mani et al. 2002). More recently, the same approach has been pursued with the CRISPR/Cas9 genome editing system, and *prodh* knock-out rice plants were obtained that showed increased proline content and displayed heat stress resistance (Guo et al. 2020).

In microalgae, however, only very few studies have investigated the feasibility of modifying proline pathways for biotechnological purposes. Two reasons may explain this delay. The first one is that most microalgal species of commercial interest are still recalcitrant to genetic transformation, mainly due to the cellulosic cell-wall surrounding the microalgal cell, and the low frequency of transgene integration into the algal genome (Cutolo et al. 2022). In fact, to the best of our knowledge, the only study in which proline synthesis has been engineered to date was carried out with the model organism *C. reinhardtii*, a cellulose cell-wall lacking species. In that case the insertion of a *P5CS* gene from mothbean allowed to obtain increased free proline content, which was paralleled by an increased tolerance to Cd ions and to salt-induced stress (Siripornadulsil et al. 2002). However, as emphasized above, some efficient transformation methods have been recently described, and this limitation could be overcome soon.

The other reason is that, contrary to plants, the metabolic pathways for proline metabolism and their regulatory switches under stress have been poorly investigated to date in microalgae. Although several efforts have been made to understand the correlation between environmental stress and fluctuations in proline metabolism, there are still insufficient information to obtain a detailed picture on how proline metabolism could protect microalgae against abiotic stress conditions, and –mainly– on the molecular mechanisms that underlie its modulation.

The selection of proline-overproducing strains, or a targeted modification of proline metabolism so as to obtain increased proline production/accumulation under stress, could increase algal capability to withstand environmental stress conditions. This would not affect production costs, and should be cost effective also for the algal cell, because the metabolic charge for increased proline synthesis would be paid off by the reduced negative effects of stress conditions. Yet, it would remain to verify whether an increased carbon flux toward proline synthesis, which requires ATP and reducing power, could be detrimental under non-stress conditions. It is quite likely that a constitutive production of proline, mainly if deriving from the overexpression of the biosynthetic genes under the control of strong promoters, can reduce the growth of overproducing strains with respect to wild-type controls in the absence of stress. But this limitation could be easily overcome by the use of stress-inducible promoters, thereby avoiding the occurrence of pleiotropic effects. On the other hand, increased proline synthesis would require increased availability of both carbon skeletons and nitrogen moieties. The first one should not be a problem for photosynthetic organisms that have been demonstrated to be able to modulate photosynthesis as a function of energy demand (Li et al. 2009). The second requirement may be more challenging, because higher nitrate/ammonia concentrations should be added to the culture medium. However, this apparent limitation could on the contrary turn into an advantage, as it would allow the (at least partial) use for algal cultivation of wastewater containing high levels of inorganic nitrogen.

The presence of increased proline content in the resulting biomass would cause no negative effects for downstream processing and product quality. If the biomass is to be used as food or feed, the taste would be substantially unchanged, since proline belongs to sweet amino

acids (Yuxiao et al. 2023) and does not contribute to bitter or umami taste. Although being not essential, increased proline level may on the contrary increase the nutritional value of the algal biomass, taking into account that emerging evidence indicates dietary essentiality of non-essential amino acids for animals and humans (Hou et al. 2015). Even in the case that the algal biomass is to be processed for the isolation of bioproducts, the presence of high levels of free proline could be beneficial because, as a kosmotropic substance, proline has been reported to increase macromolecule solubility and stability.

Investments in both basic and applied research with the aim to elucidate the metabolic pathways for proline synthesis in eukaryotic microalgae and their regulation under stress would be greatly desirable and would provide the basis for the development of future strategies for proline metabolic engineering. Hopefully, this will open new perspectives to achieve microalgae stress tolerance, with a consequent lowering of cultivation costs and enhancing of biomass yield, ensuring suitable productivity also in outdoor large-scale cultivations.

Author's contribution GF and SB identified patterns and trends in the literature and designed the structure of the review. SB prepared figures. GF and SB contributed to searching for relevant literature, and carried out a critical analysis of the literature, discussed together and wrote the manuscript. All authors read and approved the final manuscript.

Declarations

Competing interests The authors declare that they have no competing interests.

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