REVIEW

Photosynthetic machinery under salinity stress: Trepidations and adaptive mechanisms

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Abstract

Chloroplasts and photosynthesis are the physiologically fateful arenas of salinity stress. Morphological and anatomical alterations in the leaf tissue, ultrastructural changes in the chloroplast, compromise in the integrity of the three-layered chloroplast membrane system, and defects in the light and dark reactions during the osmotic, ionic, and oxidative phases of salt stress are conversed in detail to bring the salinity-mediated physiological alterations in the chloroplast on to a single platform. Chloroplasts of salt-tolerant plants have evolved highly regulated salt-responsive pathways. Thylakoid membrane remodeling, ion homeostasis, osmoprotection, upregulation of chloroplast membrane and stromal proteins, chloroplast ROS scavenging, efficient retrograde signalling, and differential gene and metabolite abundance are the key attributes of optimal photosynthesis in tolerant species. This review throws light into the comparative mechanism of chloroplast and photosynthetic response to salinity in sensitive and tolerant plant species.

Highlights

- Photosynthesis is highly sensitive to salinity stress
- An interplay of physiological and molecular determinants exists in the sensitivity of chloroplast to salinity stress
- The major tolerance and adaptive mechanisms to salinity are chloroplast membrane remodeling, ion homeostasis, and retrograde signalling

Introduction

Photosynthesis, the process that occurs in the wonderful laboratories of plants namely chloroplasts, dates back its origin to 3,800 million years ago. The Proterozoic era witnessed the origin of photosynthetic cyanobacteria as evident from the microfossils of stromatolites' reefs (Des Marais 2000). With progress in research, it was revealed that despite evolutionary differences between photoautotrophs, the reaction centers and cofactors follow the same three-dimensional structures. Moreover, the dominant carbon fixation process in all oxygenic photosynthetic organisms is the Calvin–Benson (C_3) cycle (Blankenship 2010). The process of photosynthesis that reduces gaseous carbon and fixes it to carbohydrates occurs in a biphasic manner. The biphasic mode of photosynthesis occurs in distinct regions of the chloroplast. The light-dependent reactions occur in the grana thylakoids and the carbon-reduction reactions take place in the stroma (Govindjee and Govindjee 1974).

Salinity stress is a major factor determining plant growth and yield, by significantly affecting key physiological processes, such as photosynthesis and energy metabolism (Feng *et al.* 2014). All the components and processes of photosynthesis mentioned above are directly disturbed by salt stress. Being a primary physiological process, any stress that impairs photosynthesis has a significant bearing on crop yield (Yang *et al.* 2020). Plants are forced to maintain an optimization between nutrient uptake and restriction of the entry of salinity-associated ions. The genome editing targets for such trade-offs are reported from various plants (Sathee *et al.* 2022). Salinity stress is a major global environmental challenge that degrades land and impairs production in a vast majority of crops (Soltabayeva *et al.* 2021). A global analysis of the Earth over 40 years by satellite data and machine learning showed that out of the 120.4 M km^2 of non-frigid land, 9.7% is affected by salinity. This area is not small as it encompasses 11.737 M km^2 (1,173.7 M ha) of land, of which 16.49 M ha is agricultural land (Hassani *et al.* 2020). As per the Food and Agriculture Organization (FAO), the total cultivated land is 5,301 M ha (48.01 M km^2) (http://www.fao.org/faostat/en/#data/RL) and it needs to feed the burgeoning population (Park *et al.* 2016). Across the globe, more than 424 M ha of topsoil (0–30 cm) and 833 M ha of sub soil (30–100 cm) are salt-affected (https://www.fao.org/soils-portal/data-hub/soil-maps-and-databases/global-map-of-salt-affected-soils/en/). Approximately, thirty crop species contribute to 90% of plant-based food, most of which show 50–80% yield reduction under conditions of moderate salinity (EC of 4–8 dS m^{-1}) (Zörb *et al.* 2019). Photosynthetic rate and associated traits, such as stomatal conductance, transpiration rate, internal CO_2 concentration, water-use efficiency, and stomatal density have been reported to be the major factors affecting the biomass and grain yield of crops under salinity stress (Lekklar *et al.* 2019).

In rice, the major cause of grain yield reduction under salinity stress is the photosynthetic limitation and its partitioning to spikelet (Hussain *et al.* 2017). More than a 65% reduction in photosynthetic rate has been observed in rice genotypes exposed to salinity stress of 8 dS m^{-1} during the reproductive stage (Lekklar *et al.* 2019). More than a two-fold reduction in photosynthetic rate has been observed in salt-sensitive and tolerant genotypes of durum wheat under salinity stress of 10 dS m^{-1} and has been correlated to reduced grain yield and test mass. Interestingly, in durum wheat, photosynthetic rate and stomatal conductance showed a negative correlation at high salinity stress of 15 dS m^{-1}, which suggests a C_4-type primary carboxylation using phospho*enol*pyruvate carboxylase (PEPcase) (Pastuszak *et al.* 2022). Salinity-induced reduction in photosynthetic rate, assimilate translocation to developing grains, and sink limitation are the major contributors to low grain yield in maize (Hiyane *et al.* 2010). The salinity threshold of the majority of vegetable crops ranges from 1 to 2.5 dS m^{-1}. Nearly 80% of the growth reduction in certain vegetable crops such as radish and spinach under saline conditions has been attributed to a decrease in leaf area and photosynthetic rate, and 20% due to a decrease in stomatal conductance (Machado and Serralheiro 2017). Salinity-induced reduction in photosynthesis of legume crops such as soybean and chickpea is not only a result of reduced stomatal conductance, but also due to nonstomatal limitations, such as reduced chlorophyll, chlorophyll fluorescence, and ultrastructural damage (Khan *et al.* 2015, Nadeem *et al.* 2019).

With the current annual rate of global soil deterioration, the possibility of famine in near future is looming in

the face of mankind. Deciphering how the photosynthetic apparatus gets affected and in turn responds to excess salts could provide new knowledge to enhance agricultural productivity, sustainability, and food security.

Characteristics underlying sensitivity of photosynthesis to salinity in plants

Morphological and anatomical alterations of photosynthetic tissues: Leaf anatomy is a crucial aspect in the maintenance of optimal photosynthesis levels during abiotic stresses (Longstreth and Nobel 1979). The most important anatomical traits of leaf tissue, which are strongly affected by salt stress, are mesophyll cell thickness, area of mesophylls per unit of leaf surface area, a ratio of mesophyll cell surface area to that of leaf surface area, epidermal and leaf thickness (Acosta-Motos et al. 2017). Increased leaf thickness and succulence are linked to a salt-induced decrease in the photosynthesis of salt-sensitive citrus species. This is because it reduces the surface area to volume ratio of mesophyll cells and lowers the intercellular air space, which indirectly affects CO_2 assimilation (Romero-Aranda et al. 1998).

The relative contribution of anatomical changes in the leaf to a decrease in photosynthesis is also dependent on the tolerance level of the plant genotype. Navarro and his group studied the anatomical alterations in the leaves of the strawberry tree (*Arbutus unedo*) under control and varying salt-stress conditions. The cell size of the second layer of palisade cells increased significantly with a concomitant increase in salinity levels, with no change in the cell size of the first palisade layer (Navarro et al. 2007). They also observed a reduction in intercellular spaces in the spongy mesophyll cells under salt stress which was strongly linked to reducing stomatal and mesophyll conductance to CO_2. Similarly, henna (*Lawsonia inermis* L.) plants irrigated with 75 and 150 mM NaCl showed an increase in leaf thickness to leaf area ratio (leaf specific mass) as compared to plants irrigated with normal water. This was confirmed by Fernández-García et al. (2014) as a specific investment by these plants in leaf tissue to increase leaf thickness, leaf specific mass, and thereby maximise photosynthesis under adverse conditions. Wheat plants subjected to varying degrees of salinity displayed an array of anatomical changes, such as decreased wall thickness, a diameter of hollow pith cavity, lower number of vascular bundles with reduced length and width, to survive the adverse condition (Nassar et al. 2020). In general, these anatomical changes under salt stress may not necessarily lead to efficient maintenance of photosynthetic rates, mainly due to a drastic reduction in stomatal conductance. The tolerant genotypes try to survive by anatomical alterations conserving the energy for reproduction.

Ultrastructural changes in the chloroplast: It has been demonstrated that under salt stress, chloroplasts get deformed into irregular shapes with significant compression in the grana thylakoid lamellae (Shu et al.

2015). The separation between cell membranes and chloroplasts reduced grana stacking, while a larger number of osmiophilic granules, giant starch granules, and accumulation of plastoglobules are certain key markers under salt stress (Gao et al. 2015, Guo et al. 2019a). Additionally, stroma thylakoid lamella also gets disrupted due to altered cellular ionic ratio (Shu et al. 2015). Studies from *Arabidopsis* revealed that chloroplasts of salt-treated seedlings showed deformation, reduced genome copy number, and gene expression. The organelle was enlarged, with reduced grana stacking and larger starch granules (Peharec Štefanić et al. 2013). Oi et al. (2020) described the three-dimensional structural changes in chloroplasts under salt stress in rice. They found that the cells of unstressed plants had elongated meniscus lens-shaped chloroplasts, while the stressed ones were expanded and oval-shaped. Serial sectioning also showed that the plastids in stressed cells were aggregated, but not in physical contact with each other (Oi et al. 2020). Additional observations were found in potato (Gao et al. 2015) and brinjal (Alkhatib et al. 2021), where gradient salinity stress treatment was imposed on seedlings. At the onset of stress, disintegration of outer membrane, and partial dissolution or cavitation of both grana and stroma thylakoids were observed. In the long run (six weeks of stress), there was digestion of starch granules, reduction in chlorophyll content, and the thylakoid membranes became stickier and gradually disappeared.

Compromised integrity of chloroplast membrane system: Cellular membranes are integral to all three major components of salt stress: ion imbalance and associated nutrient stress, osmotic stress, and reactive oxygen species (ROS)-induced oxidative damage. Membrane remodelling has been evident in plants exposed to salt stress *via* altered permeability and fluidity by associated changes in lipid and protein composition as well as quantity (Guo et al. 2019b). The remodelling is very prompt under salinity stress, whereas in soybean it was shown to occur within 30 min of exposure to salt (Liu et al. 2021). Studies on membrane lipid alterations are largely focused on changes in total membrane lipids with special attention to the plasma membrane. Only very few studies highlighted endomembranous lipid remodelling under salt stress.

The three-membrane chloroplast system including the outer and inner envelope membranes and the thylakoid membrane is significantly affected by altered cellular ion content (Suo et al. 2017). The chloroplast membrane network mainly comprises glycolipids, such as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG). The two major salt-mediated responses at the membrane level are changes in the total lipid content and alterations in the ratio of saturated and unsaturated fatty acids components. Salt causes changes in membrane permeability with a reduction in total lipid content and results in excess ion leakage, which is measured as a loss of membrane integrity. The reduction in lipid content and associated loss of integrity along with increased membrane

peroxidation levels has been reported to be more prevalent in salt-sensitive plant species/genotypes (Chalbi *et al.* 2013).

Salt-sensitive plant species commonly show a reduction in MGDG in the chloroplast membranes, resulting in a significant decrease in chlorophyll content (Shu *et al.* 2012, Yamane *et al.* 2015). This effect owes to the salt-induced activation of galactolipase and lipoxygenase enzymes (Hasanuzzaman *et al.* 2014). MGDG plays an important role in forming stable bilayer structures in conjunction with LHCII in the thylakoid membranes. The reduction in MGDG, thus causes serious disruption to the chloroplast membranes by altering the MGDG/LHCII ratio (Simidjiev *et al.* 2000). Decline in phospholipid (PL) component has been observed in several salt-sensitive plant species (Magdy *et al.* 1994, Lin and Wu 1996). These PLs can act as precursors for the biosynthesis of glycolipids in chloroplast membranes. A decreased phosphatidylcholine (PC) to phosphatidylethanolamine (PE) ratio has been confirmed as an indicator of salt sensitivity (Liu *et al.* 2021).

Phosphatidylglycerol (PG), an inevitable lipid of thylakoid membranes has been reported to decrease under salt stress in leaves of *Sulla* sp. which was speculated to be the major cause of ultrastructural damage to the chloroplast membranes (Bejaoui *et al.* 2016). A reduced DGDG content was also observed in *Catharanthus* sp., *Arabidopsis*, *Thellungiella halophila*, and *Sulla* sp. grown under salt stress (Sui and Han 2014, Bejaoui *et al.* 2016), which is inferred to be a possible reason for loss of membrane integrity and reduced stability of major proteins such as PS1 and LHCII (Boychova Krumova

et al. 2010). *Arabidopsis* displayed a significantly reduced DGDG/MGDG ratio in its chloroplast membranes as compared to salt-tolerant *T. halophila*. This finding indicates a direct link between this ratio and the integrity of the cellular membrane system (Sui and Han 2014). Omoto *et al.* (2013) observed that a lesser abundance of MGDG in mesophyll as compared to bundle sheath chloroplast membrane under salt stress was attributed as the major factor of difference between the salt sensitivity of the two cell types in C_4 maize.

SQDG is another glycolipid exclusively associated with chloroplast membranes. It contributes towards a more stable protein–lipid configuration in the membrane bilayer and binds with another protein called annexin involved in membrane organisation and fusion (Seigneurin-Berny *et al.* 2000). In glycophytes, SQDG contents decrease under imposed salinity stress, as evident in maize (Omoto *et al.* 2016) and *Sulla* sp. (Bejaoui *et al.* 2016). Additional findings from salt-sensitive species show that a reduction in SQDG content has been speculated to be due to decreased contents or activity of the rate-limiting enzyme UDP-glucose pyrophosphorylase under stress (Guo *et al.* 2019b).

The summarized model, which highlights the major changes in the chloroplasts of salt-sensitive plant species, is depicted in Fig. 1.

Compromised polypeptide composition of photosystems and light reaction: Chloroplast contains 30–50% of total cellular proteins, among which 80% are proteins embedded in the thylakoid membranes (Lande *et al.* 2020). Salinity stress mainly impairs photosynthesis by
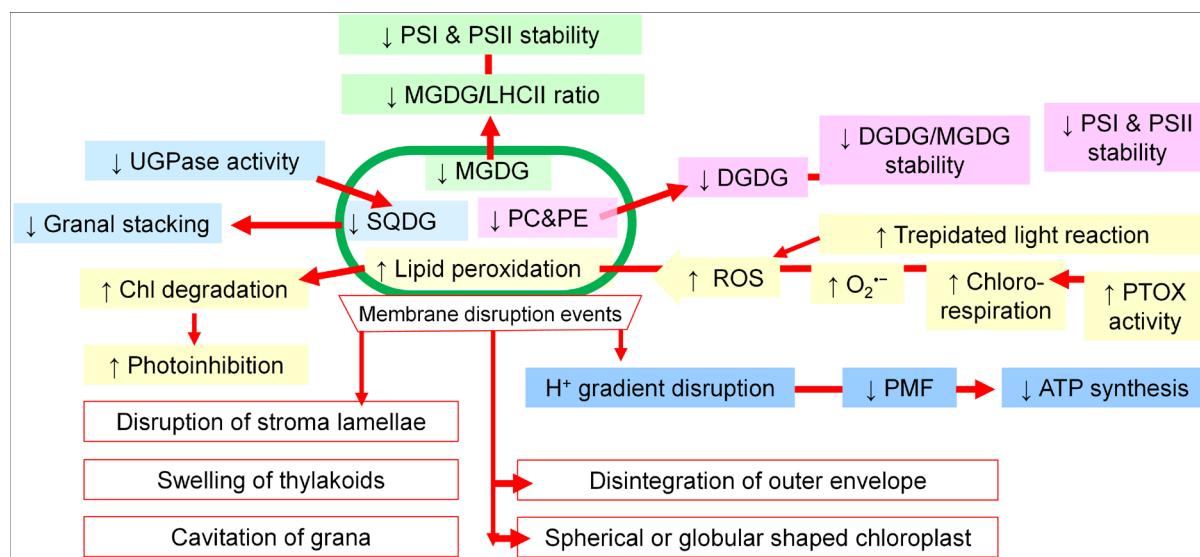
Fig. 1. Major routes of chloroplast membrane disruption and remodelling under salt stress in salinity-sensitive plant species. MGDG – monogalactosyldiacylglycerol; PC – phosphatidyl choline; PE – phosphatidyl ethanolamine; UGPase – UDP-glucose pyrophosphorylase; SQDG – sulfoquinovosyldiacylglycerol; LHCII – light-harvesting complex II; PSI – photosystem I; PSII – photosystem II; DGDG – digalactosyldiacylglycerol; PMF – proton motive force; PTOX – plastid terminal oxidase. Each colour represents different functional modules of chloroplast membrane remodelling under salinity stress.

altering the polypeptide composition of the photosystems. This degradative effect was found both in algae as well as higher plants. Salt stress causes a decrease in the number of active PSII sites, damages the oxygen-evolving complex (OEC), and impedes the electron flow to PSI (Kan *et al.* 2017). The JIP test on salinity-imposed *Dunaliella* sp. revealed that the water-splitting complex is the first site of damage by excess salts (Ghasemi and Shariati 2012).

Western blot analysis of thylakoid membrane proteins showed that the expression of LHC proteins, such as CP7, CP43, Lhcb1, and Lhcb4 was downregulated in *Chlamydomonas reinhardtii* under salt stress (Neelam and Subramanyam 2013). Studies have shown that salt stress inhibits the repair of PSII by restraining the activities of the *psbA* gene that encode the D1 protein in the cyanobacterium *Synechocystis* sp., both at transcriptional and translational levels (Allakhverdiev *et al.* 2001). Such damages specific to PSII have also been found in plant systems (Kan *et al.* 2017). In the susceptible rice cultivar Peta, PSII had lesser content of 33 and 43 kDa polypeptides and a deficiency in 23 kDa polypeptides under salt stress (Wang *et al.* 2009). Moreover, under salt stress, other proteins related to PSII and PSI, such as PsbP, PsaK, Ycf4, and OHP1 showed decreased abundance in wheat, indicating Na^+-mediated damage leading to loss of thylakoids and decreased F_v/F_m (Zhu *et al.* 2021). Salt stress also damages the transfer of electrons from Q_A to Q_B and further to the cytochrome (Cyt) b_6f complex (Akhter *et al.* 2021). In addition, the PetD protein of Cyt b_6f complex was found to be degraded under salinity stress in *Desmostachya bipinnata* (Asrar *et al.* 2017). The plastoquinone pool remains over-reduced in the process which can lead to the generation of ROS (Akhter *et al.* 2021). Studies in tomato, rice, *etc*., have shown degradation of membrane protein components of Cyt b_6f and ATP-synthase complex (Li *et al.* 2018). The physiological outcome of such damages is poor H^+ gradient and proton motive force (PMF) in thylakoids and reduced ATP-synthase activity. Chloro-respiration is a process occurring in chloroplasts that diverts electrons from PSII *via* the plastoquinone pool to molecular oxygen, producing water. It is regulated by the enzyme plastid terminal oxidase (PTOX) (Bolte *et al.* 2020). The transformation of tobacco chloroplast using the *PTOX* gene from *C. reinhardtii* showed a light-sensitive response (Ahmad *et al*. 2012). These effects are the outcome of the pro-oxidant function of the *PTOX* gene that leads to the formation of superoxide molecules (Krieger-Liszkay and Feilke 2016) (Table 1). These types of negative regulatory genes are being targeted for genome editing by CRISPR/Cas for imparting salinity tolerance in crop plants (Sathee *et al*. 2022). Thus, the various alterations in membrane proteins contribute to defective photosynthesis in chloroplasts.

Defective dark reaction processes: Several proteomic analyses as well as Western blotting studies have shown the degradation of Calvin cycle enzymes, Rubisco in particular, under salt stress (Asrar *et al*. 2017, Thagela *et al.* 2018). The activity of Rubisco was reported to be inhibited both *in vivo* and *in vitro* under salinity stress (Gong *et al.* 2018). Proteomic studies on salt-stressed alfalfa revealed upregulated expression and activity of the Rubisco activase enzyme (RCA) (Xiong *et al.* 2017). This can be inferred as the survival mechanism adopted by plants to activate the available Rubisco enzymes in the stroma. But under higher doses (600 mM NaCl), RCA content was reduced by 50% as compared to the levels at 400 mM in *Leymus chinensis* (Li *et al.* 2017a). Other Calvin cycle enzymes, which are severely affected by excess salts, include glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fructose-1,6-bisphosphatase (FBPase), and ribulose-5-phosphate kinase (RPK) (Acosta-Motos *et al.* 2017). In C_4 plants, phospho*enol*pyruvate carboxylase (PEPCase) is more sensitive to salt than Rubisco (Chiconato *et al.* 2021). The partial or complete closure of stomata also aggravates the effects of salinity stress that limits CO_2 availability (Franzisky *et al.* 2021). The storage of carbohydrates is also hampered by salt stress as evident from the microscopic observations in *Ulva prolifera*. Under salt stress, starch became smaller and lighter, albeit regained its proper structure during recovery (Huan *et al.* 2014).

Reduction in copy number of chloroplast DNA: Chloroplast has organellar DNA due to its unique endosymbiotic origin (Dyall *et al.* 2004). The plant chloroplast genomes or plastomes usually range between 120–200 kbp in size and their copy numbers can vary anywhere from 20 to several hundred depending on the species, developmental stage, and environmental conditions. Salinity stress resulted in a reduction of the average copy number of the chloroplastic genome in *Arabidopsis* by about 40% (Peharec Štefanić *et al.* 2013). Intriguingly, chloroplast DNA replication is found to be blocked by inhibition of the photosynthetic electron transport chain (ETC). This suggests the possibility that plastid DNA replication is coupled with photosynthetically supplied reducing power and is redox regulated (Kabeya and Miyagishima 2013). Thus, inhibition of photosynthetic ETC by Na^+ ion toxicity in chloroplasts might be a reason for reduced plastome replication. Nevertheless, a more direct effect of salinity stress on molecular factors involved in the rate of replication of the plastid DNA may also play an important role in the reduction (Peharec Štefanić *et al.* 2013). Indeed, previous studies have shown that the bacterial-like DNA polymerase Pol1A which plays a major role along with DNA Pol1B in the replication of the plastid genome in plants is strongly repressed by osmotic stresses (Morley and Nielsen 2016). However, the exact molecular mechanisms involved in plastome copy number reduction due to stress remains elusive.

Alterations in the transcription of chloroplast-encoded genes: Chloroplast is a semiautonomous organelle, whose genome encodes about 100–250 genes. Proteome studies showed that chloroplasts contain up to 3,000 proteins. Salinity has been shown to drastically alter organellar gene expression (Robles and Quesada 2019). Reduction in the plastome copy number leads to the concomitant decrease in transcript levels of chloroplast-encoded genes involved

Table 1. The major genes involved in imparting susceptibility or tolerance to plants under salinity stress.

Gene(s)	Protein	Plant species	Salinity stress response	Reference
Photosynthetic membrane organisation and PSII activity				
Fad6	ω-6 desaturase	*Arabidopsis*	Seedling stage salinity tolerance	Zhang *et al.* (2009)
MGD	Monogalactosyldiacylglycerol synthase	Rice (*Oryza sativa*)	Salinity tolerance; well-developed thylakoid membrane with improved grana stacking	Wang *et al.* (2014)
OEP, OECP, Chla/bBP	33 kDA oxygen-evolving protein, 23 kDa protein of oxygen-evolving complex of PSII, chlorophyll *a/b* binding protein	Rapeseed (*Brassica napus*)	Salinity tolerance	Jia *et al.* (2015)
Rrd	Rubredoxin family protein	Alkali grass (*Puccinellia tenuiflora*)	Salinity tolerance	Li *et al.* (2016)
RCI	Rare cold-inducible protein	Wheat (*Triticum aestivum*)	Salinity tolerance; maintenance of PSII stability and activity	Khurana *et al.* (2015)
GPAT	Glycerol-3-phosphate acyltransferase	Tomato (*Lycopersicon esculentum*)	Enhanced salt tolerance; unsaturation of fatty acids of PG in the thylakoid membrane	Sun *et al.* (2010)
HCF136	PSII stability/assembly factor	Wheat (*Triticum aestivum*)	Salinity tolerance; maintenance of PSII stability and assembly	Xu *et al.* (2016)
LHCB6	Light-harvesting complex protein	Indian mustard (*Brassica juncea*)	Salinity tolerance	Singh *et al.* (2019)
PsbP, Cytb$_6$f, b559-∞	23 kDa protein of oxygen-evolving complex of PSII, cytochrome b_6f complex associated proteins	Wheat (*Triticum aestivum*)	Salt tolerance	Zhu *et al.* (2021)
PSI P$_{700}$	PSI P$_{700}$ chlorophyll *a* apoprotein A2	Cotton (*Gossypium hirsutum* L.)	Salinity tolerance; high electron transfer efficiency	Gong *et al.* (2017)
Fd, NADHC	Ferredoxin, NAD(P)H dehydrogenase complex	Wheat (*Triticum aestivum*)	Salt tolerance	Zhu *et al.* (2021)
PTOX	Plastid terminal oxidase	Tobacco	Salt sensitivity; increased formation of superoxides	Ahmad *et al.* (2012)
Photosynthetic dark reactions, other stromal genes, and photorespiration				
CHL, GSAAT	Magnesium chelatase, glutamate-1-semialdehyde aminotransferase	Chickpea (*Cicer arietinum*), *Brassica napus*	Salinity tolerance	Jia *et al.* (2015), Arefian *et al.* (2019)
FBA	Fructose-1,6-bisphosphate aldolase	Cotton (*Gossypium hirsutum* L.)	Salinity tolerance	Gong *et al.* (2017)
NADP-ME	NADP-malate dehydrogenase	*Arabidopsis*	Salinity tolerance	Chen *et al.* (2019a)
ALT, AST	alanine aminotransferase, aspartate aminotransferase	Sesame (*Sesamum indicum*)	Salinity tolerance	Zhang *et al.* (2019)
NPR1	Non-expression of pathogenesis-related genes 1	Tobacco (*Nicotiana tabacum*)	Salinity tolerance; redox homeostasis	Seo *et al.* (2020)
GAPB	Glyceraldehyde-3-phosphate dehydrogenase β subunit	*Thellungiella halophila*	Salinity tolerance	Chang *et al.* (2015)
GO	Glycolate oxidase	Sugar beet (*Beta vulgaris*)	Salinity tolerance	Lv *et al.* (2019)
PEPC	Phospho*enol*pyruvate carboxylase	Sweet sorghum (*Sorghum bicolor*)	Salinity tolerance	Yang *et al.* (2020)
Chloroplast ionic and osmotic homeostasis				
KEA	K^+/H^+ antiporter	*Arabidopsis*	Salinity tolerance; chloroplast osmoregulation, integrity, and pH regulation	Kunz *et al.* (2014)
BASS2, PHT4;1, PHT4;4, PHT4;5	Bile acid sodium symporter 2, phosphate transporters	Halophyte species	Salinity tolerance; preferential Na accumulation in the chloroplast	Bose *et al.* (2017)

Gene	Protein	Organism	Effect	Reference
CHX23	Na$^+$ (K$^+$)/H$^+$ exchanger	*Arabidopsis*	Mutants displayed salt hypersensitivity and impaired photosynthetic performance	Song *et al.* (2004)
NHD1	Sodium hydrogen antiporter	*Arabidopsis*	Mutants displayed salt hypersensitivity and impaired photosynthetic performance	Müller *et al.* (2014)
OsNHAD	Putative sodium hydrogen antiporter	Rice (*Oryza sativa*)	Knockdown led to hypersensitivity to Na$^+$ ions, reduced PSII activity, and altered chloroplast morphology	Liu *et al.* (2020)
OtsA, *OtsB*	Trehalose-6-phosphate synthase, Trehalose-6-phosphate phosphatase	Rice (*Oryza sativa*)	Overexpression led to better photosynthetic rate and lower K/Na ratio in root and shoot	Garg *et al.* (2002)
TPPD	Trehalose 6-phosphate phosphatase	*Arabidopsis*	Overexpressors were tolerant to high salinity stress	Krasensky *et al.* (2014)
CodA	Choline oxidase	Tomato (*Lycopersicon esculentum*)	Improved ion homeostasis and photosynthesis	Wei *et al.* (2017)
Oxidative stress and defense				
Cu/Zn SOD	Copper/zinc superoxide dismutase	Cotton (*Gossypium hirsutum* L.)	Salinity tolerance; ROS scavenging	Luo *et al.* (2013)
GPX	Glutathione peroxidase	*Arabidopsis*	Salinity tolerance; ROS scavenging	Zhai *et al.* (2013)
γ-TMT	γ-tocopherol methyltransferase	Tobacco (*Nicotiana tabacum*)	Salinity tolerance; reduced ROS abundance and membrane injury	Jin and Daniell (2014)
GR3	Glutathione reductase	Rice (*Oryza sativa*)	Knockout led to increased salt sensitivity	Wu *et al.* (2015)
CAT	Catalase	Cotton (*Gossypium hirsutum* L.)	Salinity tolerance; ROS scavenging	Luo *et al.* (2013)
DHAR	Dehydroascorbate reductase	Rice (*Oryza sativa*)	Improved salinity tolerance; altered antioxidant metabolism	Le Martret *et al.* (2011)
WSL12	Nucleoside diphosphate kinase	Rice (*Oryza sativa*)	Salinity tolerance; increased antioxidant enzyme activities	Ye *et al.* (2016)

in photosynthesis. In *Arabidopsis*, the transcript levels in salt-stressed seedlings were reduced by 50% as compared to the control (Peharec Štefanić *et al.* 2013). DEAD-box RNA helicases are RNA-binding proteins involved in the formation of functionally processed RNAs in the nucleus, chloroplasts, and mitochondria. In *Arabidopsis*, ten members of this family are confirmed to be localised in the chloroplasts. *In silico* analysis of the chloroplast-targeted proteins showed that eight members from rice, seven from maize, and two from wheat are downregulated under salinity stress (Nawaz and Kang 2017).

In plants, mTERFs (mitochondrial transcription termination factors) are a family of nucleus-encoded proteins that are involved in the transcriptional regulation of chloroplastic and mitochondrial genes (Robles and Quesada 2019). Functional genomic approaches in *Arabidopsis* showed that *mTERF6*, *mTERF10*, and *mTERF11* function as positive regulators of salinity tolerance. Yet, a few other members, namely, *mTERF5* and *mTERF9*, negatively regulate salinity tolerance as evidenced by enhanced salt tolerance of the *mter5* and *mter9* mutants (Robles *et al.* 2018). The expression of chloroplast-encoded photosynthetic genes in *Arabidopsis*, namely, *ATPF* (ATP-synthase subunit b, F type), *NDHA* (NADH dehydrogenase subunit 1), *PETB* (photosynthetic electron transfer B), and *TRNK* (tRNA for lysine)

were found to be downregulated under salinity stress. In addition, the transcript levels of nuclear-encoded chloroplast-targeted genes, namely, *LHCA4* (light-harvesting chlorophyll–protein complex I subunit A4), *RBCS1*A (ribulose bisphosphate carboxylase small chain 1A), and *RCA* (Rubisco activase) were also down-regulated in salt-stressed sensitive plants (Peharec Štefanić *et al.* 2013). Recent advances in techniques such as cell fractionation together with the application of advanced next-generation sequencing techniques such as single-cell RNA-Seq, Chloro-Seq, global run-on (GRO)-Seq, global ribosomal profiling (Ribo-Seq) will aid in further understanding of the intricacies involved in retrograde signalling and regulation of transcription in response to salt stress (Leister *et al.* 2017).

Defects in chloroplast protein biosynthesis: Several studies indicate that salt stress, specifically Na$^+$ ion toxicity, can adversely affect protein synthesis and translational machinery including the functioning of ribosomes (Omidbakhshfard *et al.* 2012). The levels of chloroplast-encoded tRNA and rRNA were significantly reduced by salinity, indicating defects in the protein synthetic machinery (Peharec Štefanić *et al.* 2013). Salt stress was found to inhibit the transcription and translation of *psbA* genes that encode the D1 protein in

Synechocystis sp. Northern and Western blotting analysis showed time-dependent reduction in the RNA and corresponding D1 protein. Experiments using [^{35}S] Met labelling also confirmed the reduced translation of various chloroplastic proteins under salt stress (Allakhverdiev *et al.* 2002).

The Ef-Tu family proteins (eukaryotic translation elongation factor thermo unstable), namely, tufA from pea was found to be involved in protein synthesis in chloroplasts. Salinity stress reduced its transcript abundance which in turn caused alterations in the overall chloroplast protein synthesis (Singh *et al.* 2004). PSRPs (plastid-specific ribosomal proteins) are a class of proteins involved in protein synthesis, among which PSRP2 (a component of the 30S subunit) of *Arabidopsis* is chloroplast targeted. *PSRP2* overexpression lines displayed sensitivity and mutant lines showed tolerance to salinity. The protein was shown to have RNA chaperone activity and is a negative regulator of stress tolerance and germination (Xu *et al.* 2013). Salinity-induced attenuation of PSII is associated with the degradation of several important PSII reaction center proteins, mainly the D1 protein (psbA) along with the D2 protein (psbD), and the internal antenna protein CP43 (psbC) which are also essential for its repair (Nagashima *et al.* 2004).

The major components of chloroplast sensitivity to salinity in salt-susceptible plant species are depicted in Fig. 2.

How do salt-tolerant plant species sustain photosynthesis under salinity?

Salt-tolerant plant genotypes maintain high photosynthetic rates under salt stress primarily by stabilizing internal CO_2 concentration and maintaining the stability of photosynthetic systems (Sui and Han 2014). The adaptive mechanisms at the chloroplast level to cope with salt stress need to be efficiently exploited in the development of salt-tolerant crops. At the cellular level, studies on photosynthetic response under salt stress have focused on retrograde signalling and associated stress responses, chloroplast-specific ion channels and transporters, chloroplasts membrane remodelling, and maintenance of photosynthetic membrane proteins along with CO_2-fixing enzymes (Table 1). The succeeding sections deal with all these insights and the differential ability of halophytes to overcome the stomatal and nonstomatal limitations of salt stress.

Maintenance of chloroplast membrane fluidity and stability: Salt-tolerant species like barley showed no lipid reduction under salt stress, along with reduced lipolysis and peroxidation of membrane lipids which helped them maintain normal cellular expansion and growth (Chalbi *et al.* 2013). Salt stress increased the content of phosphatidyl glycerol (PG) in the photosynthetic membranes of salt-tolerant species such as buffalo grass
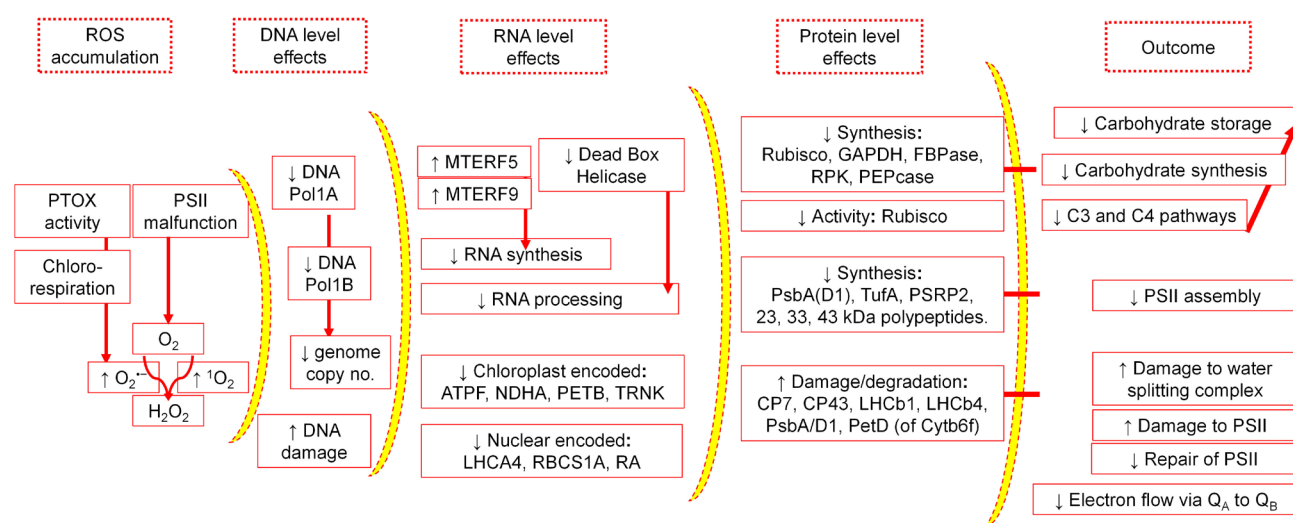
Fig. 2. Major levels of sensitivity to salinity in chloroplasts of salt-sensitive species. ROS – reactive oxygen species; PTOX – plastid terminal oxidase; $O_2^{\cdot-}$ – superoxide radical; $^1O_2$ – singlet oxygen; DNA Pol1A – DNA polymerase 1A; DNA Pol1B – DNA polymerase 1B; MTERF – mitochondrial transcription termination factor; ATPF – ATP-synthase subunit b, F type; NDHA – NAD(P)H dehydrogenase subunit 1; PETB – photosynthetic electron transfer B; TRNK – tRNA for lysine; LHCA4 – light-harvesting chlorophyll–protein complex I subunit A4; RBCS1A – ribulose bisphosphate carboxylase small chain 1A; RA – Rubisco activase; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; FBPase – fructose-1,6-bisphosphatase; RPK – ribulose-5-phosphate kinase; PEPCase – phospho*enol*pyruvate carboxylase; psbA – photosystem II reaction center protein A; TufA – eukaryotic translation elongation factor thermo unstable A; PSRPs – plastid-specific ribosomal proteins; CP – chlorophyll-binding protein; LhcB – light-harvesting complex protein subunit B; PETD – photosynthetic electron transport subunit D; PSII – photosystem II. *Upward* and *downward arrows* indicate upregulation and downregulation, respectively.

(Lin and Wu 1996) and many halophytes (Sui and Han 2014, Barkla *et al.* 2018). These reports are consistent with its critical role in oxygenic photosynthesis and the functioning of PSII and LHCII (Guo *et al.* 2019b).

Salt-induced increase in glycolipids, especially galactolipids (Wang *et al.* 2014), along with alteration and incorporation of unsaturated fatty acids into these lipids (Sui and Han 2014) is seen in the photosynthetic membranes of salt-tolerant species. Moreover, a decrease in DGDG to MGDG ratio is common under salt stress, but this decrease is lesser in salt-tolerant species leading to more stable chloroplast membranes (Sui and Han 2014). High contents of SQDG in the chloroplast membranes are often regarded as another unique attribute of salt tolerance as evident from its elevated levels in many tolerant plant species (Ramani *et al.* 2004, Hamed *et al.* 2005). Increased levels of unsaturation in the fatty acids of membrane lipids are speculated to be the reason for enhanced salt tolerance in the cyanobacterium *Synchococcus* sp., which protected the photosynthetic machinery and also activated Salt Overly Sensitive 1 (SOS1) for sodium extrusion from the cell. An increase in plastoglobule size and number has been observed in salt stress (Barkla *et al.* 2018), and this has been hypothesized to be the outcome of the mobilization of galactolipids in the thylakoid membrane and accumulation of triacylglycerols.

Chloroplast membrane transporters as saviours: Salt stress leads to an increase in Na^+ and Cl^- concentration and a decrease in K^+ concentration inside the chloroplast (Robinson and Downton 1984). In *Arabidopsis*, K^+/H^+ exchanger (KEA3) and tandem pore K^+ channel (TPK3) are involved in the influx and efflux of K^+ into and out of the thylakoid lumen, respectively, along with KEA1 and KEA2 located at the inner chloroplast envelope (Carraretto *et al.* 2013, Kunz *et al.* 2014, Finazzi *et al.* 2015). In salt-tolerant halophytic species, Na^+ is preferentially accumulated in the chloroplast (Müller *et al.* 2014), where it serves other essential functions, too, and this is mediated by Na^+-dependent transporters such as bile acid:sodium symporter 2 (BASS2) and phosphate transporters (PHT4;1, PHT4;4, and PHT4;5) (Bose *et al.* 2017). Nevertheless, excess Na^+ beyond a threshold can be detrimental to halophytes as well, and hence these salt-loving species have been speculated to possess transporters that prevent excessive Na^+ accumulation in the chloroplasts. In *Arabidopsis*, *CHX23* and *NHD1* encode putative Na^+/H^+ antiporters in the chloroplast envelope, which regulate the entry of Na^+ into the chloroplast, and the mutants of which showed significant salt hypersensitivity and impaired photosynthetic performance (Müller *et al.* 2014). Numerous research groups have also observed preferential K^+ and Cl^- accumulation in the chloroplasts of halophytes, but the corresponding transporters still need to be unveiled. Recently, a chloroplast membrane-located transporter OsNHAD encoding a putative Na^+/H^+ antiporter has been identified and correlated to enhanced salt tolerance in rice (Liu *et al.* 2020).

Salt tolerance and changes in photosynthetic membrane proteins: Several salt-responsive proteins involved in chloroplast membrane organization and associated with photosystem activities have been reported in *Arabidopsis*, rice, wheat, and tomato (Suo *et al.* 2017). The *Arabidopsis* fatty acid desaturase 6 (*fad6*) mutant displayed salt hypersensitivity, indicating its role in salt tolerance, especially during the early seedling stage (Zhang *et al.* 2009). An increase in the unsaturation levels of fatty acids in the lipids of the thylakoid membrane helps in accelerated repair and *de novo* synthesis of D1 protein in PSII (Loll *et al.* 2007). Another important protein that determines the organisation of the thylakoid membrane is the chloroplast outer envelope membrane-localized MGD. *OsMGD* gene, overexpressed in tobacco, resulted in increased contents of MGDG and DGDG and the transgenic lines displayed well-developed thylakoid membranes with significant grana stacking (Wang *et al.* 2014).

Some chloroplast-specific membrane proteins are essential for the normal functioning of PSII. Rubredoxin (Rrd), a non-heme thylakoid membrane protein, when knocked out, showed reduced activity and stability of PSII in green algae, cyanobacteria, and *Arabidopsis* (Calderon *et al.* 2013). This is indicative of its role as an electron carrier-mediated ROS scavenger (Li *et al.* 2016). Similarly, rare cold-inducible (RCI) protein is another candidate chloroplast membrane protein involved in the maintenance of PSII stability and activity under salt stress (Khurana *et al.* 2015). Two proteins closely associated with PSI and PSII, $LHCb_6$ and 10-kDa PSII polypeptide, were upregulated in the salt-tolerant genotypes of Indian mustard (CS54, variety developed by ICAR-CSSRI), indicating its higher efficiency in photoprotection under salinity (Singh *et al.* 2019). The abundance of 33-kDa oxygen-evolving protein, 23-kDa protein of OEC of photosystem II and Chl *a/b*-binding protein also increased under salt stress in salt-tolerant species of *Brassica napus* (Jia *et al.* 2015).

Photosystem II stability/assembly factor (HCF136), a hydrophilic protein in the lumen of stroma thylakoids, showed higher abundance in the salt-tolerant wheat genotype, contributing to photosynthetic stability (Xu *et al.* 2016). Expression of proteins, such as PsaA, PsaB, atpl, and LHC proteins, was found to be elevated initially and then downregulated (Jayakannan *et al.* 2015) showing that the crop tolerance to salinity stress can enhance the rate of photosynthesis in the initial phases of stress by maximizing light capture. Moreover, PsbP, PsbQ family protein, Cyt b_6f complex iron-sulfur subunit, and cytochrome b_{559}-∞ subunit also showed increased abundance in the salt-tolerant wheat isogenic line, indicating better PSII regulation, repair, and PSI assembly under salt stress (Zhu *et al.* 2020, 2021).

Oxygen-evolving enhancer proteins 1&3 along with plastocyanin-docking protein and PSI subunit VII were also differentially upregulated in tolerant genotypes of *Brassica*, cotton, and chickpea, indicating better stability of OEC and PSI under salt stress (Yousuf *et al.* 2016, Gong *et al.* 2017, Arefian *et al.* 2019). PSI reaction center subunits such as PsaK and PsaIV have also been reported

to increase under salt stress, which helps mediate the binding of antenna complexes to the PSI reaction center (Zhu *et al.* 2021). A preferential abundance of PSI-P$_{700}$ chlorophyll *a* apoprotein A2 (PSI-P700), the primary electron donor of PSI was observed in the salt-tolerant cotton genotype after being exposed to high salt stress for 4 h, indicating higher electron transfer efficiency (Gong *et al.* 2017). Similarly, an increased abundance of ferredoxin and NAD(P)H-dehydrogenase complex was reported in salt-tolerant wheat (Zhu *et al.* 2021). ATP synthase alpha and beta subunits got differentially upregulated under saline conditions in the tolerant genotype of common bean and chickpea. This highlights the possible role of these proteins in energy synthesis, as molecular chaperons, and a possible indirect role in the translocation of excess Na$^+$ and Cl$^-$ into the vacuole (Arefian *et al.* 2019).

CEST (chloroplast protein enhancing stress tolerance) is a novel thylakoid membrane-localized protein involved in chloroplast development, suppression of photooxidative damage, growth, and salt tolerance (Yokotani *et al.* 2011). Transglutaminase, a previously known protein for post-translational modification of other proteins, was found to enhance polyamine signalling and thylakoid stability in tobacco under salt stress (Zhong *et al.* 2019). Salt stress also increased the abundance of chloroplast-localized small heat shock proteins (sHSPs), sHSP70 in wheat (Zhu *et al.* 2021), which assists in the import of pre-proteins from the nucleus into the chloroplast and their maturation (Latijnhouwers *et al.* 2010). Wheat chloroplasts have a very active protein quality control machinery under salt stress *via* the activation of proteins namely carboxyl-terminal-processing peptidase 3 (CTPA3) and chaperone protein 2 (ClpC2). They are involved respectively in the assembly of PSII proteins and the degradation of damaged proteins (Zhu *et al.* 2021).

Improved stromal protein levels and activity: Reports on proteomic analysis of chloroplast in response to salinity are limited. Studies on leaf proteome in canola suggested that chloroplast proteins are key indicators of salinity (Iqbal *et al.* 2019). The abundance of magnesium chelatase and glutamate-1-semialdehyde aminotransferase, key enzymes in chlorophyll biosynthesis increased after exposure to salt stress in chickpea and *Brassica napus* seedlings, respectively (Jia *et al.* 2015, Arefian *et al.* 2019). The large subunit of Rubisco, coded by a single gene in the chloroplast genome is more sensitive to salt stress, but tolerant plant species have been able to maintain its abundance under stress conditions (Arefian *et al.* 2019, Zhu *et al.* 2021). Rubisco activase has been reported to be upregulated under salt stress, indicating its vital role in maintaining the active state of Rubisco (Jia *et al.* 2015). Overexpression of the *GAPDH* beta subunit protein in the chloroplast stroma prevented the attenuation of ROS-mediated PSII repair and maintained the efficiency of the photosynthetic apparatus in *Arabidopsis* under salinity (Chang *et al.* 2015). Concomitantly, fructose-1,6-bisphosphate aldolase activity was differentially upregulated in the tolerant genotypes of upland cotton

after 24 h of imposed salt stress (Gong *et al.* 2017). In addition, a differential abundance of other CO$_2$-fixation enzymes, such as carbonic anhydrase, phospho-ribulokinase, phosphoglycerate kinase, sedoheptulose-1,7-bisphosphatase, fructose-bisphosphate aldolase, and ketolase, has been reported in numerous salt-tolerant species or cultivars (Xu *et al.* 2016, Arefian *et al.* 2019, Zhu *et al.* 2021). The expression of NADP-malate dehydrogenase (NADP-ME) is upregulated under salt stress in salt-tolerant cultivars (Chen *et al.* 2019a). The abundance of amino acids biosynthetic enzymes such as alanine aminotransferase and aspartate aminotransferase also increased under salt stress in the tolerant genotypes of sesame (Zhang *et al.* 2019).

NPR1 (non-expressor of pathogenesis-related genes 1) protein has been shown to accumulate in the chloroplast stroma with possible roles in redox homeostasis and amelioration of salt-mediated downregulation of photosynthetic capability (Seo *et al.* 2020). Chloroplast-localized DAD1, phospholipase A, has been speculated to be responsible for the release of linolenic acid from membrane lipids, the precursory step in jasmonic acid (JA) biosynthesis (Ishiguro *et al.* 2001). In superior salt-tolerant lines of chickpea, tolerance was accredited to the salt-mediated increase of JA biosynthesis (Xu *et al.* 2016). Cold-regulated (COR) protein was upregulated in *Arabidopsis* under salt, a potential member in the ABA-dependent salt stress signalling network (Liu *et al.* 2014). Maturase K, common to all land plant chloroplasts gets activated under salt stress *via* dephosphorylation (Zörb *et al.* 2010) and plays a crucial role in primary mRNA processing/splicing (Xu *et al.* 2016). A multi-organelle-located, conserved protein OsNBL1 has been proposed to interact with plastidic caseinolytic protease OsClpP6 and is essential for enhanced salt tolerance. *Nbl1* mutants showed upregulation of several salt-inducible genes, such as *HAK1* and *HAK5*, and were highly salt tolerant. Several chloroplast ribosomal proteins, such as RPL5, RPL10, RPL14, RPL21, and RPL29, got upregulated under salt stress in wheat, which hastened the synthesis of damaged photosynthetic proteins (Zhu *et al.* 2021).

Post-translational modifications of chloroplast proteins: Post-translational modifications are key aspects of altered protein function, activity, targeting, turnover, and interactions in response to various environmental stresses (Grabsztunowicz *et al.* 2017). Phosphorylation, glycosylation, carbonylation, nitrosylation, redox modifications, ubiquitination, and SUMOylation are the commonly reported post-translational modifications of chloroplast proteins in response to salt stress (Chang *et al.* 2012, Liu *et al.* 2012). Salt stress has been reported to initiate phosphorylation cascades in rice proteins, and a total of 13 phosphorylation sites across eight proteins have been identified, which includes the PSII reaction center protein H and RBCSs (Chang *et al.* 2012). Phospoproteome studies in maize under salt stress identified several phosphoregulated proteins involved in photosynthesis (Zörb *et al.* 2010). Phosphorylation of PSII

proteins controls the functional folding of photosynthetic membranes in *Arabidopsis* and helps sustain photosynthetic activity under salinity stress (Fristedt *et al.* 2009). Moreover, Rubisco activase, RBCLs, chloroplast and mitochondrial ATP-synthase F1 were carbonylated and S-nitrosylated upon exposure to salt stress in *Citrus aurantium* seedlings (Tanou *et al.* 2009). In rice, pyruvate phosphate dikinase 1 is ubiquitinated upon salt stress treatment (Liu *et al.* 2012). Further, the enhanced photosynthetic efficiency of the salt-tolerant wheat introgression line over its parent was attributed to the putative difference in post-translational modification of Rubisco and phosphoribulokinase (Xu *et al.* 2016). Significant post-translational modifications have also been reported in the chloroplast ROS-scavenging protein, 2-Cys peroxiredoxin, which is strongly correlated with improved stability of the photosynthetic system in salt-tolerant lines (Dietz 2016).

Photorespiration as a protective mechanism under salt stress: Stomatal limitation leads to a deficit in intracellular CO_2, which causes the over-reduction of ETC. Rubisco operates as an oxygenase and photorespiration is initiated under saline conditions to channel out the excess light energy (Suo *et al.* 2017). The osmotic phase of salt stress has been reported to upregulate the expression and activities of photorespiratory genes and enzymes (Bai *et al.* 2017). Transformed rice with chloroplastic glutamine synthetase showed enhanced salt tolerance by activation of the photorespiratory pathway (Hoshida *et al.* 2000). In addition, overexpression of the *Arabidopsis* and bacterial photorespiratory pathway genes, such as serine:glyoxylate aminotransferase (*SGAT*) and serine hydroxymethyltransferase (*HMT*), respectively, enhanced the salt tolerance (Waditee-Sirisattha *et al.* 2017). Sugar beet, a highly salt-tolerant crop species showed increased contents of glycolate and serine along with enhanced expression of glycolate oxidase under salt stress (Lv *et al.* 2019).

Compatible solutes and osmotolerance: Cellular accumulation of compatible solutes, organic compounds that do not interfere with cellular enzymes and functions, is an important strategy for the protection and survival of plants under salinity. Commonly accumulated compatible solutes include glycine betaine (GB), amino acids such as proline, polyols/sugar alcohols, and quaternary amines (Munns *et al.* 2020). These solutes play an important role in osmotic adjustment and maintenance of ionic balance by minimizing the entry of salt into plants or by regulating the concentration of salts in the cytoplasm (Shabala *et al.* 2020). They also act as signalling molecules to increase ABA accumulation, affect gene expression networks, and regulate plant growth under salt stress (Marusig and Tombesi 2020).

Proline serves as one of the major osmolytes that stabilize membranes and protein and also helps in cellular redox homeostasis, ROS scavenging, and maintenance of cytosolic $NADP^+/NADPH$ ratio (Reddy *et al.* 2015). In response to salt stress, chloroplast starch reserve is rapidly catabolized to sucrose and there is a promotion

of sugar synthesis and concomitant inhibition of starch synthesis (Kumutha *et al.* 2008). This build-up of sugars (glucose, sucrose, fructose, and fructans) plays a crucial role in the adaptation to salinity and osmotic stresses (Abideen *et al.* 2021). Trehalose, a nonreducing disaccharide, is another osmolyte; overexpression of *Escherichia coli* trehalose biosynthetic genes (*otsA* and *otsB*) in rice increased trehalose biosynthesis and enhanced salt tolerance. These transgenic plants were able to maintain a higher K^+ ion content over Na^+ ion and thus have a lower K^+/Na^+ ratio both in shoots and roots which helped in ion homeostasis under salt stress. These plants also showed better photosynthetic rates than the wild type (Garg *et al.* 2002).

Increased contents of methylated inositol and myo-inositol by chloroplast-targeted overexpression of PcINO and McIMT1 improved the growth and photosynthesis of transgenic tobacco (Patra *et al.* 2010). The quaternary ammonium compounds, such as glycine betaine (GB), choline-O-sulphate, proline betaine, β-alanine betaine, hydroxyproline betaine, dimethyl sulphoniopropionate, and pipecolate betaine, are another group of efficient osmolytes (Ahmad *et al.* 2013). Amongst these betaine compounds, GB is extensively distributed over different plant species and is accumulated in response to a wide range of abiotic stresses. Apart from mediating osmotic adjustment, GB provides support in Na^+/K^+ discrimination, antioxidant defense, and protection of membranes, ultimately leading to photosynthetic viability. The exogenous application of GB is also known to protect photosynthetic machinery and improve salt tolerance (Ahmad *et al.* 2018). Biosynthesis of GB involves the oxidation of choline by choline monooxygenase and betaine-aldehyde dehydrogenase successively in the chloroplastic stroma (Sakamoto and Murata 2002). Overexpression of *E. coli betA* (choline dehydrogenase) in wheat improved the membrane stability and reduced ion leakage and sodium accumulation (He *et al.* 2010). Tomato plants overexpressing *codA* (choline oxidase) gene showed improvement in the rate of photosynthesis, antioxidant enzyme activities, decreased K^+ efflux, and increased Na^+ efflux in comparison to wild-type plants (Wei *et al.* 2017).

ROS-scavenging machinery in chloroplasts: Under unfavourable conditions, plants employ metabolic and morphological adjustments to prevent oxidative damage to photosystems. The stress-induced increase in the activity of the enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), peroxidases, and their isoforms, maintain the functional balance between the quenching of ROS and maintenance of H_2O_2 concentration required for cell signalling (Noctor and Foyer 2016). To resist the oxidative stress caused by excess salts, numerous proteins associated with anti-oxidant defence were upregulated in wheat chloroplasts that help scavenge ROS and regulate protein turnover under salt stress (Zhu *et al.* 2021). The H_2O_2 produced by plastidic Cu/Zn-SOD is scavenged by membrane-bound thylakoid ascorbate peroxidase (tAPX) in a reaction called

as water–water cycle (Edreva 2005). Overexpression of plastidic Cu/Zn-SOD improved ROS homeostasis and salinity tolerance in several plant species including *Arabidopsis*, tobacco, Chinese cabbage, and cotton (Suo *et al.* 2017). Overexpression of chloroplastic *APX* imparted salinity tolerance in tobacco (Badawi *et al.* 2004). Chloroplast targeting of CAT was found to improve salinity tolerance in cotton and Chinese cabbage (Tseng *et al.* 2007, Luo *et al.* 2013).

The stromal ascorbate–glutathione (AsA–GSH) cycle reduces H_2O_2 to H_2O in a stromal APX (sAPX) mediated reaction. sAPX utilizes AsA as the electron donor, and AsA is recycled by monodehydroascorbate reductase (MDHAR) or is spontaneously transformed into dehydro-ascorbate (DHA). DHA is then reduced to AsA by DHAR using reduced glutathione (GSH) as an electron donor. The oxidized glutathione (GSSG) is reduced by glutathione reductase (GR) where NADPH is the electron donor. Expression of sAPX, MDHAR, DHAR, and GR is responsive to salinity. Overexpression of MDHAR and DHAR increased the survival of tobacco plants under salt stress (Suo *et al.* 2017). The thioredoxin/peroxiredoxin (Trx/Prx) pathway and glutathione peroxidase (GPX) regulates H_2O_2 contents in chloroplasts. Trx-dependent peroxidase (Prx) scavenges H_2O_2, and the thioredoxin reductase (TrxR) reduces Trx/Prx system using NADPH as the electron donor. GPX uses GSH as an electron donor and reduces H_2O_2 using GSH as the electron donor. Transgenic *Arabidopsis* overexpressing wheat chloroplast GPXs showed increased tolerance to salt and H_2O_2 (Zhai *et al.* 2013).

The ROS, ˙OH, and $^1O_2$ are scavenged by nonenzymatic antioxidants, such as AsA, GSH, and tocopherol in chloroplasts. Tocopherol is a lipid antioxidant localized in the thylakoid membrane (Suo *et al.* 2017). Tocopherol biosynthesis is a finely balanced process in chloroplasts. ABC1 (activity of bc1 complex)-like kinases (ABC1K3) and AtSIA1 are associated with salinity tolerance in *Arabidopsis* (Martinis *et al.* 2013). Overexpression of γ-tocopherol methyltransferase (*γ-TMT*) reduced ROS abundance and membrane injury leading to salt stress alleviation in tobacco (Jin and Daniell 2014). Apart from the conventional antioxidant molecules, other regulatory mechanisms have also been documented concerning chloroplast ROS-scavenging under salt stress. Overexpression of chloroplast encoded nucleoside diphosphate kinase 2 (*NDPK2*) gene from *Arabidopsis* into sweet potato led to the induction of an array of compounds having antioxidant activity (Kim *et al.* 2009). The *Arabidopsis* AtTSPO involved in the transport of photoreactive tetrapyrrole intermediates protected chloroplasts from ROS accumulation in response to 150 mM NaCl (Balsemão-Pires *et al.* 2011). Methionine sulfoxide reductase reduces the methionine sulfoxide back to methionine during episodes of salinity stress. Overexpression of chloroplast-localized OsMSRA4.1 improved plant survival under salinity (Guo *et al.* 2009).

Transcriptional regulation of chloroplast genome: Several transcriptomic studies have been undertaken to identify key genes which respond to salinity stress at various stages. The comparative analysis of the transcriptome of a salt-tolerant wheat cultivar (Arg) in comparison to a susceptible variety (Moghan3) revealed several genes involved in photosynthesis such as plastidial chlorophyll-binding proteins (Amirbakhtiar *et al.* 2021). In salt-tolerant rice, most of the upregulated genes were associated with photosynthetic electron transport (Razzaque *et al.* 2019). Observations by Fan and coworkers in the response of *Salicornia europaea* to salinity stress showed the gene expression of several plastidial photosystem-associated proteins, such as PSI and PSII-binding proteins, Cyt b_6f complex proteins, and the ATP synthase CF1 subunit (Fan *et al.* 2013).

In salinity-tolerant sweet sorghum cultivars, PEPC and NADP-ME were upregulated to impart tolerance to the photosynthetic system (Yang *et al.* 2020). The most significant pathway upregulated in salinity-stress response was the photosynthetic light reaction pathway in a salinity-tolerant chickpea cultivar JG-62 relative to susceptible ICCV-2 (Garg *et al.* 2016). The tolerant barley genotype (Boulifa) showed differential upregulation of PSI assembly proteins, PSII reaction center proteins, D2, 10-kDa polypeptide, PSI P700 chlorophyll apoproteins, and iron-sulphur center proteins (Ouertani *et al.* 2021). In a comparative study between the salinity-tolerant peach cultivar GF677 and the susceptible Maotao, the integrity of chloroplast structure and upregulation of photosynthetic genes were identified as critical to salinity tolerance (Sun *et al.* 2020). In grapes, transcriptome analysis of the moderately salinity-tolerant 'Thompson seedless' cultivar showed the overexpression of several chloroplastic genes involved in maintaining photosynthesis, such as *APX*, Chl *b* reductase, and Mg-binding proteins among others (Das and Majumder 2019). *Ulva compressa* is a green alga adapted to a moderately saline environment such as brackish waters. A transcriptomic study of *Ulva* showed that several plastidial genes associated with photosystem maintenance and photosynthetic acclimation, such as high chlorophyll fluorescence 244 (*HCF244*), maintenance of photosystem II under high light 2 (*MPH2*), hypothetical chloroplast reading frame 4 (*YCF4*), hypersensitive to high light 1 (*HHL1*), and vesicle-inducing protein in plastids (*VIPP*) were constantly upregulated in both short- (1, 6 h) and long-term (24, 72 h) salinity treatment. This affirms the critical role of genes associated with maintenance of the photosynthetic assembly in salinity tolerance mechanisms (Xing *et al.* 2021).

Metabolic reshuffle to optimize photosynthesis under salt stress: Global metabolic changes are a reflection of cellular protein activities and physiological alterations under abiotic stresses in plants. Increased accumulation of organic acids was observed in salt-tolerant barley genotypes (Wang *et al.* 2019). Similarly, salt-tolerant wild Tibetan barley cultivars (XZ16 and XZ169) accumulated more metabolites related to photosynthesis than their cultivated counterparts (CM72 and Gairdner) (Wu *et al.* 2013). Decreased glutamic acid was observed in salt-stressed *S. persica*, which indicates that more of it was

channelled for the synthesis of Chl *a* and Chl *b* (Kumari and Parida 2018).

On exposure to salinity, plants showed reduced contents of glutamine, D-alanyl-D-alanine, cystine tyrosine, L-alanyl-L-glutamate, D-alanyl-D-serine, and elevated contents of some amino acids, such as phenylalanine, glutamate, methionine, dihydroxyisoleucine, ornithine, L-methionine, 3-methoxytyramine, tetra-homomethionine, 1-(3-aminopropyl)-4-aminobutanal, and L-homomethionine. Moreover, contents of branched-chain amino acids, which act as stress modulators, got increased under salt-stress conditions (Benjamin *et al.* 2019). Several sugar and sugar alcohols, namely, arabinose, inositol, sorbitol, and mannitol accumulated in the chloroplasts and extra chloroplastic space of salt-stressed sugar beet plants. It reflects that safeguarding the internal osmotic environment of chloroplast is key to survival under salt stress (Lv *et al.* 2019). Polyamines, such as spermine, putrescine, spermidine, tricaffeoyl spermidine, and coumaroyl spermidine also increased under salt stress in salt-tolerant crops/genotypes (Kumari and Parida 2018). Putrescine interacts with negatively charged thylakoid membranes, increases the lipid accumulation in chloroplasts, and thereby minimises thylakoid membrane degradation (Shu *et al.*

2012). Secondary metabolites such as terpenes and flavonoids were found to accumulate under salinity stress, which possibly protects the photosynthetic machinery from oxidative stress (Chen *et al.* 2019b, Qin *et al.* 2022).

A cumulative model highlighting the major attributes of salt tolerance at the chloroplast level is shown in Fig. 3.

Salt-mediated retrograde signals from the chloroplast: Chloroplast is probably the most sensitive organelle to salt stress. At the same time, it is also one of the key cellular sensors of climatic fluctuations. Reduction in photosynthetic efficiency and energy supply during salt stress forces the plant to divert energy from growth to stress response. Efficient coordination between organelles and nuclear transcriptional machinery becomes inevitable. This communication, namely retrograde signalling, is highly imperative under stress conditions (Crawford *et al.* 2018).

PSII is the main site of $^1O_2$ generation in the chloroplast, which further triggers two different downstream retro-signalling cascades (Cruz de Carvalho 2014). β-carotene quenches singlet oxygen produced in PSII and nonenzymatically gets cleaved in to β-cyclocitral. This secondary messenger interacts with methylene blue
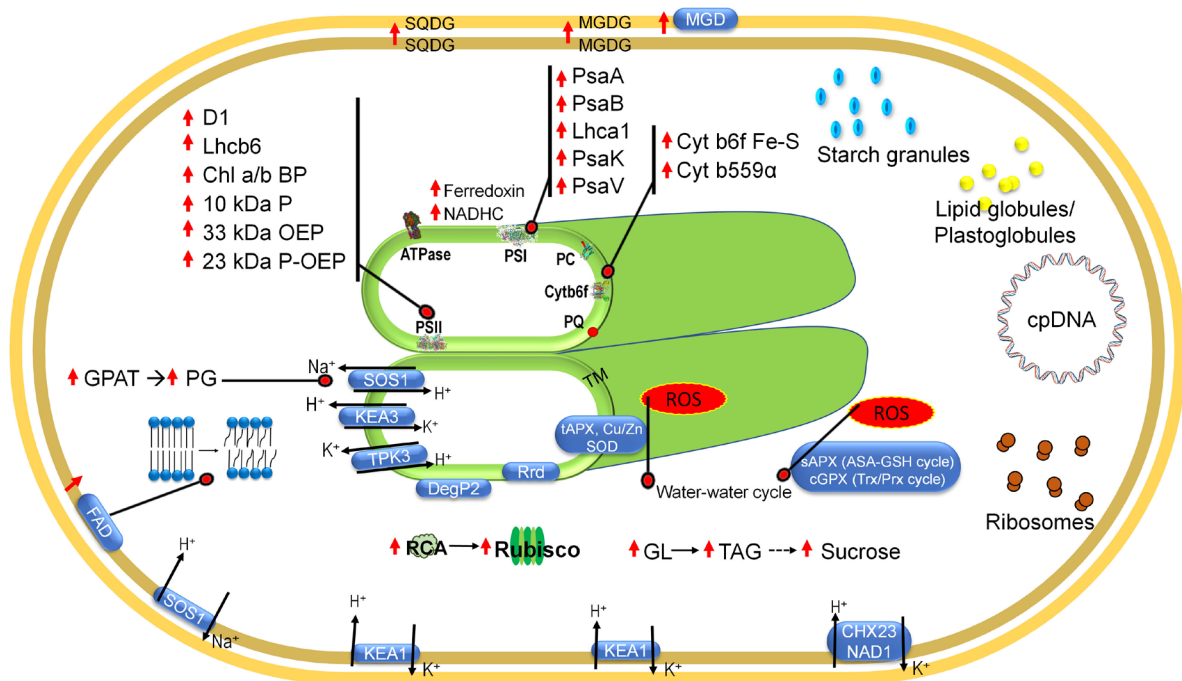
Fig. 3. The major components of tolerance to salinity stress at chloroplast level. MGD – monogalactosyldiacylglycerol synthase; MGDG – monogalactosyldiacylglycerol; SQDG – sulfoquionovosyldiacylglycerol; cpDNA – chloroplast DNA; TM – thylakoid membrane; GPAT – glycerol 3-phosphate acyl transferase; PG – phosphatidylglycerol; ROS – reactive oxygen species; GL – galactolipids; TAG – triacylglycerol; RCA – Rubisco activase; FAD – fatty acid desaturase; NHAD – putative Na^+/H^+ antiporter; KEA1 & 2 – K^+/H^+ exchanger; CHX23 & NAD1 – Na^+/H^+ antiporters; SOS1 – salt overly sensitive 1; TPK – tandem pore K^+ channel; DegP2 – trypsin type serine protease; Rrd – rubredoxin; SOD – superoxide dismutase; tAPX – thylakoid membrane-localized ascorbate peroxidase; sAPX – stromal ascorbate peroxidase; AsA–GSH; ascorbate–glutathion; Trx/Prx – thioredoxin/peroxiredoxin; NADHC – NADH dehydrogenase complex; Lhc – light-harvesting complex; Chl *a/b* BP – chlorophyll *a/b*-binding protein; OEP – oxygen-evolving protein; Cyt – cytochrome; PSI – photosystem I; PSII – photosystem II; PC – plastocyanin; PQ – plastoquinone. *Upward arrows* and *dotted lines* represent upregulation and hypothetical proteins/pathways, respectively.

sensitivity 1 protein (Shao *et al.* 2013) and increases the expression of a set of nuclear encoded singlet oxygen-responsive genes (SORGs) (Ramel *et al.* 2012). This retro-signalling pathway is initiated from the PSII reaction centers of the grana core (Wang *et al.* 2016). The second pathway operates from the grana margins, with the help of two nuclear-coded, chloroplast-targeted proteins, Executer 1 and 2. They transfer the $^1O_2$-mediated signals to the nucleus and affect the activation of SORGs (Wang *et al.* 2016). In the nucleus *DREB2A*, *CYT P450*, *GST 6*, *WRKY*, *bHLH*, *HSPs*, and *MAP* kinase are known to be the common target genes for these two signalling pathways (Fig. 4) (Dogra *et al.* 2017).

Another retro-signal from the plastid under any kind of oxidative damage is linked to the tetrapyrroles (*e.g.*, chlorophyll, heme, siroheme, and phytochromobilin)

biosynthesis pathway (Crawford *et al.* 2018). This pathway is highly regulated, and any alteration in the metabolic flux of the pathway has been shown to affect the expression of nuclear-coded photosynthetic genes through retrograde signalling (Ibata *et al.* 2016). Recent evidence suggests that 3'-phosphoadenosine-5'-phosphate (PAP), a by-product of secondary sulphur metabolism also acts as a second messenger in chloroplast retrograde signalling in response to oxidative stress caused by drought or high light (Estavillo *et al.* 2011). Oxidative stress inhibits the activity of inositol polyphosphate-1-phosphatase enzyme (SAL1) (Chan *et al.* 2016), which increases the accumulation of PAP. PAP moves from the plastid to the nucleus *via* the directional transporter PAPST1 and induces the expression of nuclear-coded stress-response proteins such as early light-induced
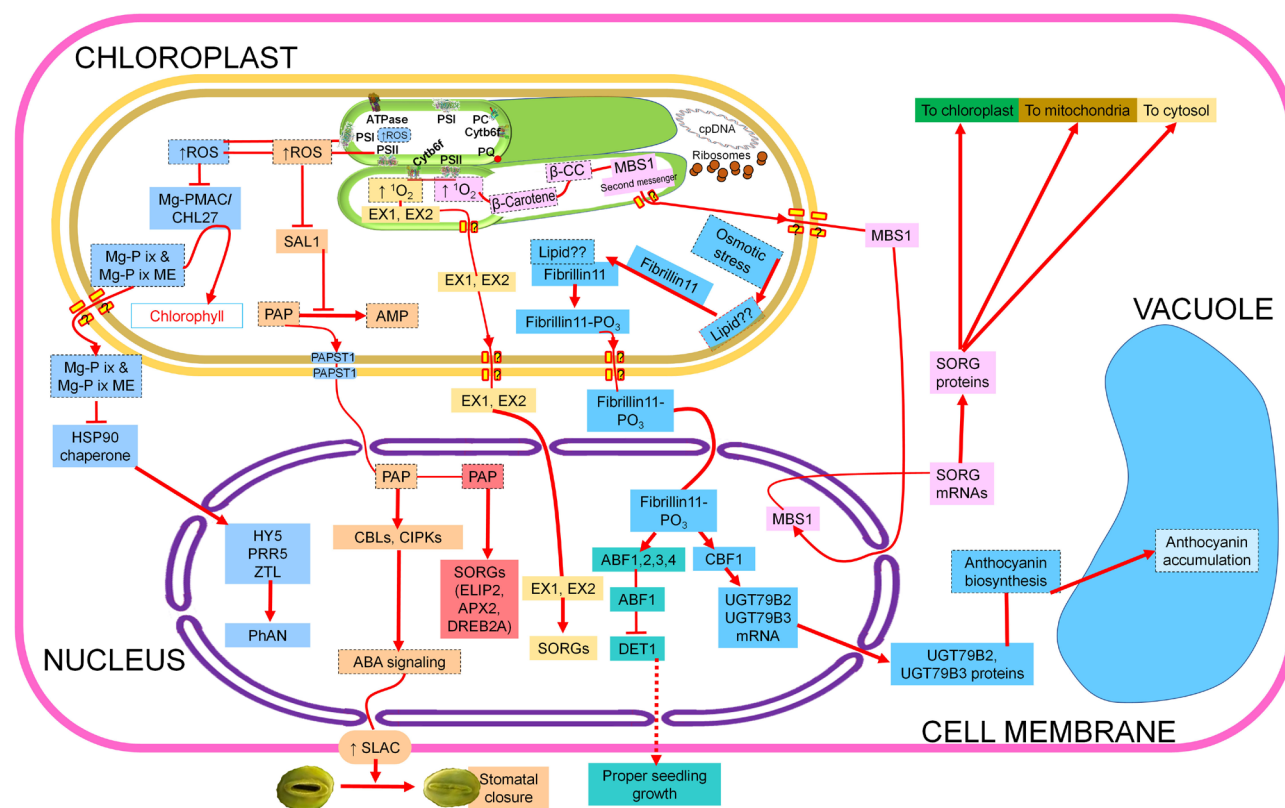

Fig. 4. Major components of salinity-mediated retrograde signalling from the chloroplast. PSI – photosystem I; PSII – photosystem II; PC – plastocyanin; $^1O_2$ – singlet oxygen; β-CC – β-cyclocitral; MBS1 – methylene blue sensitivity 1; SORGs – singlet oxygen-responsive genes; EX1/EX2 – EXECUTER 1/EXECUTER 2; ROS – reactive oxygen species; MgPMAC/CHL27 – Mg-protoporphyrin monomethylester aerobic cyclase; MgPIX/ME – Mg protoporphyrin IX and its monomethyl ester derivative; HY5 – LONG HYPOCOTYL 5; PRR5 – PSEUDO RESPONSE REGULATOR 5; ZTL – ZIETLUPE; PhANGs – photosynthesis-associated nuclear genes; SAL1 – inositol polyphosphate-1-phosphatase; PAP – 3'-phosphoadenosine-5'-phosphate; AMP – adenosine monophosphate; PAPST1 – bidirectional PAP transporter; ELIP2 – EARLY LIGHT-INDUCED PROTEIN 2; APX2 – ascorbate peroxidase 2; DREB2A – DEHYDRATION-RESPONSIVE ELEMENT BINDING 2A; CBLs – calcineurin B-like protein; CIPKs – CBL-interacting protein kinase; ABA – abscisic acid; SLAC – slow anion channel associated 1; Lipid?? – unknown lipid molecule; Fibrillin11 PO_3 – phosphorylated fibrillin11; ABF1-4 – ABA-responsive element-binding factor 1-4; CBF1 – dehydration-responsive element binding factor 1; SA – salicylic acid; JA – jasmonic acid; DET1 – DEETIOLATED 1; UGT79B2 & UGT79B3 – *Arabidopsis* UDP glycosyl transferases. The five different retrograde signalling pathways are shown in different coloured boxes. Further, the branching in individual pathways is also coloured differently. ⬜❓ depicts unidentified transporters. The unidentified signalling routes are depicted in *red dotted arrows* and non-protein, non-RNA components are depicted in *black dashed boxes*. *Upward arrows* indicate increased accumulation/overexpression.

protein 2 (ELIP2) and ascorbate peroxidase 2 (APX2) (Gigolashvili *et al.* 2012). The PAP-SAL1 retro-signalling system was shown to interact with the ABA-signalling network to regulate stomatal closure in response to osmotic stress through alternate ABA-signalling components (Fig. 4) (Pornsiriwong *et al.* 2017).

Another chloroplast protein, fibrillin11 has been shown to play a major role in osmotic stress tolerance during germination as part of retrograde signalling in *Arabidopsis*. This chloroplast-generated protein signal regulates the expression of ABF (ABA-responsive element-binding protein/factor) and CBF1 (C-repeat binding factor 1) transcription factors in the nucleus (Choi *et al.* 2021). CBF1 controls the expression of glycosyltransferase genes (*UGT79B2* and *UGT79B3*), involved in anthocyanin synthesis and thereby confers salt tolerance (Fig. 4) (Li *et al.* 2017b). To elicit an appropriate stress response, these stress-induced retrograde signals must be integrated into stress-responsive pathways in the cytosol. It is hypothesized that the mediator transcriptional co-activator complex in the nucleus might play a key role in integrating stress-signalling pathways operating in different organelles (Crawford *et al.* 2018).

Photosynthetic apparatus of halophytes and salt tolerance: Though halophytes share salinity tolerance mechanisms with glycophytes, these species have evolved certain additional characteristics to adapt to highly saline soils. These adaptive mechanisms can be exploited to achieve improved salt tolerance in major glycophytes (Volkov 2015). Halophytes maintain their operational and maximum PSII efficiency under progressive salt stress. This is the most important attribute of halophytes which protects them from photoinhibition and associated oxidative stress (Duarte *et al.* 2014). Nevertheless, a quantitative analysis of halophytic PSII efficiency shows a significant difference between species. For instance, *Suaeda fruticosa* has a system where PSII can absorb light even under high salt stress. This mechanism is inactive in *Halimione portulacoides*, leading to lower levels of light absorption and photosynthetic efficiency (Rabhi *et al.* 2012, Duarte *et al.* 2014). This put forth an interesting pattern of salt tolerance even across halophytic species of the same family. Moreover, the coordination of both photosystems in terms of the rapid response of the PSII acceptor site and PSI stability contributed towards lowered levels of oxidative stress in the photosynthetic apparatus of halophytic soybean (Yan *et al.* 2020).

In certain halophytes like *Desmostachya bipinnata*, moderate salt stress did not significantly affect chlorophyll content, net photosynthetic rate, ETR, and q_P. In contrast, these parameters improved under varying degrees of salinity in obligate halophytic species, namely, *Sesuvium portulacastrum* and *Tecticornia indica* (Asrar *et al.* 2017). Halophytes also show spatially different gene expression pattern as evident from the transcriptomic analysis of quinoa. Lowered expression of photosynthesis-related genes in the epidermal bladder cells as compared to mesophyll cells was recorded (Böhm *et al.* 2018). Another mechanism by which halophytes, such as

Suaeda fruticosa and *Halimione portulacoides*, overcome salt-induced photoinhibition, is the enhanced activation of the xanthophyll cycle as evident from the increased de-epoxidation state index coupled with increased Chl/Car ratio (Wang *et al.* 2006). Enhanced expression of photosynthetic genes, such as Mn-stabilizing proteins of the oxygen-evolving complex of PSII, *CP47* (Chl *a*/*b* protein), *RBCL*, and *Rubisco activase* has been reported as protective mechanisms in halophytic rice (*Porteresia coarctata*) and wheat (*Thinopyrum ponticum*) genotypes (Sengupta and Majumder 2009). The shift in the mode of carbon assimilation is another common mechanism among halophytes under salt stress. For instance, C_3 (*Mesembryanthemum crystallinum*) and C_4 (*Portulaca oleracea*) halophytes have been reported to shift to the CAM pathway under salinity stress (Bose *et al.* 2014). Moreover, *T. halophila* showed increased activities of proteins, such as starch synthase, amylase, and starch branching enzymes, complemented by increased starch accumulation in the chloroplasts under salt stress (Wang *et al.* 2013).

Conclusions and future trends

Photosynthesis is highly sensitive to salt stress. Na$^+$ influences photosynthesis by disrupting the chloroplast membrane system and chloroplast function and by interfering with the stromal CO_2-fixing enzymes. Extensive membrane damage disrupts the PMF and causes irregularities at different stages of photophosphorylation. Chloroplasts have evolved highly regulated salt-responsive pathways. This review tried to collate different characteristics of chloroplast sensitivity to salt stress, followed by the tolerance mechanisms, such as thylakoid membrane remodelling, ion homeostasis regulation, chloroplast membrane and stromal proteins and their turnover, osmoprotectants, chloroplast ROS-scavenging system, as well as chloroplast gene expression and metabolic turnover. Halophytes possess unique response salt-responsive traits which help them maintain their photosynthetic levels under high salinity. These provide vital physiological and molecular know-how on chloroplastic salt response and adaptation of photosynthetic machinery to salinity stress. However, the chloroplast salt-response network and its retrograde communication with the nucleus is a too complex process to be fully interpreted by this information. Further investigations on the biological functions of already identified putative chloroplast transporters, genes, proteins, and metabolites along with retrograde signalling components are needed for a better comprehension of the photosynthetic salt response. The genes validated and characterized serve as valuable inputs for sustaining the photosynthesis of sensitive crop cultivars under salt-stress conditions.

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