Chloroplast function and ion regulation in plants growing on saline soils: lessons from halophytes

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Highlight: This review critically examine whether membrane transport processes and ionic relations are differentially regulated between glycophyte and halophyte chloroplasts and whether this contributes to the superior salt tolerance of halophytes.
Salt stress impacts multiple aspects of plant metabolism and physiology. For instance it inhibits photosynthesis through stomatal limitation, causes excessive accumulation of sodium and chloride in chloroplasts and disturbs chloroplast potassium homeostasis. Most research on salt stress has focused primarily on cytosolic ion homeostasis with few studies studying how salt stress affects chloroplast ion homeostasis. This review asks the question whether membrane-transport processes and ionic relations are differentially regulated between glycophyte and halophyte chloroplasts and whether this contributes to the superior salt tolerance of halophytes. The available literature indicates that halophytes can overcome stomatal limitation by switching to CO$_2$ concentrating mechanisms and increasing the number of chloroplasts per cell under saline conditions. Furthermore, salt entry into the chloroplast stroma may be critical for grana formation and PSII activity in halophytes but not in glycophytes. Salt also inhibits some stromal enzymes (e.g. fructose-1,6-bisphosphatase) to a lesser extent in halophyte species. Halophytes accumulate more chloride in chloroplasts than glycophytes and appear to use sodium in functional roles. We propose the molecular identities of candidate transporters that move sodium, chloride and potassium across chloroplast membranes and discuss how their operation may regulate photochemistry and PSI and II activity in chloroplasts.

**Key words:** Charge balance, chloride, CO$_2$ fixation, electron transport, ion homeostasis, photosynthesis, photosynthetic enzymes, potassium, proton motive force, reactive oxygen species, sodium.
Introduction

Salt stress reduces plant productivity partly through its inhibition of photosynthesis. Reduced CO$_2$ uptake due to stomatal closure (stomatal limitation) at the early stages of salt stress has been suggested to be the primary cause of poor photosynthetic performance (Chaves et al., 2009). However, increasing external CO$_2$ concentration to very high levels in an attempt to overcome stomatal limitation did not lead to increase in photosynthetic rate in salt-stressed plants indicating that there is a non-stomatal component to salt-induced decreases in photosynthesis (Cheeseman, 2013). This non-stomatal limitation has been proposed to originate from: 1) excessive accumulation of Na$^+$ and Cl$^-$, and deficiency of K$^+$, inside the cytosol; and, 2) increased reactive oxygen species (ROS) production (Munns and Tester, 2008; Bose et al., 2014a). Until now, most research on salt stress has focused primarily on cytosolic ion homeostasis, with little attention paid to the question of how salt stress affects chloroplast ion and volume homeostasis and whether membrane-transport processes are differentially regulated between glycophyte and halophyte chloroplasts. This is the main focus of this review.

Chloroplasts are the specialised plant organelles responsible for photosynthesis; they harvest light energy to make ATP, fix atmospheric CO$_2$ into sugars and produce oxygen. Photosynthesis underpins plant growth and crop yield, but chloroplasts also carry out other essential functions such as amino acid synthesis, fatty acid synthesis, and stress signalling in plants (Chan et al., 2016). The coordinated trafficking of ions across the three chloroplast membrane systems (Fig. 1) – the outer- and inner-envelope membranes and the thylakoid membrane – is essential for creating an optimal environment for these processes. This trafficking includes the establishment of ionic gradients within each chloroplast compartment (e.g. the stroma and thylakoid lumen) (Finazzi et al., 2015; Pottosin and Shabala, 2016). In the light, a pH gradient between the stroma (pH 8) and thylakoid lumen ($\approx$ pH 6) facilitates ATP synthesis and ultimately CO$_2$ fixation (Mitchell, 1966; Werdan et al., 1975; Kramer et al., 2003).

Maintenance of optimal potassium (K$^+$) and chloride (Cl$^-$) concentrations within the chloroplast is pivotal for pH regulation, volume regulation, thylakoid stacking, electron transport properties, and eventual photosynthetic efficiency (Hind et al., 1974; Barber, 1982; Beebo et al., 2013; Finazzi et al., 2015; Pottosin and Shabala, 2016). Salt stress increases Na$^+$
and Cl⁻ concentrations and decreases K⁺ concentration inside chloroplasts (Robinson and Downton, 1984; Robinson and Downton, 1985), and imposes osmotic, ionic and oxidative (ROS) stresses (Munns and Tester, 2008; Flowers et al., 2015). All these components of salt stress ultimately upset the ionic balance within chloroplasts, resulting in poor photosynthetic performance and reduced growth and yield. However, the specific chloroplast Na⁺, Cl⁻ and K⁺ transporters that affect these ion concentrations during salt stress have not been identified.

Land plants differ dramatically in their sensitivity to salinity and are divided into two major groups: halophytes and glycophytes (Flowers et al., 2015). For glycophytes, an external salt concentration of 0.5 % (≈86 mM NaCl) has been taken as a general limit above which plant yield is severely reduced (Chapman, 1942), while for halophytes, yield penalties are usually only observed at external concentrations exceeding 200 mM NaCl (Flowers and Colmer, 2008; Santos et al., 2016). External salt concentrations (40–100 mM NaCl) severely inhibit photosynthesis in glycophytes (Munns and Tester, 2008), while many halophytes maintain or have enhanced photosynthesis at these salt concentrations (Stepien and Johnson, 2009; Flowers et al., 2015). The superior ability of halophytes to maintain photosynthesis during salt stress may be attributed to halophytes’ capacity to overcome stomatal and non-stomatal limitations (Shabala, 2013). Ion homeostasis and functioning of chloroplasts is dependent upon ionic status of the cytosol. It could be assumed that halophytes are superior in cytoplasmic salt exclusion via vacuolar sequestration, leading to lower cytoplasmic salt concentrations in halophytes than glycophytes (Shabala, 2013), but there are no data on this. A meta-analysis suggested that the leaf mesophyll cell cytoplasm of halophytes (largely occupied by chloroplasts) contains 100–200 mM NaCl (Flowers et al., 2015), and isolated halophyte chloroplasts exposed to high salt (100 mM NaCl) and low K (50 mM KCl), were better able to maintain photosynthesis than glycophyte chloroplasts (Percey et al., 2016). Therefore, it is possible that the ability of halophyte chloroplasts to regulate Na⁺, Cl⁻ and K⁺ transport differentially to glycophytes may be an important attribute contributing to the superior salt tolerance of halophytes.

Here we analyse how ion transport processes and photosynthesis are regulated in halophyte and glycophyte chloroplasts. Special attention has been given to addressing the following questions: (1) What is the role of Na⁺, Cl⁻ and K⁺ ions in chloroplast function? (2) How are Na⁺, Cl⁻ and K⁺ transported in or out of the chloroplast during salt stress?; and, (3) Where do the potential differences lie between glycophyte and halophyte chloroplast ion transport mechanisms?
Halophytes overcome stomatal limitation by switching to CO$_2$ concentrating mechanisms

Salt stress decreases stomatal conductance thereby reducing the CO$_2$ available for photosynthesis. This stomatal limitation increases the rate of photorespiration in C3 plants. There is some evidence to suggest that photorespiration may be a positive component of salinity tolerance in C3 plants by acting as an energy sink preventing the collapse of the photosynthetic electron transport chain that occurs via photoinhibition (Wingler et al., 2000). However, a comparison between the glycophyte Arabidopsis thaliana and the halophyte Eutrema salsugineum revealed that photorespiration is not a strategy adopted by either plant to enhance salt tolerance (Stepien and Johnson, 2009). Despite this there are halophytes (e.g. Salvadora persica, Arthrocnemum macrostachyum, Sarcocornia fruticosa) that increase photorespiration under high (>500 mM NaCl) salt concentrations causing a growth reduction (Redondo-Gómez et al., 2006; Redondo-Gómez et al., 2010; Rangani et al., 2016). From these observations, one can speculate that increased photorespiration may protect the systems but still imposes growth penalties because of the net loss of CO$_2$. On the other hand, CAM (Crassulacean Acid Metabolism) and C4 plants tend to be able to deal with stomatal limitation and photorespiration better by employing CO$_2$ concentrating mechanisms. Perhaps as a result, CAM and C4 plants are generally more salt tolerant than C3 plants (Stepien and Klobus, 2005; Vasquez et al., 2006). There is evidence suggesting that halophytes are able to employ CO$_2$ concentrating mechanisms during salt stress (Bose et al., 2014a). For instance, Portulacaria afra and Mesembryanthemum crystallinum are able to switch from C3 photosynthesis into CAM photosynthesis upon salt treatment (Ting and Hanscom, 1977; Cushman et al., 1990). Similarly, other halophytes such as Aeluropus littoralis, Salicornia europaea and Puccinellia nuttalliana switch from C3 to C4 photosynthesis during salt stress (Shomer-Ilan and Waisel, 1973; Guy et al., 1980). Interestingly, the operation of C4 photosynthesis within a single cell through biological sorting of chloroplasts that mimics mesophyll and bundle sheath cells has also been discovered in halophyte species such as Bienertia cycloptera, Bienertia sinuspersici, and Suaeda aralocaspica (Chuong et al., 2006; von Caemmerer et al., 2014). Further, C4 species represent about ≈3% of higher plants (Sage et al., 2012), but are over represented by halophytes, which comprise 30–45%. This suggests that saline environments apply selection pressure favouring C4 photosynthesis (Feldman et al., 2008; Bromham and Bennett, 2014; Santos et al., 2016).
Salt tolerance of CO$_2$ fixing enzymes

Are the enzymes involved in CO$_2$ assimilation in halophytes more tolerant of Na$^+$ and Cl$^-$ than glycophytes? If this were the case, it would imply that ion concentrations within the stroma could be higher in halophytes than glycophytes, and protein structure could be modified accordingly. If photosynthetic enzymes from halophytes and glycophytes are equally sensitive, then it is likely that ion concentrations in the stroma are similar in halophytes and glycophytes. Few studies have measured the sensitivity \textit{in vitro} of photosynthetic enzymes to NaCl, and fewer still have compared sensitivity of enzymes extracted from halophytes to those from glycophytes. Three enzymes have been studied in some detail.

Chloroplastic fructose-1,6-bisphosphatase (FruP$_2$ase) purified from rice (\textit{Oryza sativa}) was more sensitive to NaCl \textit{in vitro} than that from the close halophytic relative \textit{Porteresia coarctata} (Ghosh et al., 2001). The enzyme from the salt-sensitive rice cultivar IR26 was 50\% inhibited by 25 mM NaCl, while the equivalent enzyme from \textit{P. coarctata} was only inhibited by 10\% over a broad NaCl range from 25 to 400 mM. Addition of 20 mM of osmolytes, particularly mannitol, inositol and proline (betaine was not tested) relieved any inhibition up to 400 mM NaCl in IR26. The FruP$_2$ase enzymes from rice differs from \textit{P. coarctata} by only five amino acid residues: Glu$^{14}$, Thr$^{24}$, Ala$^{48}$, Ala$^{163}$ and Arg$^{296}$ of rice have been replaced by Ser$^{14}$, Ile$^{24}$, Ser$^{48}$, Ser$^{163}$ and Lys$^{296}$ in \textit{P. coarctata} (Chatterjee et al., 2013). Bacterial expression of the recombinant protein showed that FruP$_2$ase from \textit{P. coarctata} was not inhibited by even 500 mM NaCl but the activity of that from cultivated rice declined linearly over this range (Chatterjee et al., 2013). It remains to be tested whether all these substitutions are important for improved salt tolerance.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) from glycophytes does not seem as salt sensitive as FruP$_2$ase. In bean (\textit{Phaseolus vulgaris}) and maize (\textit{Zea mays}), Rubisco activity was reduced below 50\% only at salt concentrations over 250 mM NaCl, similar to that for the halophyte \textit{Atriplex spongiosa} (Osmond and Greenway, 1972). Likewise, Rubisco from the halophyte \textit{Tamarix jordanis} as well as seagrasses (\textit{Halophyla stipulacea}e and \textit{Halodule uninervis}) was 50\% inhibited by 200 mM NaCl (Beer et al., 1980; Solomon et al., 1994). Addition of compatible solutes reduced the inhibition for the \textit{T. jordanis} Rubisco (Solomon et al., 1994). Rubisco from the halophytic cyanobacterium \textit{Aphanothece halophytica} was 50\% inhibited at 200 mM KCl (and one would presume NaCl
as well) but the inhibition could be completely abolished by the addition of compatible solutes such as betaine (Incharoensakdi et al., 1986). Given that synthesis of compatible solutes are higher in halophytes than glycophytes (Bose et al., 2014a), it is reasonable to assume that Rubisco activity may not be inhibited in halophytes. Furthermore, some halophytes (e.g. Porteresia coarctata, Eutrema halophila, Suaeda salsa) showed a higher Rubisco activation state than in glycophytes by (1) increasing the amount of Rubisco activase, and (2) subsequent attachment of Rubisco activase to the thylakoid membrane (Sengupta and Majumder, 2009; Li et al., 2011; Wiciarz et al., 2015). The later step ensures restoration of the Rubisco active form in the close proximity of photosystem I (PSI), which has been suggested to improve CO₂ fixation by Rubisco in an ATP limiting environment (Chen et al., 2010).

Phosphoenolpyruvate carboxylase (PEPC) is the enzyme responsible for the initial fixation of CO₂ in C₄ photosynthesis and is located in the cytosol of mesophyll cells. It is more sensitive to salt in vitro than Rubisco (Osmond and Greenway, 1972; Manetas et al., 1986) consistent with a lower salt concentration in the cytosol than in the chloroplasts. PEPC extracted from leaves of Atriplex spongiosa was inhibited by 50% in 50 mM NaCl and was more sensitive than PEPC from maize (Osmond and Greenway, 1972). Proline and betaine influenced the in vitro sensitivity to salt of four C₄ halophytes but in different ways (Manetas, 1990). For instance, betaine protected PEPC against NaCl in two Salsola species while proline was inhibitory, whereas proline was protective to PEPC from Cynodon dactylon (Manetas et al., 1986). Complex interactions take place between the nature and the concentrations of the enzyme, compatible solutes, PEP, and ionic strength of the assay medium. Optimising all these factors showed that inhibition of PEPC from Salsola soda by 100 mM NaCl could be partly overcome by the addition of betaine, glycerol and glucose-6-phosphate up to 0.6 M (Manetas, 1990).

It is difficult to make generalisations about the different responses of enzymes from halophytes versus glycophytes from such sparse information, and also to draw conclusions about sensitivity of enzymes in intact plants from experiments done in vitro. There is good evidence that chloroplastic FruP₂ase from a halophytic rice relative is more salt-tolerant than in cultivated rice, implying the chloroplasts of the halophyte rice can tolerate higher NaCl concentrations in the stroma, so studies with other close relatives are warranted. However, there is not sufficient evidence for the differential sensitivity of other photosynthetic enzymes available in the literature to draw a broad conclusion.
Halophytes maintain optimal Photosystem II and I activity during salt stress

In the thylakoid membrane, photosystem II complexes (PSII) are physically separated from those of PSI to avoid spontaneous energy spill over (Anderson, 1981). PSII is enriched in appressed regions of thylakoid stacks (grana) whereas PSI is enriched in non-appressed regions (stroma lamellae) of the thylakoid membrane (Fig. 1; Anderson, 1981; Caffarri et al., 2014). The ionic composition of the stroma is critical for grana formation (Barber, 1982). Entry of Na\(^+\) and Cl\(^-\) into the stroma and excessive ROS production during salt stress would alter stromal ionic composition, which in turn could affect grana stacking. Indeed, in C3 glycophytes such as rice and Arabidopsis, salt stress caused unstacking of grana resulting in poor PSII activity (Rahman et al., 2000; Peharec-Štefanić et al., 2013). By contrast, in Arthrocnemum macrostachyum (a C3 halophyte), no salt in the external medium caused grana unstacking and poor PSI activity but adding salt up to 1 M enhanced grana formation and PSII activity (Redondo-Gómez et al., 2010; Trotta et al., 2012). These observations indicate that salt entry into the stroma may be critical for grana formation and PSII activity in halophytes but not in glycophytes.

C4 plants have dimorphic chloroplasts; the chloroplast ultrastructure as well as PSI and PSII activities are different between mesophyll and bundle sheath cells and also between dimorphic chloroplasts in single cells in some species (Offermann et al., 2011). C4 plants are divided into three basic biochemical subtypes based on their primary decarboxylating enzymes, Nicotinamide Adenine Dinucleotide Phosphate-dependent Malic Enzyme (NADP-ME), NAD-dependent Malic Enzyme (NAD-ME), and Phosphoenolpyruvate Carboxy-Kinase (PCK). The chloroplasts in the bundle sheath cells of NADP-ME subtype plants are deficient in grana and PSII activity whereas the NAD-ME subtype plants are enriched with granal chloroplasts and PSI activity in these cells. Furthermore, in the NADP-ME subtype, the mesophyll cells are dominated by granal chloroplasts containing both PSII and PSI. The NAD-ME subtype mesophyll cells are low in grana chloroplasts and are rich in PSI activity (Voznesenskaya et al., 1999; Omoto et al., 2009; Omoto et al., 2010). In glycophyte plants of the NADP-ME subtype, such as maize, salt stress causes grana unstacking in mesophyll cells (similar to C3 mesophyll cells) and induces grana formation in bundle sheath cells (Hasan et al., 2006). Interestingly, in halophytes of the same NADP-ME subtype, the grana are constitutively present in bundle sheath cells and grana unstacking is absent in mesophyll cells (Voznesenskaya et al., 1999; Omoto et al., 2009). Similarly, within the NAD-ME subtype, glycophytes show grana unstacking and an increase in grana formation during salt stress but...
such effects are absent in halophytes (Voznesenskaya et al., 1999; Omoto et al., 2010). From these observations, it appears that; (1) having high grana chloroplasts constitutively in the bundle sheath cells is advantageous during salt stress, and (2) the bundle sheath cells could be more tolerant to salt stress than mesophyll cells. This later notion is supported by a comparison of ROS production between bundle sheath and mesophyll cells of maize during salt stress in which ROS accumulation is higher in mesophyll cells than bundle sheath cells (Omoto et al., 2013). Given that bundle sheath cells are adjacent to the xylem, one could assume higher salt concentrations in these than in mesophyll cells. However, there is no information on how ion transport is regulated between these two cell types.

PSI could play a vital role during salt tolerance by increasing cyclic electron flow generating ATP while avoiding the build-up of toxic reducing species (Munekage et al., 2004; Johnson, 2011). The excess ATP generated though cyclic electron flow around PSI has been suggested to prevent Na$^+$ over accumulation into the chloroplasts of soybean (He et al., 2015). Salt stress has been shown to enhance PSI content and cyclic electron flow in a halotolerant cyanobacterium (Aphanothece halophytica) (Hibino et al., 1996). A proteomic comparison of cultivated rice with its halophytic wild relative (Porteresia coarctata) revealed that PSI reaction centre subunit IV protein (PsaE) was upregulated in halophyte rice (Sengupta and Majumder, 2009). This PsaE is a stromal subunit essential for cyclic electron transport by facilitating (1) the binding of ferredoxin to PSI, and (2) the cross-linking of ferredoxin-NADP$^+$ oxidoreductase (FNR) (Chitnis et al., 1995). Interestingly, maintenance of thylakoid surface charge (through ion binding or protonation of amino acid residues) is key to both the aforementioned processes (Lushy et al., 2002; Matthijs et al., 2002). We have shown that thylakoid surface charge balancing around PSI is superior in halophyte (Chenopodium quinoa) chloroplasts in comparison with glycophytes: Pisum sativum and Vicia faba (Percey et al., 2016). These observations suggest that halophyte chloroplasts are able to maintain higher PSI activity compared to glycophytes. Future research should focus on identifying how halophyte chloroplasts maintain thylakoid surface charge during salt stress.

**Number of chloroplasts and salt tolerance: Energy supply and salt compartmentalisation**

In leaf mesophyll cells, more than half of the volume of the cytoplasm is occupied by chloroplasts (Winter et al., 1993). The number of chloroplasts per leaf cell is not definite and
can vary from few to hundreds (Kubínová et al., 2014). Interestingly, Na$^+$ has been shown to participate in chloroplast multiplication in salt tolerant plants (spinach and sugar beet) (Marschner and Possingham, 1975). This could suggest that halophytes have greater capacity to increase chloroplast numbers than glycophytes. Indeed, under salt stress halophytes [e.g. *Eutrema* (Wang et al., 2013b), sugar beet (Marschner and Possingham, 1975)] are able to increase the number of chloroplasts per cell (Fig. 2) compared to many glycophytes [e.g. wheat (Aldesuquy et al., 2014), rice (Flowers et al., 1985), *Arabidopsis* (Peharec-Štefanić et al., 2013)]. This difference in chloroplast number suggests that halophytes may have either; (1) increased succulence resulting in bigger cells containing more chloroplasts, or (2) a greater demand for energy to support salt tolerance mechanisms such as ion pumping and osmotic adjustment, or (3) chloroplasts have become less efficient because of salt accumulation and therefore the cells require additional chloroplasts to maintain energy supply. These mechanisms are not mutually exclusive. On the other hand, chloroplasts of halophytes can store significant quantities of Na$^+$ and Cl$^-$ (Fig. 3) without compromising photosynthesis (Flowers et al., 2015). Thus chloroplasts can form a significant component of salt compartmentalisation that may ultimately translate into higher tissue tolerance in halophytes than glycophytes.

Chloroplasts multiply by binary fission and the genes involved in such chloroplast division (e.g. *Multiple Chloroplast Division* site 1, MCD1; *Accumulation and Replication of Chloroplasts* 3, RC5, ARC6; *Paralog of ARC6, Plastid Division* 1, PDV2; *Filamentous Temperature Sensitive Z1, FTSZ2*) are well characterised (Yoshida et al., 2012; Jarvis and López-Juez, 2013; Osteryoung and Pyke, 2014). It would be interesting to examine how these chloroplast division genes are modulated in glycophyte and halophyte model plants during salt stress.

**Operation of Na$^+$, Cl$^-$ and K$^+$ transporters across chloroplast membranes are key to membrane potential maintenance, pH and osmo-regulation**

The light-driven water-splitting reaction in the lumen and subsequent generation of proton motive force (PMF) across the thylakoid membranes of plants is one of the most important bioenergetic process on Earth. The PMF has two components namely proton gradient ($\Delta$pH) and transmembrane electric potential difference ($\Delta\psi$) (Kramer et al., 2003). The $\Delta\psi$ component determines the turnover rate of H$^+$-ATP synthase and transmembrane electron transport (Fischer and Gräber, 1999). High $\Delta\psi$ will favour photo-damage through the formation of singlet oxygen from the P680 triplet state of photosystem II (Bennoun, 1994;
For optimal CO₂ fixation, the pH of the stroma should be maintained around pH 8 (Werdan et al., 1975) and hence the extent of lumen acidification defines the magnitude of the ΔpH component. The lumen acidification must also be limited to around pH 6 in order to maintain electron transport from the cytochrome b6f complex to photosystem I (PSI), non-photochemical quenching (NPQ), and to prevent loss of Ca²⁺ from water-splitting complex and loss of Cu²⁺ from plastocyanin (Kramer et al., 2003; Niyogi et al., 2005). Thus, a fine balance between Δψ and ΔpH is pivotal for optimal energy transduction and CO₂ fixation during photosynthesis.

The ion channels and transporters located in the thylakoid membrane play a role in PMF partitioning between Δψ and ΔpH components. The efflux of Cl⁻ from the stroma to the lumen and K⁺ influx from the lumen to stroma have been demonstrated to be the major counter ion fluxes balancing light-driven H⁺ efflux into the lumen thereby maintaining the Δψ component of PMF while allowing luminal acidification (Kramer et al., 2003; Pottosin and Shabala, 2016).

On the other hand, the uncoupling activity of electroneutral K⁺ (Na⁺)/H⁺ exchangers may dissipate ΔpH with a compensatory increase in Δψ thereby avoiding excessive luminal acidification (Pottosin and Shabala, 2016). Apart from membrane potential and pH regulation, Cl⁻, Na⁺ and K⁺ ions are also essential for chloroplast volume regulation in conjunction with aquaporins (Beebo et al., 2013). Indeed, Cl⁻ influx from the stroma to lumen is essential for thylakoid swelling. Conversely, K⁺ (or Na⁺) efflux from the lumen to stroma would cause the lumen to shrink (Pottosin and Dobrovinskaya, 2015).

Involvement of chloroplast K⁺ transporters in PMF partitioning, pH and volume regulation is starting to emerge (Kunz et al., 2014). In Arabidopsis, a thylakoid membrane localised tandem-pore K⁺-channel (TPK3) involved in K⁺ efflux from the lumen (Fig. 1) has been shown to affect PMF partitioning by affecting the ΔpH component of PMF. The TPK3 silenced plants showed a decrease in non-photochemical quenching (NPQ) due to reduced ΔpH between the stroma and lumen (Carraretto et al., 2013). Preliminary characterisation of TPK3 suggested that this channel is not perfectly selective for K⁺ over Cl⁻ (Carraretto et al., 2013) and its relative selectivity for other ions (especially Na⁺) and regulatory mechanisms (pH or Ca²⁺) are not established. By contrast to TPK3, loss-of-function of a thylakoid membrane localised K⁺/H⁺ exchanger (KEA3) involved in K⁺ influx into the lumen from stroma (Fig. 1) increased the ΔpH component of PMF (Finazzi et al., 2015). Intriguingly, even inner envelope localised K⁺ /H⁺ exchangers (KEA1 and KEA2) increased the ΔpH
component of PMF when knocked out (Kunz et al., 2014). Salt stress will affect K+ homeostasis of chloroplasts (e.g. Robinson et al., 1983; Robinson and Downton, 1984), hence disturbance of the ΔpH component of PMF is expected. Characterisation of TPK3, KEA1, KEA2 and KEA3 transporters (Table. 1) during salt stress in representative halophyte and glycophyte species will pave the way to dissect how excessive Na+ and Cl− affect PMF partitioning in these species.

**Halophyte chloroplasts use sodium in functional roles and have transport mechanisms to regulate sodium concentrations.**

In general, halophytes are considered as salt includers compared with glycophytes. Participating in osmotic adjustment has been suggested as the main function of Na+ in halophytes (Shabala, 2013). However, a comparison of Na+ concentrations within glycophyte and halophyte chloroplasts grown under low salt (≤ 1 mM Na+) conditions (Fig. 3) revealed that halophytes can preferentially accumulate more than 20-fold higher Na+ than glycophytes (Robinson and Downton, 1984; Robinson and Downton, 1985; Cosentino et al., 2010; Müller et al., 2014). This observation suggests chloroplasts of halophytes require high Na+ and that Na+ may participate in other essential functions in chloroplasts. Indeed, Na+ plays essential roles in chloroplast functioning of all CAM plants and several C4 plant species, if not all (Subbarao et al., 2003). For example, Na+ deficiency (<0.1 µM Na+ in the growth media) caused: (1) chlorosis in Atriplex vesicaria (a C4 halophyte), even though leaves had a high amount of K+ (Brownell and Wood, 1957; Brownell, 1965), (2) a reduction in PSII activity and modification in ultrastructure of mesophyll chloroplasts but not bundle sheath chloroplasts of two C4 halophytes species Amaranthus tricolor and Kochia childsii (Grof et al., 1989; Johnston et al., 1989) and, (3) alanine and pyruvate (C3 metabolites) to accumulate and PEP, malate, and aspartate (C4 metabolites) to decrease in A. tricolor (Johnston et al., 1988). Resupplying Na+ (0.1 to 1 mM) reversed the aforementioned effects (Brownell, 1965; Johnston et al., 1988; Grof et al., 1989). The Na+ ion also participates in the transport of pyruvate (Furumoto et al., 2011; Zhao et al., 2016), ascorbate (Miyaji et al., 2015) and phosphate (Guo et al., 2008) into chloroplasts. The molecular identity of these Na+-dependent transporters is starting to emerge (Fig. 1, Table. 1), but how these Na+-dependent transporters (e.g. BASS2- bile acid: sodium symporter2, phosphate transporters-PHT4;1, PHT4;4; PHT4;5) are regulated during salt stress needs to be established because salt in the growth media would alter the root to shoot Na+ gradient. Also, glycophytes and halophytes may differ in their ability to use Na+ in their transport processes. For example, the
The majority of the C4 halophytes are the NAD-ME type and pyruvate import to the chloroplast is Na⁺-dependent, whereas C4 glycophytes (e.g. sugarcane, maize) are primarily NADH-ME type and have H⁺-dependent pyruvate import (Aoki et al., 1992).

Elevated Na⁺ concentrations in chloroplasts are associated with enhanced photosynthetic performance of halophytes such as *Mesembryanthemum crystallinum*, and *Suaeda maritima* (Hajibagheri et al., 1984; Stepien and Johnson, 2009; Cosentino et al., 2010; Flowers et al., 2015). In contrast, a slight increase in chloroplast Na⁺ (2.1–3.8 mM Na⁺) in *Arabidopsis* (a glycophyte) correlated with a significant reduction in photosynthetic efficiency (Müller et al., 2014). Furthermore, halophytes appear to possess a mechanism to prevent Na⁺ concentrations from exceeding an upper threshold, because at higher salinities (300 mM or more in the growth media) can lead to high leaf Na⁺ concentrations (494–683 mM), but halophyte chloroplast Na⁺ concentrations are kept much lower (103–234 mM) (Flowers et al., 2015). From these observations, it can be hypothesised that chloroplasts of halophytes have Na⁺ transporters that preferentially take up Na⁺ under growth conditions with low external salt concentrations in the soil yet prevent excessive Na⁺ accumulation during high salt loads. The types of transporters that could mediate this response and their molecular identities are discussed further below.

**Maintenance of chloroplast K⁺ homeostasis is pivotal for photosynthesis.**

Potassium is the major counter ion balancing light-driven H⁺ influx into the lumen (Finazzi et al., 2015; Pottosin and Shabala, 2016) and is essential for chloroplast development, ultrastructure, light utilisation and volume regulation (Haswell and Meyerowitz, 2006; Carraretto et al., 2013; Kunz et al., 2014; Aranda-Sicilia et al., 2016). Despite this, a general assumption has been that in halophyte chloroplasts K⁺ can be replaced by Na⁺ without any deleterious effect on photosynthesis (Subbarao et al., 2003). This may not be the case because; (1) treating leaf segments of bean, barley and sugar beet with 25 mM NaCl for several hours induced greater K⁺ efflux from bean chloroplasts than barley but no K⁺ efflux was observed from sugar beet chloroplasts even though a strong influx of Na⁺ was evident (Marschner and Mix, 1973), (2) halophytes have been shown to retain at least 20 % more K⁺ in chloroplasts compared with the leaf sap (Flowers et al., 2015), and (4) K⁺ cannot be replaced by Na⁺ in chlorophyll synthesis (Knyp and Chylinska, 1972; Marschner and Possingham, 1975) and Rubisco synthesis (Hanikenne et al., 2014). Hence, it appears that optimal K⁺/Na⁺ ratio must be maintained within the chloroplasts to maintain photosynthesis.
Salt stress can induce cell death by depleting cytosolic K\(^+\) beyond a threshold level. Thus, under saline environments, maintaining optimal K\(^+\)/Na\(^+\) ratio inside the cytosol is critical for normal functioning of cytoplasm (Bose et al., 2015; Shabala et al., 2015). Chloroplasts being in the cytoplasmic compartment are influenced by K\(^+\)/Na\(^+\) ratio in the cytoplasm. The $F_o/F_m$ ratio and the normalised fluorescence transients ($F_t$; a measure for charge balancing between PSII and PSI) measurements involving isolated chloroplasts revealed that the halophyte (Chenopodium quinoa and Carpobrotus rossii) chloroplasts were able to tolerate high Na\(^+\) (100 mM Na\(^+\)) and low K\(^+\) (50 mM K\(^+\)) in the cytosol better than the glycophyte (Pisum sativum and Vicia faba) counterparts (Percey et al., 2016). Analogous to the chloroplast’s dependence on cytosolic concentrations, oxygen evolution from isolated halophytic (Avicennia marina and Sarcocornia quinqueflora) thylakoids had a preference for high Na\(^+\) and low K\(^+\) in the stroma, but such a preference was absent in glycophytic (Spinacia oleracea) thylakoids (Preston and Critchley, 1986). From these observations, it appears that chloroplasts of halophytes have machinery to operate in high Na\(^+\) and low K\(^+\) environments and research should focus on identifying and introducing similar mechanisms in glycophyte species.

Under optimal conditions, chloroplast K\(^+\) concentration is \(\approx 90–200\) mM with the average chloroplast volume of 25 µl per mg Chl (Fig. 3). Similar to the cytoplasm, salt stress induces K\(^+\) loss from chloroplasts of glycophytes and halophytes (Wignarajah and Baker, 1981; Robinson et al., 1983; Hajibagheri et al., 1984; Robinson and Downton, 1984; Robinson and Downton, 1985; Demmig and Winter, 1986). However, no information is available regarding; (1) the threshold K\(^+\) concentration below which chloroplast function is disrupted; (2) differences in salt-induced K\(^+\) loss between glycophyte and halophyte chloroplasts; and (3) the molecular identity of the transporters mediating this K\(^+\) loss. Future research should focus on providing this missing information. Since the aforementioned phenomena also occurs at the cellular level when exposed to high NaCl concentration (e.g. Bose et al., 2015), one can suggest plasma membrane and chloroplast envelope membranes may be under similar constraints arising from how the transporters respond to Na\(^+\) to regulate K\(^+\) homeostasis within the respective compartments.

**Halophytes and glycophytes differ markedly in Cl\(^-\) homeostasis within chloroplasts.**

 Preferential accumulation of Cl\(^-\) within chloroplasts, even if plants are grown in Cl\(^-\) deficient media, suggests that Cl\(^-\) is pivotal for normal chloroplast function (Robinson and Downton, 1984). Indeed, Cl\(^-\) is a co-factor in the PSII complex, can act as a counter anion to

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stabilise chloroplast envelope and thylakoid membrane potentials, and is involved in volume and pH regulation (Pottosin and Dobrovinskaya, 2015; Herdean et al., 2016b; Pottosin and Shabala, 2016). Although excessive Cl\(^{-}\) in chloroplasts during salt stress affects the photosynthetic performance of many crop species (Teakle and Tyerman, 2010), some halophytes (e.g. Limonium vulgare, Avicennia marina) require high concentrations of Cl\(^{-}\) in chloroplasts to enhance electron transport and oxygen evolution during salt stress (Critchley, 1982; Preston and Pace, 1985; Preston and Critchley, 1986). Closer examination reveals that halophytes accumulate more Cl\(^{-}\) than glycophytes under low salt (\(\leq 1\) mM Cl\(^{-}\)) conditions (Fig. 3); while at higher salinities some halophytes maintain steady Cl\(^{-}\) concentrations and others show a slight increase within the chloroplasts irrespective of external Cl\(^{-}\) (Larkum, 1968; Robinson and Downton, 1985; Demmig and Winter, 1986; Wang et al., 2007; Flowers et al., 2015). This indicates that chloroplasts of halophytes have mechanisms to regulate Cl\(^{-}\) concentrations. However, the candidate transporters that regulate Cl\(^{-}\) during salt stress remain uncharacterised.

An interesting difference between halophytes and glycophytes may lie in the ability of chloroplasts to sense and incorporate Cl\(^{-}\) ions into the PSII oxygen evolving Mn-Ca-Cl complex. The PSII (Psb) complex has three extrinsic proteins namely, PsbO (33 kDa), PsbP (23 kDa), and PsbQ (17 kDa). Of these, PsbP and PsbQ proteins have high affinity Cl\(^{-}\) binding sites (e.g. His144 along with Arg48, Lys143, and Lys160 in PsbP), consequently increasing the binding affinity of Cl\(^{-}\) of the Mn-Ca-Cl complex (Seidler, 1996; Kakiuchi et al., 2012; Nishimura et al., 2014). In vitro studies have demonstrated that in the presence of 30 mM Cl\(^{-}\) or higher, these extrinsic proteins are dispensable from the Mn-Ca-Cl complex without compromising maximal activity (Seidler, 1996). A comparison of PSII complexes between halophytes (Salicornia veneta, S. emerici, Arthrocnemum macrostachyum, Halocnemum strobilaceum, Eutrema salsugineum) and glycophytes (Spinacia oleracea, Arabidopsis thaliana) revealed that the PsbQ protein is completely absent from halophytes and the PsbP protein levels decreased in halophytes without the loss of functional activity of Mn-Ca-Cl complex whereas this occurs in glycophytes (Gong et al., 2005; Pagliano et al., 2009; Trotta et al., 2012). This suggests that halophyte chloroplasts use high Cl\(^{-}\) to drive PSII activity. On the other hand, glycophytes use Cl\(^{-}\) enrichment by the Mn-Ca-Cl complex thorough PsbQ and PsbP. Salt stress would saturate the Cl\(^{-}\) binding of PSII, which may lead to higher ROS generation and photoinhibition in glycophytes compared to halophytes. This
hypothesis must be validated by measuring light-saturated oxygen evolution rate, Fv/Fm ratio and ROS generation in glycophyte and halophyte chloroplasts during high Cl\textsuperscript{–} stress.

**Excess ROS production in chloroplasts during salt stress is well established, but its effect on ion homeostasis and retrograde signaling within and by this organelle has never been examined.**

Salt stress increases ROS production (Bose et al., 2014a), with chloroplasts and peroxisomes producing 20-fold more ROS than mitochondria during the day (Wrzaczek et al., 2013). The major ROS produced in chloroplasts are hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), superoxide radical (O\textsubscript{2}\textsuperscript{•}), hydroxyl radical (\textsuperscript{•}OH) and singlet oxygen (\textsuperscript{1}O\textsubscript{2}) (Bose et al., 2014a). Salt-stress-induced stimulation of electron transport has been observed in some halophytes (Critchley, 1982), including *Eutrema* (Stepien and Johnson, 2009), and often these excess electrons have been observed to be dissipated by alternative electron sinks such as plastid terminal oxidase (PTOX) (Stepien and Johnson, 2009; Uzilday et al., 2015). Inhibition of PTOX increased the H\textsubscript{2}O\textsubscript{2} levels inside thylakoids of *Eutrema* (Wiciarz et al., 2015), suggesting alternate oxidase acts as a safety valve controlling ROS production. However, a direct comparison of chloroplast-derived H\textsubscript{2}O\textsubscript{2} between Arabidopsis and Eutrema revealed that isolated thylakoids of *Eutrema* produced higher H\textsubscript{2}O\textsubscript{2} in control media grown plants. Salt stress (100 mM NaCl for Arabidopsis; 300 mM NaCl for Eutrema in growth media) significantly increased H\textsubscript{2}O\textsubscript{2} production in Arabidopsis but not in Eutrema, suggesting that halophyte species have efficient mechanisms to control excessive H\textsubscript{2}O\textsubscript{2} levels during salt stress (Wiciarz et al., 2015). Another interesting difference between glycophytes and halophytes comes from the kinetics of H\textsubscript{2}O\textsubscript{2} accumulation that appears to be much faster in halophytes. For example, the salt-induced H\textsubscript{2}O\textsubscript{2} accumulation peaked at 4 h upon salt (400 mM) stress onset in the leaves of a halophyte *Cakile maritime* and declined rapidly afterwards. However, in a glycophyte Arabidopsis, H\textsubscript{2}O\textsubscript{2} continued to accumulate even after 72 hours in 100 mM NaCl (Ellouzi et al., 2011). Similarly, salt-stress induced H\textsubscript{2}O\textsubscript{2} production was higher in the leaves of a halophyte (*Populus euphratica* Oliv.) in comparison with the leaves of a glycophyte (*P. popularis*) for up to 24 h of salinity treatment but the opposite was true during long-term salt exposure (Ding et al., 2010). This suggests halophytes may use elevated H\textsubscript{2}O\textsubscript{2} as a salt-stress signal at the early stages and have efficient detoxification mechanisms (enzymatic and non-enzymatic antioxidant) to decrease H\textsubscript{2}O\textsubscript{2} levels on signal completion (Bose et al., 2014a).
The activity of some plasma membrane ion transporters is modulated by ROS, and this regulates cytosolic Na\(^+\) and K\(^+\) (Bose et al., 2014b; Shabala et al., 2014; Shabala et al., 2015). Similar to the plasma membrane, ROS may modulate ion fluxes across chloroplast membranes during salt stress. There is an indication that salt-induced thylakoid swelling (due to net ion influx) is associated with H\(_2\)O\(_2\) production and an H\(_2\)O\(_2\) scavenger was shown to abolish such thylakoid swelling (Yamane et al., 2012). However, no study has ever examined the extent to which ROS regulates K\(^+\), Na\(^+\) and Cl\(^-\) transporters on the chloroplast membranes during salt stress, and whether there is a clear difference between halophytes and glycophytes.

The aforementioned increases in ROS in response to salt stress could activate chloroplast retrograde signaling pathways [e.g. 3¢-phosphoadenosine 5¢-phosphate (PAP) phosphatase (SAL1) pathway] that are stimulated by hydrogen peroxide (H\(_2\)O\(_2\)), superoxide radical (O\(_2\cdot\)), hydroxyl radical (·OH) and singlet oxygen (\(^1\)O\(_2\)) (Chan et al., 2016). However, one study that has considered the transcriptome changes regulated by high light and the SAL1-PAP pathway found very little overlap with salt-induced transcripts (Wilson et al., 2009). Consideration of direct impacts of salt stress, if any, on retrograde communication would need to carefully consider the confounding effects of indirect stimulation of said pathways due to the changes to ROS, electron transport rates, PMF and redox balance of the chloroplast: all of which are altered by salt stress, and all of which in turn have impacts on chloroplast to nuclear communication (Chan et al., 2016).

### Candidate ion transporters mediating Na\(^+\), K\(^+\) and Cl\(^-\) transport in chloroplasts.

The outer envelope membrane was considered to be freely permeable to Na\(^+\), K\(^+\) and Cl\(^-\) ions due to the presence of large (600 Da) pores (Bölter and Soll, 2001). This view is now challenged, because some members of the outer envelope porins (OEP) showed from very weak (OEP16, OEP21, OEP24) to high (OEP23, OEP37) cation selectivity when they were reconstituted in planar lipid bilayers (Carraretto et al., 2016; Pottosin and Shabala, 2016). The P\(_{K^+}:P_{Cl^-}\) values, voltage dependence, conductance and pharmacology for these OEPs are reviewed in (Carraretto et al., 2016; Pottosin and Shabala, 2016). However, there is no information available in the literature about how these OEPs could function in planta and alter salt tolerance.

A search through literature (Hanikenne et al., 2014; Pfeil et al., 2014; Finazzi et al., 2015; Pottosin and Dobrovinskaya, 2015; Carraretto et al., 2016; Pottosin and Shabala, 2016)
and online databases for proteins targeted to chloroplasts (Ferro et al., 2010; Hooper et al., 2014), revealed several nucleus-encoded candidate ion channels and transporters (verified with MS/MS data) that may mediate Na\(^+\), K\(^+\) and Cl\(^-\) transport through the chloroplast envelope and thylakoid membranes (Fig. 1 and Table 1) (Kleffmann et al., 2004; Ferro et al., 2010; Tanz et al., 2012; Hooper et al., 2014). For the majority of these transporters, transport function and their role during salt stress have not been established. Further, it is not well established how the respective orthologue transporters are regulated in halophyte species. Hence, the following sections discuss, with some speculation, how these chloroplast Na\(^+\), K\(^+\) and Cl\(^-\) transport mechanisms could alter salt tolerance in plants.

**Non-selective ion channels and aquaporins**

Two members of *Arabidopsis* mechanosensitive channels like (MSL) family namely MSL2 and MSL3 are reported to be in the chloroplast envelope (Haswell and Meyerowitz, 2006). Characterisation of *msl2; msl3* double-mutant revealed that these two MSLs are necessary for the maintenance of osmotic balance, shape and size of the chloroplast (Haswell and Meyerowitz, 2006). Under control conditions, the *msl2* mutant and *msl2 msl3* double-mutant had enlarged plastids, but increasing cytoplasmic osmolality by supplying sugars, mannitol and NaCl in the growth medium supressed this phenotype, suggesting MSL2 and MSL3 may act as safety valves during hypo-osmotic swelling by releasing ions out of the plastid stroma (Veley et al., 2012; Wilson et al., 2014). In general, MSLs can vary in ion selectivity from non-selective to Cl\(^-\), or K\(^+\), or Na\(^+\) or Ca\(^2+\)-selective channels (Pottosin and Dobrovinskaya, 2015). Hence, ion flux characterisation of chloroplasts isolated from *msl2* and *msl2 msl3* mutants is essential to establish what ion(s) are transported and the roles that these MSLs play during salt stress.

Among the aquaporins, *Arabidopsis* Plasma membrane Intrinsic Proteins, namely PIP2;1, PIP2.3, PIP2.7, PIP1.3 and PIP1.2 (Kleffmann et al., 2004; Ferro et al., 2010) are indicated to be localised to the chloroplast envelope according to MS/MS data in the SUBA3 database (Kleffmann et al., 2004; Ferro et al., 2010; Tanz et al., 2012; Hooper et al., 2014). Both PIP2;1 and PIP2;7 have rapidly reduced expression in roots and are withdrawn from the plasma membrane under salinity (Boursiac et al., 2005; Pou et al., 2016). It is not clear if their presence on the chloroplast envelope is also affected by salinity. In other membranes it is proposed that PIP2;1 transports CO\(_2\) as well as water (Hanba et al., 2004; Kaldenhoff et al., 2014; Wang et al., 2016). Some aquaporins also have the capacity to transport ions (Yool and Campbell, 2012). PIP2;1 has been shown to transport cations non-selectively when expressed...
in *Xenopus* oocytes and yeast (Byrt *et al.*, 2016). But little is known about how PIP2;1 may regulate Na\(^+\) or indeed K\(^+\) and Cl\(^-\) concentrations inside the chloroplasts and how it may interact with co-located aquaporins. Co-expression of PIP2;1 with PIP1;2 abolished cation conductance (Byrt *et al*. 2016). Thus, elucidating the ion, water and CO\(_2\) transport properties of chloroplasts in the *Arabidopsis* pip2;1 mutant during salt stress is warranted.

The TIP2;1 (Tonoplast Intrinsic Protein 2;1) aquaporin has also been shown to be located in the thylakoid membrane in addition to the tonoplast (Tanz *et al.*, 2012; Hooper *et al.*, 2014). Apart from water transport, TIP2;1 transports NH\(_3\)/NH\(_4^+\) efficiently (Holm *et al.*, 2005) with a unique mechanism of coupling H\(^+\) in a water-filled side-pore that can deprotonate NH\(_4^+\) (Kirsch et *al.*, 2016). This is very important in the context of regulation of PMF components across the thylakoid membrane since NH\(_3\) is an uncoupler and will have the effect of dissipating the pH gradient. The NH\(_4^+\) formed in the acidic lumen may permeate through the K\(^+\) channels (KEA3 TPK3, see below) depending on their selectivity, alternatively the deprotonation that can occur in the TIP2;1 pore may allow depletion of NH\(_4^+\) from the lumen. Under salinity stress there is an increase in photorespiration (Miller *et al.*, 2010), which generates NH\(_3\) in the mitochondria, though it is possible that the NH\(_3\) is re-assimilated in a way that protects the chloroplast from NH\(_3\) exposure (Linka and Weber, 2005). Thus, the control of TIP2;1 on the thylakoid membrane may be critical under salinity in regulation of thylakoid PMF. Mutants of TIP2;1 would also be interesting to investigate in relation to salt stress and chloroplast energetics.

The NRT2;6 (Nitrate Transporter 2;6), GLR3.4 (Glutamate Receptor like 3.4) and CNGC13 (Cyclic Nucleotide-Gated Channel 13) of *Arabidopsis* are localised in envelope and thylakoid membranes (Fig. 1) (Finazzi *et al.*, 2015; Pottosin and Dobrovinskaya, 2015), suggesting that these may transport Na\(^+\), K\(^+\) and Cl\(^-\) ions during salt stress, which needs to be tested using their respective mutants.

**Na\(^+\) transporters**

The sodium ion can be transported into the chloroplasts through an inner envelope membrane localised Na\(^+\)-dependent pyruvate (*BASS2* bile acid: sodium symporter2) transporter. Crude protein extract from the leaves of different *Flaveria* species revealed that BASS2 abundance is higher in halophyte (*F. brownii, F. campestris, F. australasica, F. trinervia*) species than glycophyte (*F. pringlei*) species (Furumoto *et al.*, 2011). Recently, a wheat *TaBASS2* isolated from a salt-tolerant wheat cultivar Shanrong No.3 (bred using
asymmetric somatic hybridization between bread wheat and a C4 halophyte *Agropyron elongatum* was shown to improve salt tolerance (Zhao et al., 2016), suggesting halophyte BASS2 can be introduced into glycophyte chloroplasts to improve salt tolerance.

The inorganic phosphate transporters (thylakoid membrane localised-PHT4;1 and inner envelope localised-PHT4;4 and PHT4;5) can use Na\(^+\) or H\(^+\) as the co-transporting ion (Finazzi et al., 2015) and hence, they can alter Na\(^+\) concentrations inside the chloroplasts. Recently, PHT4;4 has been shown to mediate Na\(^+\)-dependant inorganic phosphate as well as ascorbate uptake dependent on membrane potential and Cl\(^-\) concentrations (Miyaji et al., 2015); this suggests that the nature of the substrate, co-transported ions and regulatory mechanisms can vary within the PHT transport family. Hence, characterisation of each of the members is critical to establish their specific role in chloroplasts during salt stress.

The existence of Na\(^+\)/H\(^+\) antiporter (NhaD; here after NHD)-type transporters at the chloroplast envelope membrane mediating Na\(^+\) efflux out of the stroma has long been suggested (Huber and Maury, 1980). Although this type of transporter has been reported in several halophilic eubacteria, the first NHD-type transporter (PeNHD1) in higher plants was cloned from a halophytic tree *Populus euphratica* and characterised in *Escherichia coli* (Ottow et al., 2005). Later, NHD1 orthologues were cloned from a moss *Physcomitrella patens* (Barrero-Gil et al., 2007), halophyte *Mesembryanthemum crystallinum* (Cosentino et al., 2010), as well as plants showing Na\(^+\)-dependent pyruvate uptake into their chloroplasts such as *Flaveria bidentis, Cleome gynandra* (Furumoto et al., 2011). Interestingly, transcript levels of NHD1 were sustained during salt stress only in the halophyte *P. euphratica* but not in a salt-sensitive *Populus x canescens* species (Ottow et al., 2005). In *Arabidopsis*, salt stress did not alter the expression of NHD1 but silencing NHD1 resulted in high Na\(^+\) within chloroplasts causing poor growth and photosynthetic performance (Müller et al., 2014). In contrast, in *Mesembryanthemum crystallinum* (a halophyte), salt stress increased NHD1 expression and the pattern of expression correlated with the Na\(^+\) accumulation pattern suggesting that NHD1 may mediate Na\(^+\) import into the chloroplasts instead of Na\(^+\) export (Cosentino et al., 2010). Such opposite regulation of transport mechanism must be verified through simultaneous Na\(^+\) and H\(^+\) flux measurements involving isolated chloroplasts of the respective species.

**K\(^+\) transporters**
Two K\(^+\) efflux antiporters (KEA1 and KEA2) located at the envelope membrane of Arabidopsis have been suggested to be functioning as K\(^+\)/H\(^+\) exchangers mediating K\(^+\) export out of the stroma (Kunz et al., 2014). However, the K\(^+\):Na\(^+\) selectivity needs to be investigated because half-length KEA2 has been demonstrated to transport Na\(^+\) in exchange with H\(^+\) in an in vitro assay (Aranda-Sicilia et al., 2012). The Arabidopsis double loss-of-function kea1kea2 mutant showed a severe growth phenotype under control conditions; but surprisingly, salt stress rescued this mutant (Kunz et al., 2014). However, the mechanism responsible for this salt alleviation linked to KEA1 and KEA2 is not established. One possibility is that salt stress induces K\(^+\) efflux from the stroma. Such K\(^+\) efflux will be low in kea1kea2 mutant resulting in increased K\(^+\) retention as well as maintenance of pH in stroma leading to improved photosynthetic performance and growth. This notion needs to be supported by experimental evidence.

Arabidopsis KEA3 located in the thylakoid membrane, has been suggested to import K\(^+\) into the lumen in exchange with H\(^+\) (Armbuster et al., 2014; Kunz et al., 2014) and has been shown to be involved in downregulation of luminal acidification-dependent heat dissipation leading to faster recovery of PSII quantum efficiency and increased CO\(_2\) assimilation during transitions to low light (Armbuster et al., 2014). However, there is no information on how KEA3 would function during salt stress. Another K\(^+\)/H\(^+\) exchanger from Arabidopsis, CHX23 was initially thought to operate in chloroplast envelope membrane (Song et al., 2004) but later studies proved that this transporter is targeted to the endoplasmic reticulum; never detected in proteomic studies targeted to the chloroplasts and preferentially expressed in pollen (Finazzi et al., 2015).

The two-pore potassium channel, TPK3 has been suggested to be involved in K\(^+\) export out of the lumen (Carraretto et al., 2013). A member of the two-pore potassium channel family, cloned from a halophyte Populus euphratica (PeTPK1) has been shown to increase the salt-induced K\(^+\) efflux, presumably from the vacuole (Wang et al., 2013a). If a similar scenario is considered for the chloroplast, an increase in salt concentration in the stroma will increase TPK3 mediated K\(^+\) efflux from the lumen, which in turn would affect ΔpH maintenance between stroma and lumen. Thus, alteration in ATP synthesis and CO\(_2\) fixation is expected during salt stress. This notion must be evaluated by exposing over-expressor and antisense lines of TPK3 to high salinity.

**Cl\(^-\) transporters**
Patch-clamp studies have shown the existence of Cl\(^-\) permeable channels on the chloroplast envelope and thylakoid membranes (Pottosin, 1992; Heibert et al., 1995; Pottosin and Dobrovinskaya, 2015). The molecular identity of those channels is starting to emerge. *Arabidopsis* has seven chloride channel family members (CLCa–g), of which only two members (CLC-e and -f) are localised to the thylakoid membrane and envelope membrane, respectively (Teardo et al., 2005; Marmagne et al., 2007; Herdean et al., 2016a). These CLC transporters can function as either anion (Cl\(^-\) or NO\(_3^-\)) channels or anion/H\(^+\) exchangers (Accardi et al., 2005). The Cl\(^-\) or NO\(_3^-\) selectivity is determined by the presence of specific glutamate and proline residues (Accardi et al., 2005; Wege et al., 2010). Anion transport activity is also regulated by ATP binding to the C terminus of AtCLCa (De Angeli et al., 2009). The Glu203 residue provides the pH sensitivity and the Glu148 abolishes the pH sensitivity (Pottosin and Dobrovinskaya, 2015). Since CLCe is localised to the thylakoid membrane and has only Glu148 residue, this suggests CLCe may function as a chloride channel responsible for ion compensation during the generation of PMF across the thylakoid membrane (Herdean et al., 2016a). The loss-of-function *clce* mutant showed altered photosynthesis (Herdean et al., 2016a) presumably due to altered Cl\(^-\) homeostasis within the chloroplast, but no gross phenotypic differences. The selectivity, pharmacology, gating, pH regulation of CLCe and CLCf must be assessed to establish the role of these CLCs in Cl\(^-\) homeostasis maintenance within the chloroplasts during salt stress.

A bestrophin-like protein from *Arabidopsis* has been discovered and shown to alter PMF portioning by functioning as a voltage-dependent Cl\(^-\) channel (AtVCCN1) on the thylakoid membrane (Herdean et al., 2016b). This channel is permeable to both Cl\(^-\) and NO\(_3^-\) but showed higher selectivity for Cl\(^-\) than NO\(_3^-\) in electrophysiological experiments. What role this channel could play during salt stress needs to be established.

**Summary and Future work**

Given the narrow nature of genetic diversity for salt tolerance within crops and their landraces (Colmer et al., 2005), and the lack of major breakthroughs and practical progress in plant breeding for salinity tolerance (Gilliham et al., 2017), there is renewed research on halophytes as a potential source of salt tolerance genes and a valuable source of knowledge for mechanisms underlying this complex trait (Shabala, 2013; Shabala et al., 2014; Flowers and Colmer, 2015). However, at the very best, comparisons are generally done at the cellular level; the subtle difference in operation of specific organelles between halophytes and glycophytes remains largely unknown. It appears that the classic dogma, that enzymatic
activity of halophytes and glycophytes are equally sensitive to salinity (Flowers et al., 1977),
does not hold in the case of chloroplasts. Halophyte chloroplasts may have intrinsically
higher levels of oxidative protection, more stable photosynthetic apparatus, and/or better
control over chloroplast membrane transport. It would be interesting to know what proportion
of halotolerant genes are encoded by the chloroplast genome or the nuclear genome.
Although chloroplasts could be exposed to relatively high levels of Na\(^+\) and Cl\(^-\) in the cytosol
during salt stress, the current knowledge on how chloroplasts regulate Na\(^+\), Cl\(^-\) and K\(^+\)
homeostasis during salt stress is limited. Further, the ionic concentrations could be different
between different compartments (stroma, lumen and intermembrane space) of the
chloroplasts but due to technological constraints this has not been measured. Proteomic
approaches targeted to the chloroplast membranes have identified several putative Na\(^+\), Cl\(^-\)
and K\(^+\) transporters, and some of the transporter’s roles are starting to emerge. However,
much work is still needed to link transporter expression patterns with functions, and to
understand the modes of regulation of chloroplast-based transporters. Gaining such
knowledge may be crucial for understanding the mechanisms by which halophytes optimise
ion transport and photosynthesis, and is essential for translation of this knowledge into
breeding for more salt-tolerant cultivars in farmers’ fields.

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**Figure Legends**

**Fig. 1** Putative chloroplast Na\(^+\), Cl\(^-\) and K\(^+\) transporters with a proposed role in salt tolerance (Hooper *et al.*, 2014; Finazzi *et al.*, 2015; Pottosin and Dobrovinskaya, 2015; Pottosin and Shabala, 2016). Abbreviations: BASS (bile acid:sodium symporter), CLC (chloride channel family), CNGC (cyclic nucleotide gated channel), F0F1 (thylakoid ATP synthetase F-type H\(^+\)-ATPase), GLR (Glutamate receptor like), KEA (cation/proton antiporter), MSL (mechanosensitive MscS-like channel), NHD (sodium/proton antiporters), NRT (nitrate transporters), OEPs (outer envelope porins 16, 21, 23, 24, 37), PHT (phosphate transporters), Pyr (pyruvate), TPK3 (tandem-pore K\(^+\) 3 channel), VCCN1 (voltage-dependent Cl\(^-\) channel 1). ? indicates uncertain transport functions. TM, IE, and OE are thylakoid, inner envelope, and outer envelope membranes. Transporters are grouped by different colour based on their suggested transport function: pink – Na\(^+\), grey – K\(^+\), blue – Cl\(^-\), black – non-selective, yellow – water channels.

**Fig. 2** Number of chloroplasts per cell increase in halophytes but not in glycophytes in response to salt exposure. The data are from (Flowers *et al.*, 1985; Peharec-Štefanič *et al.*, 2013; Wang *et al.*, 2013b; Aldesuquy *et al.*, 2014).

**Fig. 3** Chloroplast Na\(^+\), K\(^+\) and Cl\(^-\) concentration in glycophytes and halophytes grown under low salt (<1 mM) growth conditions. The plant species appear on the x-axis from left to right in order of increasing salt tolerance. The data are from aqueously isolated chloroplasts (Robinson and Downton, 1984; Robinson and Downton, 1985; Müller *et al.*, 2014). For a similar comparison see Larkum (1968) (which used non-aqueous isolation of chloroplasts) and Wang *et al.*, 2007 (that used X-ray microanalysis of chloroplasts).
Table 1. *Arabidopsis thaliana* chloroplast envelope and thylakoid membrane Na\(^+\), K\(^+\), and Cl\(^-\) transporters and their orthologue transporters in *Eutrema salsugineum* that have potential to alter salt tolerance of respective glycophyte and halophyte species.

<table>
<thead>
<tr>
<th>Arabidopsis protein name and locus number</th>
<th>Orthologue with the highest similarity in <em>Eutrema salsugineum</em></th>
<th>Similarity</th>
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<td>AtNHD1 (At3g19490)</td>
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<td>AtPHT4;5 (At5g20380)</td>
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<td>AtBASS2 (At2g26900)</td>
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<td>AtKEA1 (At1g01790)</td>
<td>EsKEA1 (XP_006418459.1)</td>
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<tr>
<td>AtKEA2 (At4g00630)</td>
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<td>AtCLC-f (At1g55620)</td>
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<td>AtCNGC13 (At4g01010)</td>
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<td>90%</td>
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References


Pagliano C, La Rocca N, Andreucci F, Déák Z, Vass I, Rascio N, Barbato R. 2009. The extreme halophyte *Salicornia veneta* is depleted of the extrinsic PsbQ and PsbP proteins of


Fig. 2