Linking Detrimental Effects of Salinity on Leaf Photosynthesis with Ion Transport in Leaf Mesophyll

by

William John Percey

B.Agr.Sc. (Hons)

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

University of Tasmania

July 2014

Declaration of Originality

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William John Percey

University of Tasmania

July 2014

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Statement of Co-Authorship

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

William John Percey, School of Land and Food

Sergey Shabala, School of Land and Food

Rosanne Guijt, School of Pharmacy

Michael Breadmore, School of Chemistry

Jayakumar Bose, School of Land and Food

Lana Shabala, School of Land and Food

Andrew McMinn, Institue of Marine and Antartic Science

Author details and their roles:

Paper 1: **Ion transport in broad bean leaf mesophyll under saline conditions**

Published in *Planta*; *Located in chapter 2*

William Percey and Sergey Shabala conceived and designed the research and wrote the manuscript. William Percey conducted the experiments with the assistance and advice of Lana Shabala. William Percey analysed data. Michael Breadmore, Rosanne Guijt and Jayakumar Bose contributed to results interpretation and provided critical analysis of the manuscript.

Paper 2: **Salinity effects on chloroplast photosynthetic performance in glycophytes and halophytes**

Submitted to *Physiologica Plantarum*; Located in chapter 3

William Percey and Sergey Shabala conceived and designed the research and wrote the manuscript. William Percey conducted the experiments and analysied the data. Andrew McMinn, Michael Breadmore, Rosanne Guijt and Jayakumar Bose contributed to results interpretation and provided critical analysis of the manuscript.

Paper 3: **Amelioration of the effects of salinity on chloroplasts**

To be submitted to *Enivronmental and Experimental Botany*; Located in chapter 4

William Percey and Sergey Shabala conceived and designed the research and wrote the manuscript. William Percey conducted the experiments and analysied the data. Andrew McMinn, Michael Breadmore, Rosanne Guijt and Jayakumar Bose contributed to results interpretation and provided critical analysis of the manuscript.

Paper 4: **Linking growth with mesophyll ion transport under saline conditions in three species contrasting salinity tolerance**

Submitted to *Plant and Soil*; Located in chapter 5

William Percey and Sergey Shabala conceived and designed the research and wrote the manuscript. William Percey conducted the experiments and analysied the data. Andrew McMinn, Michael Breadmore, Rosanne Guijt and Jayakumar Bose contributed to results interpretation and provided critical analysis of the manuscript.

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Signed: _____ _____

Sergey Shabala

Supervisor

School of Land and Food

University of Tasmania

Dugald Close

Deputy Head of School

School of Land and Food

University of Tasmania

Date:_____

Acknowledgements

This research would not have been possible without the supervision and guidance given to me by my supervisory team. I would like to specially thank Sergey Shabala for his commitment and dedication to providing the right support and encouragement, initially in my honours project and all throughout my PhD. On multiple occasions he was left to pick up the pieces and stick them back together. I would also like to thank all my other supervisors, Rosanne Guijt, Michael Breadmore and Jayakumar Bose, for providing much help and encouragement. Lana Shabala and Andrew McMinn also provided invaluable technical support and for this I am very thankful.

I would like to thank all the people from the School of Chemistry for all their support and friendship as well as their insights into the world of separation science.

I would also like to thank all my cell (lab) mates in Sergey's stress physiology lab for making my work entertaining and enjoyable.

The Tasmanian University Union White Water Rafting Club, Tasmanian University Dive Club and their members have helped keep me sane. There is nothing like a good days rafting or diving to clear the mind and set you up for another week of work.

I would like to thank my girlfriend, Min Zhu, for her support and help over the final 2 years of my PhD. This has been very valuable to me and kept me focused on my work.

My parents have supported me immensely through this journey, that has taken the better part of 5 years to complete. Without their constant support there is no way that I would have made it through my PhD in one piece.

The funding for this work was provided by an Australian Research Council grant DP0987402 awarded to Sergey Shabala and Rosanne Guijt and a Grain Research and Development Corporation (GRDC) grant to Sergey Shabala.

Abstract

Soil salinity is defined as a situation when the electrical conductivity of the saturated paste extract from the upper layers of the soil is in excess of 4 dSm^{-1} (which equates to ~40 mM NaCl). Soil salinisation is an ever-growing problem, resulting in vast losses of agricultural land and costing industry in excess of $12 billion in the lost production globally. The only practical way of dealing with increasing land salinisation is to develop ways of growing plants in these saline environments, either through breeding/ engineering of salinity tolerant plants or through the application of chemicals that have ameliorative effect on plant performance under saline conditions. Both of these approaches have been tried in the past, but with a rather limited success. To increase the efficiency of breeding/ engineering and the development of chemical applications, the processes by which salinity affects key intracellular structures and processes, and physiological mechanisms behind salinity tolerance, should be understood.

Salinity causes osmotic and ionic limitations to growth. While osmotic effects causing reduced growth within minutes, ionic limitations take longer but are predominate long lasting effects. Less tolerant plants (glycophytes) tend to be Na^+ excluders while naturally more tolerant plants (halophytes) tend to include Na^+. Na^+ has long been believed to be toxic in the cytosols of cells, however, its uptake into the shoot is necessary to provide low carbon-cost osmotic adjustment, which is essential for plants to maintain tissue turgor and expansion growth under saline conditions. The exact biochemical/ physiological targets of Na^+ stress in the cytosol are not well understood, despite many years of research. It has long been suggested that the maintenance of high K^+/Na^+ ratios in the cytosol is more important than the Na^+ concentration itself. Despite being a long-standing paradigm in salinity tolerance, this notion has not been well tested either *in vitro* or *in vivo*.

The major aim of this work was to investigate mechanisms of non-stomatal limitation to photosynthesis caused by salinity stress and link them with ionic relations and ion transport across mesophyll cell plasma and chloroplast membranes. By doing this the following objectives were addressed:

- To understand effects of salinity on ionic homeostasis and light-induced changes in ion transport in leaf mesophyll, in the context of leaf photosynthetic and growth responses.
- To elucidate effects of altered ionic homeostasis and cytosolic osmolality on photosynthetic performance in chloroplasts.
- To reveal differences between chloroplast responses to salinity from species contrasting in salinity tolerance, e.g. halophytes and glycophytes.
- To the link ion transport in the chloroplast and their resilience to perturbed ionic homeostasis.
- To investigate effects of ameliorative chemicals (polyamines, compatible solutes and antioxidants) on salinity-induced disturbance to leaf photosynthetic machinery, at the chloroplast level.
- To understand the differences in mesophyll ion transport between species with contrasting salinity tolerance and link them with primary photosynthetic processes in green leaf tissues.

For plants to be truly salinity tolerant they need to be able to handle elevated Na^+ concentrations in leaves. The ability to handle Na^+ in leaves is called tissue tolerance. Using non-invasive microelectrode ion flux measuring (the MIFE) technique it was found that an increase in salinity within the apoplast of bean leaves results in a large K^+ efflux from mesophyll tissue. This efflux was mediated predominately by K^+ outward rectifying channels (84%), with the remainder of the efflux being through non-selective cation channels. The reduction in K^+ concentration associated with this efflux was linked to a decline in the photochemical efficiency of photosystem II (chlorophyll fluorescence F_V/F_M values). In addition to K^+ efflux, Na^+ has also induced a vanadate sensitive H^+ efflux presumably mediated by the plasma membrane H^+-ATPase. This H^+ efflux is essential for the maintenance of membrane potential and ion homeostasis in the cytoplasm of bean mesophyll. Salinity also caused reductions in the ability of bean mesophyll tissue to respond to light with both K^+ and H^+ fluxes. This decline in response was both time- and NaCl concentration-dependant.

The effects of salinity-induced altered ionic conditions in the cytoplasm of mesophyll cells on photosynthesis were assessed. Isolated chloroplasts were subject to a range of

ionic conditions and the F_V/F_M and relative electron transport rates (rETR) were measured. Decreased K^+ concentrations caused declines in F_V/F_M. K^+/Na^+ ratios and did not affect F_V/F_M; rather at very low K^+ concentrations Na^+ was beneficial to photosynthesis. This suggests that damage to photosynthesis is more dependent on K^+ concentration and is not strongly related to the concentration of Na^+ in the cytosol. Salinity treatment (including reduced K^+) resulted in reductions in both F_V/F_M and rETR; these reductions were time-dependant. We found that chloroplasts isolated from the halophyte species quinoa showed an overall decline in F_V/F_M of 18% under salinity treatment while the chloroplasts of the two-glycophyte species tested, pea and bean, declined by 31% and 47 % respectively. To the best of my knowledge, this is the first report of differential sensitivity of leaf photosynthetic machinery to salinity between halophyte and glycophyte species. One possible explanation for the difference between the chloroplast's ability to deal with altered ionic environments is a difference in the way they control ion transport across chloroplast membranes. Vanadate, a P-type ATPase inhibitor, significantly reduced the F_V/F_M of both control and salinity treated chloroplasts with salinity treated chloroplasts being much more affected. The blockage of K^+ channels in the chloroplast with tetraethyl ammonium (TEA) resulted in an increase in the F_V/F_M of salinity treated chloroplasts of 16%. The inhibition of chloroplast monovalent cation/ H^+ exchanges with amiloride also resulted in increased F_V/F_M (17%) under salinity treatment.

The amelioration of salinity damage with chemicals provides a viable alternative to producing tolerant plants. There are a few different classes of ameliorative chemicals that can be used to try and remediate the effects of salinity on plants. These include compatible solutes, polyamines and antioxidants. When tested, all classes of these chemicals resulted in different levels of amelioration from salinity-induced reductions in F_V/F_M or rETR to isolated chloroplasts. In the short term (25 min), both compatible solutes and polyamines caused increases in the rETR of salinity treated chloroplasts. However, after 100 min treatment they caused negative effects, most likely as a result of their over-accumulation in chloroplast stroma. This demonstrates a clear need for further research into the effects of these ameliorative substances on photosynthesis.

The retention of K^+ in mesophyll tissue in halophytes has been hypothesised to be an important part of their salinity tolerance. To test this hypothesis, we investigated K^+

retention in species with contrasting salinity tolerance. It was found that halophytic quinoa and salt-tolerant sugar beet lose much less K^+ from mesophyll tissue when subjected to 100 mM NaCl stress over a 72 h period, compared with salt-sensitive broad beans. Changes in the ability of quinoa and sugar beet mesophyll to respond to light under salinity stress seemed to be related to changes in the steady state efflux of both H^+ and K^+. Unlike bean, the two salinity tolerant species quinoa and sugar beet did not have vast increases in H^+ flux with the onset of Na^+ stress. This suggests that the more tolerant species rely more heavily on vacuolar sequestration of Na^+ rather than pumping Na^+ back out into the apoplast in a futile cycle.

In conclusion, this work has found that control of K^+ transport in leaves at tissue, cellular and chloroplast levels is very important to conferring salinity tolerance in plants. Maintaining high K^+ concentrations in the cytosol is more important than the exclusion of Na^+ from the cytosol. The long-held belief that cytosolic K^+/Na^+ ratios are important for salinity tolerance did not hold when tested on isolated chloroplasts. Halophytes have the ability to retain more K^+ in their mesophyll tissue without the need for high levels of energy dependant H^+ efflux (unlike in glycophytes). The application of ameliorative substances to leaves to provide salinity tolerance needs to be tightly controlled as oversupply of both compatible solutes and polyamines causes declines in electron transport rates under salinity stress. Overall, the reported findings highlight the paramount role of intracellular ionic relation on leaf photosynthetic machinery and provide insights into fundamental mechanisms of leaf photosynthetic machinery to salinity stress, both at the tissue and organelle levels.

List of publications

Percey WJ, Shabala L, Breadmore MC, Guijt RM, Bose J, Shabala S (2014) Ion transport in broad bean leaf mesophyll under saline conditions. *Planta*. DOI 10.1007/s00425-014-2117-z

Percey WJ, McMinn A, Bose J, Breadmore MC, Guijt RM, Shabala S (2014) Salinity effects on chloroplast photosynthetic performance in glycophytes and halophytes. *Physiologica Plantarum* (submitted)

Percey WJ, McMinn A, Bose J, Breadmore MC, Guijt RM, Shabala S (2014) Amelioration of the effects of salinity on chloroplasts. *Environmental and Experimental Botany* (to be submitted)

Percey WJ, Shabala L, Breadmore MC, Guijt RM, Bose J, Shabala S (2014) Linking growth with mesophyll ion transport under saline conditions in three species contrasting salinity tolerance. *Plant and Soil* (submitted)

Chapter 1: General introduction

Salinity as an issue

Salinity is defined as the build-up of salts in the root zone of the soil resulting in the electrical conductivity of saturated soil paste being in excess of 4 dS m^{-1}, or about 40 mM NaCl. The global area of agricultural land affected by soil salinity is 800 million hectares and growing (Rengasamy 2006). Salinity negatively impacts crop growth and yield, resulting in vast losses in agricultural production (in excess of $12 billion/year; Qadir et al. 2008). Moreover, salinity takes out of production approximately 3 ha of currently arable every minute (Ghassemi et al. 1995), limiting conventional crop production. This is occurring as the world population is increasing. The global population is expected to increase by 50% by 2050 and food production will also need to increase to meet this growing demand (Flowers 2004; Tester and Langridge 2010). Taken together, this puts a strong pressure on shifting agricultural crop production into saline areas.

There are two options for reducing the effects of salinity. The first is a large-scale removal of salts from the soil via remediation practices. The second is breeding crop plants that are able to live in these hostile conditions (Pannell and Ewing 2004).

Large scale removal of salt can be through irrigation with high quality water to leach the salt out of the soil, if the soil is free draining. This is a very expensive option given the acute shortage of good quality irrigation water. Moreover, in most cases, this is not an effective remediation practice. As saline soil is often not free draining and expensive drainage needs to be installed (Pannell and Ewing 2004).

The second option of using salinity tolerant plants is much cheaper and thus preferable. However, despite decades of research, conventional breeding techniques and genetic engineering to date have not resulted in the creating of any truly salt tolerant crops (Flowers 2004; Shabala 2013). Salinity stress tolerance is very complex, both physiologically and genetically, and breeding for this trait requires an understanding of the physiological mechanisms of salt stress and the best way to overcome these.

Effect of salinity on plant growth and development

Plants differ in their ability to tolerate high NaCl concentrations in the soil (Flowers et al. 1977; Munns and Tester 2008). Some species (termed glycophytes) are highly susceptible to salinity, while others (termed halophytes) can even benefit from moderate levels of salinity (100 - 200 mM NaCl). Most crops fall into the glycophyte category and, as such, are severely affected by salinity. Halophytes will also show signs of reduced growth and toxicity once salinity reaches a certain threshold (Flowers et al. 1977).

From germination to reproduction salinity exhibits negative effects on plant performance regardless of the stage of plant development (Flowers 2004). Salinity causes reductions in overall seed germination in all species of plants including halophytes (Khan and Ungar 1984a) and can cause uneven and delayed germination in some species (Kent and Läuchli 1985; Hampson and Simpson 1990a; Hampson and Simpson 1990b). Salinity also impacts upon vegetative growth causing reduced leaf expansion (Munns and Termaat 1986), accelerated leaf mortality (Yeo et al. 1991) and an increase in the root to shoot ratio (Munns and Termaat 1986). Salinity also induces earlier flowering and reproductive development (Rawson et al. 1988), with a reduced number of seeds produced where plants manage to live through their life cycle.

Salinity-induced reductions in seed germination are caused by multiple mechanisms including reduced soil water potential (Khan and Ungar 1984b; Khan and Ungar 1984a), ion toxicity to the embryo (Khan and Ungar 1984b; Khan and Ungar 1984a) and changes in protein synthesis (Dell'Aquila and Spada 1993; Almansouri et al. 2001). However the effects of salinity on seed germination and early plant development are poor indicators of the effects of salinity on overall plant performance (Flowers 2004).

Salinity impacts the vegetative growth of all plants to varying extents (Flowers et al. 1977; Munns and Tester 2008). Salinity negatively impacts many physiological functions in plants including photosynthesis (Seemann and Critchley 1985; Brugnoli and Lauteri 1991; Belkhodja et al. 1999; Parida et al. 2003; Wang et al. 2007), respiration (Jacoby et al. 2011), nutrient uptake (Yildirim et al. 2009), protein

synthesis (Hall and Flowers 1973) and oxidative homeostasis (Bose et al. 2013; Baxter et al. 2014). The growth response under salinity occurs in two phases. The first phase is attributed to osmotic stress; the second is due to specific ion toxicity (Munns and Termaat 1986). The onset of the osmotic phase of salinity stress is very rapid. The first effects of salinity stress are observed as a reduction in elongation growth, transpiration rate and reduced stomatal aperture which occur almost instantly (Munns and Termaat 1986). The osmotic effects are closely tied to stomatal limitations of plant growth. The specific ion toxicity effect occurs due to the uptake of Na^+ and associated changes in plant ionic composition and compartmentation. The ionic effects are believed to dominate during prolonged salinity stress (Munns and Termaat 1986; Munns and Tester 2008).

Osmotic effects of salinity

The osmotic effects of salinity occur due to the increased ionic concentration of soil water reducing water potential and therefore water uptake through roots (Munns and Termaat 1986). This reduced water availability results in lost turgor pressure, which is an essential component of cell elongation. It also translates into less water transport to the shoot, reducing transpiration rates and ultimately causing stomatal closure (Brugnoli and Lauteri 1991). Stomatal closure causes stomatal limitations to photosynthesis through reduced CO_2 availability leading to reduced CO_2 assimilation and the detrimental processes of photorespiration (Noctor et al. 2002; Geissler et al. 2008) and ROS production (Kuvykin et al. 2011).

Na^+ toxicity

Most of the breeding and research effort in to salinity tolerance so far has concentrated its attention on Na^+ exclusion from roots (reviewed in Greenway and Munns 1980; Gevaudant et al. 2007; Munns and Tester 2008). As a result specific ion toxicity has received more research attention than the osmotic effects of salinity (Ashraf and Khanum 1997; Tester and Davenport 2003; Garthwaite 2005). Na^+ has long been considered toxic within the cytosol of cells through disrupting K^+ nutrition and by direct interference with enzymes. Na^+ may block the activation sights of enzymes (Nitsos and Evans 1969; Hall and Flowers 1973; Benito et al. 2014), reduce cytosolic and tissue K^+ content (Chen et al. 2005; Wu et al. 2013), and increase

intracellular ionic strength disrupting protein structure (Kronzucker et al. 2013). In light of the above, it has been traditionally thought that the K^+/Na^+ ratio within the cytosol of cells and, indeed, whole plants, is an essential component of salinity tolerance (Munns and Tester 2008; Benito et al. 2014). Surprisingly, there have been very few direct measurements of cytosolic K^+/Na^+ ratios, instead many authors have opted to use whole tissue K^+/Na^+ ratios as a proxy for the cytosolic ratios (Shabala and Mackay 2011). Such an approach is highly misleading and methodologically flawed, as it fails to take into account the plant's ability to sequester toxic Na^+ in vacuoles a key component of the tissue tolerance mechanism. The main reason for these proxies being used is the technical challenges that come with measuring ion contents in such a small non-uniform space. The few measurements that do exist vary greatly (Britto and Kronzucker 2008; Kronzucker and Britto 2011) and there is no K^+/Na^+ ratio that has ever been considered a threshold of salinity damage. Furthermore, there is also no clear suggestion of an upper concentration limit of Na^+ within the cytosol (Cheeseman 2013; Kronzucker et al. 2013), with the general consensus being rather vague ("less is better").

There are a few different strategies plants may employ to reduce cytosolic Na^+ concentrations. This includes (Munns and Tester 2008): (i) exclusion Na^+ from root uptake, (ii) removal of Na^+ from cells via SOS1 antiporter, (iii) recirculation Na^+ in the phloem, (iv) direct Na^+ uptake to areas of low metabolic activity, and (v) Na^+ sequestration into the cell vacuoles. The first option of excluding Na^+ from uptake has received the most attention; yet, this approach has not led to a single field-ready salt-tolerant variety of any species. The most likely explanation is that excluding Na^+ from uptake allows avoiding cytosolic toxicity but fails to account for the osmotic component of salinity stress. The strategy of Na^+ recirculation in the phloem also neglects an osmotic adjustment component. The localisation of Na^+ into certain tissues within the plant that have low metabolic activity partially addresses the issue of osmotic stress by loading some of the Na^+ into the plant. The best strategy available is the sequestration of Na^+ in vacuoles; this has advantages over the other strategies mentioned above. However, even in this case many unresolved issues remain and, there are no published reports of any transgenic plant that can match (naturally-evolved) halophyte species at salinity stress tolerance.

Osmotic problems with Na^+ exclusion

There have been many attempts to show that Na^+ exclusion from plants is correlated with salinity tolerance. Over the past decade there has been much work on increasing Na^+ exclusion from the shoot, including the mapping of Na^+ exclusion traits (Lindsay et al. 2004; James et al. 2006; Huang et al. 2008; Munns et al. 2012). The overall progress was, however, very modest. In the later work, Munns et al. (2012) have shown that the presence of TmHKT1;5-A gene (Nax2 locus), encoding a Na^+-selective transporter located on the plasma membrane of root cells surrounding xylem vessels, increased durum wheat grain yield by 25% compared to near-isogenic lines without Nax2. However, these results were observed only in one of the three tested field sites; even within one where it worked, the grain yield of transformed plants under saline conditions was still 50% of the control. It was argued that Na^+ inclusion in the shoot may actually be beneficial to achieve osmotic adjustment, (assuming it is sequestered away from metabolically active compartments). Increased Na^+ in the xylem stream of barley has been associated with increased salinity tolerance (Shabala et al. 2010), and overexpression of HvHKT2;1 led to higher xylem and leaf Na^+ content in saline-grown plants (Mian et al. 2011) and was correlated with increased salt tolerance in barley. Finally, all halophytes are known to be salt includers and accumulate higher concentrations of Na^+ within their leaves than glycophytes (Flowers and Colmer 2008). This enhanced tolerance with the accumulation of Na^+ most likely comes from its use in osmotic adjustment.

In saline conditions there are two possible ways for plants to adjust to osmotic stress. One is through de novo synthesis of compatible solutes, and another one is by means of inorganic ions (mainly Na^+ and Cl^-). Compatible solutes are relatively low molecular weight organic compounds that do not interfere with metabolic reactions at normal concentrations. Enhanced compatible solute production has been a growing area of research, with many over expression mutants produced (Hayashi et al. 1997; Huang et al. 2000; Holmström et al. 2000; Chen and Murata 2002; Ashraf and Foolad 2007; Fan et al. 2012). These mutants increase the accumulation of compatible solutes and are normally found to increase salinity tolerance under the test conditions (Hayashi et al. 1997; Huang et al. 2000; Holmström et al. 2000; Fan et al. 2012). However these transformed plants generally exhibit reduced growth under normal

non-saline conditions (Huang et al. 2000; Wang et al. 2013). The reason for reduced growth under normal non-saline conditions is most likely due to the energetic cost of compatible solute production diverting energy away from growth (Shabala and Shabala 2011). The other option is to use inorganic ions – the option that all naturally salt tolerant plants use. In the pseudo-cereal halophyte species quinoa, over 90% of leaf osmotic adjustment under saline conditions came from increased Na^+ and Cl^- concentrations within the plant (Hariadi et al. 2011). However, as mentioned above, the use of Na^+ in the shoot brings with it its own problems and results in a need for tissue tolerance to increased Na^+ concentrations.

Tissue tolerance

As mentioned previously, accumulation of Na^+ in the shoot for osmotic adjustment purposes should be complemented by mechanisms conferring tissue tolerance in the mesophyll tissue. Traditionally, tissue tolerance has been always associated with increased vacuolor sequestration of Na^+. Increased vacuolar sequestration of Na^+ has benefits for both turgor maintenance and reducing cytosolic Na^+ concentrations by relocating Na^+ from the cytosol. The sequestration of Na^+ is achieved by the operation of the tonoplast Na^+/ H^+ exchangers (NHX). The overexpression of these transporters has been shown to increase salinity tolerance (Apse et al. 1999; Zhang and Blumwald 2001); importantly, this was achieved with a concurrent increase in the overall leaf Na^+ content. These exchangers need to be energised by a H^+ gradient, generated by the operation of tonoplast H^+-PPases and H^+-ATPases. The increased expression of these active transporters has been shown to increase salinity tolerance in some species; again, it was accompanied by increased Na^+ concentrations in the shoots (Colombo and Cerana 1993; Zingarelli et al. 1994; Fukuda 2004). Dual overexpression, of both NHX and one of the H^+ active transporters confers higher salinity tolerance than the expression of either transporter by itself (Bhaskaran and Savithramma 2011; Bao et al. 2014; Shen et al. 2014).

K^+ nutrition

While Na^+ exclusion from the cytosol has received much attention, the ultimate aim of this exclusion is to reduce its competition with K^+ and the disturbance to cytosolic K^+ homeostasis. K^+ is the most abundant inorganic cation in plants and is vital for

plant growth (Marschner 1995; Dreyer 2014). It has many functions in plants including osmoregulation, cell extension, stomatal movement, respiration (Marschner 1995), phloem loading (Marten et al. 1999; Lacombe et al. 2000), ribosome function (Foucher et al. 2012), carbon fixation via RuBisCo (Viitanen et al. 1990), photoreduction, photophosphorylation (Pflüger and Mengel 1972), and the activation of type 1 H^+PPases (Belogurov and Lahti 2002). Upon the application of salinity stress K^+ is lost from cells; however, the cytosolic concentration is buffered by the K^+ in the vacuole being relocated to replenish lost K^+ from the cytosol (Shabala et al. 2006). K^+ retention in cells is likely a large component of tissue tolerance (Shabala et al. 2013; Wu et al. 2014). Cuin et al. (2003) found that under 200 mM NaCl treatment that the K^+ activity in the vacuoles of barley mesophyll dropped by 36 % while cytosolic concentrations only dropped by 19 %. Wu et al. (2014) found that 10 % of the variation in salinity tolerance in barley could be explained by leaf K^+ content in plants grown in normal non-saline conditions. This non-salinity treated K^+ content would be essentially measuring vacuolar K^+ concentration as the vacuole makes up over 90 % of the cell volume in mesophyll. So plants with a greater ability to relocate K^+ from the vacuole to the cytosol to replenish the K^+ lost due to salinity treatment were more salt tolerant. This is in accordance with previous studies that have correlated K^+ retention in roots of various species (Chen et al. 2005; Smethurst et al. 2008; Cuin et al. 2008), as well as more recently studies that have correlated K^+ retention in leaves with salinity tolerance (Wu et al. 2013; Wu et al. 2014).

High cytosolic K^+ concentrations are also essential to prevent programmed cell death (PCD) (Hughes et al. 1997; Lam and del Pozo 2000). Caspase-like enzymes are responsible for cell death under PCD conditions (Lam and del Pozo 2000). K^+ represses the activity of caspase enzymes in animal cells and caspase-like proteases and endonucleases in plant cells (Demidchik et al. 2010). K^+ loss is an essential part of PCD (Peters and Chin 2007). *Arabidopsis gork1-1* lacking functional K^+ outward rectifying channels showed lower almost non-existent Na^+ induced K^+ efflux and consequently much lower rates of PCD (Demidchik et al. 2010).

The Na^+ induced K^+ efflux from plant tissues is dominated by potassium outward rectifying channels (KOR) and non-selective cation channels (NSCC) (Shabala and Cuin 2008). When Na^+ enters the cell, the plasma membrane becomes depolarized

resulting in activating the depolarisation-activated KOR channels and initiating efflux of K^+ from the cell (Shabala et al. 2006). Salinity stress also induces a vanadate sensitive H^+ efflux (Wu et al. 2013), presumably mediated by H^+-ATPases. This efflux is generated to repolarise the cell therefore leading to K^+ retention by closing depolarisation activated KOR channels. The H^+ efflux can also be used to generate a H^+ gradient across the membrane to energise Na^+ efflux via the SOS1 antiporter (Apse and Blumwald 2007).

In addition to voltage gating, NSCC and KOR can be both activated by reactive oxygen species (ROS)(Demidchik et al. 2003; Demidchik et al. 2010). ROS activation of NSCC is a double-edged sword, as NSCC is also responsible for Na^+ entry into the cell (Demidchik and Tester 2002; Demidchik and Maathuis 2007). Uptake of Na^+ via NSCC leads to further membrane depolarisation and K^+ loss through KOR, resulting in a positive feedback as K^+ deficient plants increase ROS production (Kim et al. 2010; Hafsi et al. 2010).

Ion imbalance and light responses

The ability of mesophyll tissue to respond to light with ion fluxes is central to plant growth and development. These responses control a range of different process in plants. It is not understood how salinity affects these responses. Normal responses are characterised by a Ca^{2+} influx (Shacklock et al. 1992) causing membrane depolarisation and in turn K^+ efflux (Shabala and Newman 1999). There is also an observed H^+ uptake under illumination; this results from CO_2 uptake for photosynthesis and therefore a reduction in the carbonic acid concentration in the solution (Shabala and Newman 1999). It may also be expected that more tolerant plants have a better ability to maintain normal flux responses under saline conditions. The already altered ion fluxes from mesophyll may result in changes in these light induced fluxes and therefore light induced fluxes may provide a measure of salinity damage to the tissue.

Ion imbalance and photosynthesis

As commented above, most research efforts were focused on mechanisms of salt stress signalling and Na^+ exclusion from uptake in roots, and Na^+ sequestration in leaf

vacuoles. At the same time, a very limited number of studies have addressed the effects of elevated cytosolic Na^+ and depleted K^+ on primarily photosynthetic processes. There have been previous reports describing the effects of altered ionic homeostasis on individual enzymes involved in photosynthesis, for instance RuBisCO (Viitanen et al. 1990) and the enzymes involved in photoreduction and photophosphorylation (Pflüger and Mengel 1972). Other papers investigated the effects of reduced K^+ concentrations on the photosynthetic performance of isolated chloroplasts (Bulychev and Vredenberg 1976; Demmig and Gimmler 1983). These latter reports, however, have failed to account for the increased Na^+ concentrations observed under salinity stress conditions. The effects of altered cytosolic ion homeostasis are hard to study in whole plants, as non-stomatal limitations and other effects make it hard to distinguish between different mechanisms of salinity damage. It is also technically challenging to measure cytosolic Na^+ and K^+ concentrations. Understanding the relationship between cytosolic ion homeostasis and damage to photosynthesis is central to our understanding of salinity damage and therefore of strategies to mitigate this damage.

Salinity and ROS production

Salinity causes increases in ROS production, with the majority of ROS in photosynthesising tissues being produced in the chloroplast (Gill and Tuteja 2010; Bose et al. 2013). ROS production leads to the damage of proteins, lipids, carbohydrates and DNA (Gill and Tuteja 2010). K^+ depletion is also known to increase ROS production (Kim et al. 2010; Hafsi et al. 2010). Increased ROS production can also be due to limited CO_2 availability (Kuvykin et al. 2011) and damage to photosynthetic apparatus (Pospíšil 2009). As mentioned earlier K^+ is essential for photosynthesis particularly CO_2 fixation (Viitanen et al. 1990) and photophosphorylation (Pflüger and Mengel 1972). If these two processes are ineffective, other non-target species are oxidised by PSII and PSI due to limits imposed on the electron transport chain (Pospíšil 2009). The notion that the maintenance of K^+ is essential to prevent over production of ROS under salinity stress further adds to the need for K^+ retention.

Plant breeding and engineering of non ion transport related traits

In addition to modifying various Na^+ transport systems, breeding and engineering for salinity tolerance has been strongly focused on the up-regulation of certain metabolites to overcome salinity stress. These metabolites include compatible solutes (Chen and Murata 2002; Ashraf and Foolad 2007), polyamines (Groppa and Benavides 2008; Hussain et al. 2011) and antioxidants (Pilon et al. 2011). The increased production of these metabolites has been shown to increase salinity tolerance under certain conditions; however in many cases transgenic plants grown under normal non-saline conditions exhibited reduced growth (Huang et al. 2000; Wang et al. 2013). This reduced growth has often been attributed to the increased metabolic costs of the over production of these metabolites. Attempts to use stress promoters to make the increased expression of these genes have somewhat mitigated the reduced growth exhibited by transgene plants while still providing greater salinity tolerance under test conditions (Wang et al. 2013). Also, negative changes in plant morphology associated with transgenically increased production have been observed at many occasions (Yeo et al. 2000), and negative feedback inhibition of photosynthesis in plants accumulating excessive concentrations of compatible solutes was reported (Szabados and Savouré 2010).

Chemical amelioration

A practical way of overcoming salinity-induced yield losses maybe by exogenous applications of chemicals to ameliorate detrimental effects of salinity on plants (Shabala and Munns 2012). This approach is especially attractive in light of the limited progress of traditional breeding and the generally negative public perception of GMO technologies. The application of a variety of different chemicals has resulted in increased salinity tolerance under test conditions (Ashraf and Foolad 2007; Shabala et al. 2012). The work has been largely empirical (largely a trial and error approach - “spray and pray”). Very little attention has been paid to the direct effects of these chemicals on photosynthesis under saline conditions. There have been varying levels of success with these approaches. While most papers report beneficial results, in some cases the application of different ameliorative substances including compatible solutes (Garcia et al. 1997; Nanjo et al. 1999; Yamada et al. 2005; Chang et al. 2014) and

polyamines (Suleiman et al. 2002; Ndayiragije and Lutts 2006) have had negative effects on plant performance, or were deemed as non-effective.

It is clear that a better understanding of the physiology involved in ameliorating salinity stress with chemicals is needed if these treatments are to become cost effective and widely adopted (Ashraf and Foolad 2007; Shabala and Munns 2012). It is known that polyamines (Shabala et al. 2007) and compatible solutes (Cuin and Shabala 2005) have been shown to reduce Na^+ induced K^+ efflux. These three classes of chemicals also have other roles within plants. Polyamines have the ability to stabilise a range of proteins including those that are involved in photosynthesis (Kotzabasis et al. 1993; Hamdani et al. 2011). Compatible solutes have multiple roles under stress responses, they provide osmotic adjustment, act as molecular chaperones (Hare et al. 1998; Chen and Murata 2002) and in some cases can detoxify ROS (Smirnoff and Cumbes 1989). Antioxidants can detoxify ROS preventing ROS induced damage to proteins and lipids that are essential for photosynthesis. What is not well understood is how these chemicals act in chloroplasts under saline conditions, when cytosolic Na^+ concentrations are elevated and K^+ concentrations are reduced. These chemicals may be able to directly mediate the effects of altered ionic homeostasis on chloroplasts increasing photosynthetic parameters or may in fact interfere with processes in chloroplasts under saline conditions.

Overview

It is clear that for plants to be truly tolerant to salinity, they need to be able to accumulate higher concentrations of Na^+ in the shoot to provide osmotic adjustment, at the minimal energetic cost. However, many adverse effects may be potentially associated with Na^+ accumulation in the shoot. This implies an orchestrated regulation of many signalling cascades and downstream effectors, including a large number of plasma-, tonoplast- and chloroplast- based membrane transporters.

One of the major side effects of increased salinity is K^+ loss from the photosynthesising mesophyll tissues. A better understanding of the mechanisms of K^+ loss from leaf mesophyll, therefore, maybe instrumental in developing better strategies for K^+ retention under salinity treatment and minimising detrimental effects of salinity on cell metabolism. To further investigate these mechanisms the non-

invasive MIFE technique is used to measure the ion fluxes of K^+ and H^+ from bean mesophyll in response to salinity treatment. A variety of pharmacological agents were used to determine the contributions of different channels and H^+ gradients to salinity induced K^+ leak.

Ion homeostasis in the cytosol of mesophyll cells is altered under salinity stress. Little is known about how these altered ionic conditions affect chloroplasts. In chapter 3 chlorophyll fluorescence measurements were used to assess the effect of Na^+: K^+ ratios on photosynthesis in isolated chloroplasts. It has often been assumed that halophytes and glycophytes are similarly affected by altered ionic homeostasis; however, there are no published reports of this being tested in direct experiments. Chlorophyll fluorescence was used to determine if there were any differences between chloroplasts from glycophytes and halophytes in their ability to deal with altered ionic homeostasis. The effect of chloroplast ion channels in maintaining photosynthesis in altered ionic environments was also assessed with a range of pharmacological agents.

As mentioned above the evidence for the effects of ameliorative chemicals on plants under salinity stress has been largely empirical and based on trial and error approach. In chapter 4 the effect of different ameliorative chemicals on leaf photochemistry and primary photosynthetic processes in chloroplasts, was assessed under saline conditions, using broad beans as a model species. A variety of compatible solutes, polyamines and antioxidants were tested and used to explain controversies reported in the literature and a failure of these substances to ameliorate detrimental effects of salinity in some experiments.

While it is clear that halophytes have a better ability to control ion homeostasis under saline conditions the different mechanisms that halophytes apply in mesophyll to maintain ion homeostasis are less clear. In chapter 5, the MIFE technique was used investigate these mechanisms. Differences in ion transport between glycophytes and halophytes were investigated and linked to differences in growth and photosynthesis.

The major aim of this work was to investigate mechanisms of non-stomatal limitation to photosynthesis caused by salinity stress and link them with ionic relations and ion transport across mesophyll cell plasma and chloroplast membranes. By doing this the following objectives were addressed:

- To understand effects of salinity on ionic homeostasis and light-induced changes in ion transport in leaf mesophyll, in the context of leaf photosynthetic and growth responses.
- To eludicate effects of altered ionic homeostasis and cytosolic osmolality on photosynthetic performance in chloroplasts.
- To reveal differences between chloroplast responses to salinity from species contrasting in salinity tolerance, e.g. halophytes and glycophytes.
- To the link ion transport in the chloroplast and their resilience to perturbed ionic homeostasis.
- To investigate effects of ameliorative chemicals (polyamines, compatible solutes and antioxidants) on salinity-induced disturbance to leaf photosynthetic machinery, at the chloroplast level.
- To understand the differences in mesophyll ion transport between species with contrasting salinity tolerance and link them with primary photosynthetic processes in green leaf tissues.

The thesis is written in a "by publication" format, with each of four experimental chapters being a stand-alone paper. One of these papers has already been published (Chapter 2; Percey et al (2014) *Planta.* DOI 10.1007/s00425-014-2117-z), two are currently under review with *Photosynthesis Research* (Chapter 3; submitted on 12/06/14), and *Plant and Soil* (Chapter 5; submitted on 29/07/14), and one will be submitted shortly to *Envirnmental and Experimental Botany* (Chapter 4)

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Chapter 2: Ion transport in broad bean leaf mesophyll under saline conditions

William J. Percey[1], Lana Shabala[1], Michael C. Breadmore[2], Rosanne M. Guijt[3], Jayakumar Bose[1], Sergey Shabala[1]

[1]School of Land and Food, University of Tasmania, Hobart, Tasmania 7001, [2]Australian Centre for Research on Separation Science (ACROSS), School of Chemistry, University of Tasmania 7001 and [3]School of Pharmacy, University of Tasmania, Hobart, Tasmania 7001

Abstract

Modulation in ion transport of broad bean (*Vicia faba* L.) mesophyll to light under increased apoplastic salinity stress was investigated using vibrating ion selective microelectrodes (the MIFE technique). Increased apoplastic Na^+ significantly affected mesophyll cells ability to respond to light by modulating ion transport across their membranes. Elevated apoplastic Na^+ also induced a significant K^+ efflux from mesophyll tissue. This efflux was mediated predominately by potassium outward rectifying (KOR) channels (84%) and the remainder of the efflux was through non-selective cation channels (NSCC). NaCl treatment resulted in a reduction in photosystem II efficiency in a dose- and time-dependent manner. In particular, reductions in Fv'/Fm' were linked to K^+ homeostasis in the mesophyll tissue. Increased apoplastic Na^+ concentrations induced vanadate-sensitive net H^+ efflux, presumably mediated by the plasma membrane H^+-ATPase. It is concluded that the observed pump's activation is essential for the maintenance of membrane potential and ion homeostasis in the cytoplasm of mesophyll under salt stress.

Keywords: Potassium, Flux, Photosynthesis, H^+-ATPase, Membrane potential

Abbreviations: DMSO, Dimethyl sulfoxide; KOR, Potassium outward Rectifying channels; NHX, Sodium/proton exchanger; NSCC, Non selective cation channels; PCD, Programmed cell death; ROS, Reactive oxygen species; TEA, Tetraethylammonium

Published in: *Planta* (2014) DOI 10.1007/s00425-014-2117-z

Introduction

Elevated NaCl concentrations in the soil solution observed under saline conditions result in significant limitation to photosynthesis. Two major components – stomatal and non-stomatal – are distinguished (Brugnoli and Lauteri 1991). A salt concentration of 4 dSm^{-1} (or 40 mM NaCl) is considered as the threshold of salinity and has an osmotic pressure of about 0.2 MPa, which affects the ability of plants to take up water (Shabala and Munns 2012). This imposes an osmotic stress on the plant causing stomatal closure leading to reduced CO_2 assimilation, additionally slower formation of photosynthetic leaf area reduces the flow of assimilates to growing leaf tissues (Munns and Sharp 1993). Moreover, reduced CO_2 assimilation is a double edged sword not only directly reducing the amount of photosynthesis but also increasing undesirable photorespiration (Noctor et al. 2002). Apart from osmotic stress, salinity stress also results in significant accumulation of Na^+ in the shoot, with apoplastic leaf Na^+ concentrations often exceeding 80 mM which is at least 30 times that which occurs in non-saline environments (Speer and Kaiser 1991). As a mesophyll cells' ability to pump Na^+ out of cytosol is limited, this (or a significant part of) Na^+ will enter the cytosol potentially inhibiting primarily photosynthetic reactions in chloroplasts. This phenomenon is defined as a non-stomatal limitation of photosynthesis. While most research so far has focused on the stomatal limitations (Sirault et al. 2009; Rajendran et al. 2009) specific details of inhibitory salinity effects on leaf photochemistry remain largely unexplored.

Despite the ability of plants to prevent Na^+ uptake by roots and to reduce Na^+ xylem loading being considered as the most essential features of salinity tolerance in glycophytes, many plants have only a limited ability to do this (Bose et al. 2013). Once the limitation of Na^+ exclusion from the transpiration stream is reached, the apoplastic concentration of Na^+ rises dramatically and plants will be left to deal with the consequences of increased apoplastic salinity. The latter includes, among other things, massive K^+ leak from the cytosol (Shabala 2000), reductions in photosynthetic viability (Shabala et al. 2010), increased ROS production (Miller et al. 2010), and reduced stomatal aperture (Brugnoli and Lauteri 1991).

Mesophyll cells need to do at least three things to overcome the high salinity levels reached in leaf apoplast: (i) sequester Na^+ in leaf vacuoles; (ii) prevent K^+ from leaking out and (iii) deal with stress-induced ROS production. The reasons for this are given below.

(i) Increased Na^+ pumping into the vacuole has two major positive effects: reducing the effects of otherwise phytotoxic cytosolic Na^+ (Na^+ increases the ionic strength of the cytosol disturbing the tertiary and quaternary structures of proteins; it also displaces K^+ from sites of enzyme activation rendering the enzyme inactive) and providing cells with a cheap osmoticum required for turgor maintenance (and, hence, tissue growth). Sequestration of Na^+ in vacuoles is achieved by operation of tonoplast Na^+/H^+ exchangers (NHX transporter in Arabidopsis). NHX expression has been shown to provide increased levels of salinity tolerance in several species (Apse et al. 1999).

(ii) K^+ is an essential nutrient for many cellular functions including enzyme activation, osmoregulation, cell extension, stomatal movement, phloem loading, photosynthesis, protein synthesis and respiration (Dreyer and Uozumi 2011; Marschner 1995). With over 50 metabolic enzymes activated by K^+ (Marschner 1995), plant ability to maintain cytosolic K^+ homeostasis is considered to be a critical feature of salinity tolerance in plants (Anschütz et al. 2014). Given that K^+ transport across cellular membranes is mediated by a large number of membrane transporters (at least 75 in Arabidopsis; Véry and Sentenac 2003; Dreyer and Uozumi 2011), prevention of K^+ leak is not a trivial task. Indeed, multiple K^+ leak conductances may be activated by salinity stress; among these, K^+ outward rectifying channels (KOR, depolarisation activated unidirectional K^+ selective channels) and non-selective cation channels (NSCC; a diverse group of bidirectional channels; the most important in this case are the ROS activated channels) are the major ones (Shabala et al. 2007). Na^+ entry into the cell causes membrane depolarisation instigating the K^+ leak through depolarization-activated KOR channels (Shabala et al. 2006). Na^+ accumulation in the cytosol also triggers ROS production, with ROS-activated NSCC providing an additional avenue for further K^+ leakage (Shabala et al. 2007; Rodrigo-Moreno et al. 2012).

(iii) As mentioned above, salinity stress results in a dramatic rise in ROS levels in leaf mesophyll (Mittler 2002). The major sources of ROS production are chloroplasts, mitochondria, peroxisomes and NADPH oxidase (Mittler 2002). This increase in ROS results in damage to DNA, RNA, proteins, enzymes, lipids and can lead to cell death either though Programed Cell Death (PCD) or through oxidative damage. There are two possible ways to prevent damage by ROS: prevention of ROS production *per se*, or ROS scavenging. Reduction of K^+ losses and/or Na^+ accumulation may control ROS production. Similar to mammalian cells, reduction in cytosolic K^+ concentration leads to the activation of caspase-like enzymes, resulting in a programmed cell death (Lam and del Pozo 2000). It was also shown that ROS scavenging involves the production of enzymes and antioxidants to protect vital cellular processes (Mittler 2002; Bose et al. 2013). However, the effects of ROS on light responses and ion fluxes in mesophyll under salinity stress are not fully understood.

All three above components rely heavily on tight control over the ionic exchange across the mesophyll plasma membrane under saline conditions. Tightly controlled ion exchange is essential for growth, photosynthesis and signalling. K^+ is essential to drive expansion growth in new leaves to provide the turgor pressure for cellular expansion through osmotic potential (Van Volkenburgh 1999). Maintenance of K^+ in the cytosol becomes critical under stress conditions as it is linked to PCD (Lam and del Pozo 2000), phloem loading (Lacombe et al. 2000), ribosome function (Foucher et al. 2012), and ribulose-1,5- bisphosphate carboxylase activity (Viitanen et al. 1990).

Apart from K^+ and Na^+, other ions were shown to play important roles in plant survival under stress conditions. Proton efflux is critical in providing the driving force for many other active transport processes. It also loosens the cell wall through acidification activating expansins which break hydrogen bonds between celluloses microfibrials and hemicellulose allowing turgor pressure to expand the cell (Van Volkenburgh 1999). H^+ pumping is also a major electrogenic source defining cell membrane potential (Sze et al. 1999); it is also required for the uptake of essential nutrients (including K^+), as well as for pH homeostasis (Sze et al. 1999). There is also tight control over Ca^{2+} transport and cytosolic Ca^{2+} homeostasis, as Ca^{2+} is used as a key second messenger for signalling during all forms of stresses, both abiotic and biotic (Dodd et al. 2010). Ca^{2+} is also important in growth regulation (Hepler et al.

2001), maintenance of stomatal aperture and for light and circadian signals (Ng et al. 2001).

Fluctuations in light intensity are known to not only modulate the rate of net CO_2 assimilation resulting from altered electron transport rate but also change stomatal conductance characteristics. Light is also essential for the initiation and synchronisation of gene expression and many physiological processes (Chen et al. 2004). Signal transduction and the processes themselves cause significant perturbations to ion transport in mesophyll cells. For example, light-induced changes in membrane-transport activity have been reported for various leaf tissues such as epidermis (Elzenga et al. 1995; Shabala and Newman 1999), mesophyll (Elzenga et al. 1995; Shabala and Newman 1999) and guard cells (Kinoshita and Shimazaki 1999), as well as in chloroplast envelope (Kreimer et al. 1985) and thylakoid membranes (Spetea and Schoefs 2010). Chlorophyll pigments are used for adsorbing and transferring light energy. While the vast majority of chlorophyll is used to transfer energy to the chlorophyll in the reaction centres of photosystems I & II there are also many other pigments in chloroplasts that are used for light and heat dissipation under high light stress to prevent oxidative damage of the chloroplasts (Green and Durnford 1996). Additional to the pigments are the photoreceptors: phototropins and cryptochromes which are responsible for UV-A/blue light perception (Christie 2007) while phytochromes are responsible for red light and far red light perception (Quail 2002). Although these receptors are more sensitive to certain types of light there is also some overlap in sensitivity (Chen et al. 2004). The phytochromes and cryptochromes are responsible for the regulation of timing growth in response to light while phototropins are responsible for growth in response to the direction of light, i.e. chloroplast movement in response to light intensity (Quail 2002). While the light receptors are well known, the downstream signalling and responses to light are less well understood. The light receptors seem to be highly tissue specific eliciting a reduction in growth in the hypocotyl (Chen et al. 2004) and increasing growth in the epidermis (Živanović et al. 2005). These differences in response occur due to differences in downstream signal transductions from the photoreceptors. Different receptors also elicit different responses as demonstrated by red and blue light having two separate activation methods of the proton pump in leaf epidermal cells of pea independent of photosynthesis (Staal et al. 1994). Red light has been shown to cause a

short lived spike in cytosolic Ca^{2+} concentration (Shacklock et al. 1992); this is believed to be responsible for the observed light-induced membrane depolarisation (Shabala and Newman 1999). K^+ and Cl^- fluxes have been shown to have a delayed response to light as they start at the peak of membrane depolarisation (Shabala and Newman 1999). All these results suggest that light-induced modulation of ionic exchange across the plasma membrane is absolutely essential for normal plant metabolism and functioning, and that the extent of their modification by apoplastic Na^+ may be taken as a measure of salt-induced damage to the mesophyll tissue.

Cytosolic Na^+ is known to cause K^+, H^+ and Ca^{2+} efflux from mesophyll (Shabala 2000). As discussed above these ions are all important for photosynthesis and light signal transduction. Perturbations to the homeostasis of these ions by Na^+ will affect the normal response of these tissues to light. In this work, we have hypothesised that exposing plants to elevated salinity levels will affect their ability to respond to light. We propose that by measuring the extent of the impact of salinity on the magnitude of light-induced net ion fluxes may offer a novel tool for assessing damage to mesophyll tissues. To the best of our knowledge, such an approach has not yet been reported in the literature.

The non-invasive MIFE technique provides the ability to measure ion fluxes of interest with high temporal (a few seconds) and special (several μm) resolution, providing a contribution to understanding ionic relations in mesophyll tissue under saline conditions. In this study transient K^+ and H^+ ion fluxes from bean mesophyll tissue are measured in response to salt treatment as well as exploring the effects of salt treatment on light response in K^+ and H^+ fluxes. The aim of this study was to validate the above hypothesis and quantify the effect of apoplastic Na^+ on ion fluxes and light responses in the mesophyll tissue.

Materials and methods

Plant material

Broad beans (*Vicia faba* L cv Oswald; Hollander Imports, Hobart, Australia) were grown from seed in 0.5 L plastic pots under controlled greenhouse conditions (temperature between 19 ^{o}C and 26 ^{o}C; day length 12-14 h; average humidity ~65%) at the University of Tasmania between August and November 2012. The potting mixture included 70% composted pine bark, 20% course sand, 10% sphagnum peat (pH 6.0) which was fertilised (1.8 kg m^{-3} Limil, 1.8 kg m^{-3} dolomite, 6.0 kg m^{-3} Osmocote Plus and 0.5 kg m^{-3} ferrous sulphate). Plants were irrigated twice daily with tap water to maintain potting mix at full field capacity. Plants were grown for four to six weeks. The newest fully expanded leaves were used for all measurements.

Ion flux measurements

Net fluxes of H^{+} and K^{+} were measured non- invasively using vibrating ion-selective microelectrodes (the MIFE technique; University of Tasmania) as described previously (Shabala and Newman 1999). Microelectrodes were prepared from borosilicate glass capillaries (GC 150-10, Harvard apparatus Ltd, Kent, UK) by pulling capillaries on a vertical puller (PP-830, Narishige, Tokyo, Japan) and oven drying them overnight at 230°C. The dried electrodes were silanised in the oven for 10 min at 230 °C using 55 µl tributylchlorosilane (Fluka, catalogue no. 90796) added to 1.5 L volume under the cover, and then heated at the same temperature for a further 30 min. Electrode tips were broken to achieve external tip diameters of 2 to 3 µm by moving electrode blanks against a flat glass surface using a micromanipulator. Electrodes were then backfilled with corresponding back filling solutions as specified in Table 1 followed by front-filling with appropriate ion selective cocktail (Table 1).

Table 1 Details of back-filling solutions and liquid ionic exchangers (LIX) used in this study

Ion	Back filling solution	LIX
H^+	15 mM NaCl+40 mM KH_2PO_4, adjusted to pH 6.0 using NaOH	Hydrogen ionophore II - cocktail A (95297, Fluka)
K^+	0.2 M KCl	Potassium ionophore I - cocktail A (60031, Fluka)

Once prepared electrodes were calibrated in an appropriate set of standards encompassing measured ranges of particular ions using a three-point calibration. Electrodes with a Nerst slope of less than 50 mV per decade and correlation less than 0.999 were discarded from measurements. One hour prior to measurements leaf segments were immobilised in the measuring chamber, and electrode tips were positioned 40 μm above the leaf segment, with their tips aligned and separated by 1-3 μm, using a 3D micro manipulator (MMT-5, Narishige). For flux measurements, a computer-controlled stepper motor moved electrodes in a 6 s square wave cycle between two positions, 40 μm and 80 μm from the specimen. The CHART software (see Shabala and Newman 1999 for details) recorded the electrochemical potential difference between the two positions, and the MIFEFLUX software was used to convert this potential differences into net ion fluxes using the previously recorded calibration files.

Ion flux measuring protocols

The first fully expanded leaf was removed and brought into laboratory in a sealed plastic bag. The abaxial epidermis was pealed off using a fine forceps. Pealed leaves were immediately cut into 5x7 mm segments and placed pealed side down floating on a shallow layer of Basic Salt Media (BSM) solution (1 mM NaCl; 0.5 mM KCl; 0.1 mM $CaCl_2$; pH 5.7 non-buffered) in 35 mm Petri dishes. The segments were left

floating in the dark overnight and used for measurements next day. This time (10 to 12 h) was sufficient for all possible confounding wounding responses to have ceased (see Živanović et al. 2005 for justification), ensuring high reproducibility of all results.

Segments were mounted in a Perspex sample holder and placed into a 6 ml-measuring chamber. Mounted samples were left to acclimatise to ionic and light conditions for 1 h prior to commencing recordings. Two different types of measurements were conducted. For transient flux measurements, the sample was placed in 4 ml of BSM solution, and net ion fluxes were recorded under control conditions for 5 to 10 min, under dim green microscope light (10 Wm^{-2}). Then 2 ml of the respective Na^+ solution made up in BSM was added to the container, thoroughly mixed with a pipette, and net ion fluxes measured for another 60 min.

Another experimental protocol, defined as "light cycles" in this work, implied exposing the mesophyll segment to periodical light/dark fluctuations. Samples were left floating on the surface of appropriate treatment solution (containing various amounts of salts or chemical agent) for the prescribed amount of time before being mounted in the measuring chamber with 5ml of their respective solution. After 1 h of acclimation period, leaf segments were exposed to rhythmical (5 min light; 5 min dark) light cycles provided by white optic fibre light source. The light intensity at the surface of the specimen was 100 W m^{-2}. The overall duration of light/dark exposure was between one and three h. Net ion fluxes were recorded at 6 s intervals, and then plotted against the time.

Membrane potential measurements

Leaf specimens were prepared, mounted and treated as in MIFE experiments. Membrane potential (MP) measurements were made using a method previously described (Shabala and Newman 1999). In brief, glass microelectrodes (GC 150-10F, Harvard apparatus Ltd) were pulled to achieve a tip diameter of ~ 0.5 μm. Electrodes were back filled with 0.5 M KCl and connected to the MIFE amplifier via an Ag/AgCl bridge. Membrane potentials were measured by impaling microelectrode into mesophyll tissue to achieve the stable recordings for at least 30 s.

Pharmacology

The following metabolic inhibitors (agents) were used:

- Dimethyl sulfoxide (DMSO; VWR 23486.322; effective working concentration 0.03% w/v), a known antioxidant. DMSO is equally soluble in both water and lipid systems and can easily cross membranes without the use of trans-membrane proteins (Sanmartín-Suárez et al. 2011). DMSO has been used previously as a ROS scavenger in planta at the 0.03% w/v (Demidchik et al. 2010, Pottosin et al. 2014);
- Tetraethylammonium (TEA; 20 mM working concentrations); a known K^+ channel blocker. TEA inhibits K^+ outward rectifying (KOR) channels (shabala et al. 2006) without effecting NSCC (Demidchik 2014);
- Gadolinium chloride ($GdCl_3$; 100 μM); a known blocker of non-selective cation channels (NSCC) (Pottosin et al. 2014);
- Vanadate (Na_2VO_4, 1 mM) a known inhibitor of H^+-ATPase (Michelet & Boutry 1995);

For transient measurement, the pharmacological agent was added at the time of mounting the sample in the chamber, e.g. 1 h before commencing salinity treatment. When using "light cycle" protocols, an appropriate pharmacological agent was added at the time of pealing.

Chlorophyll fluorescence measurements

Leaf segments were prepared in the same manner as for MIFE experiments and then left floating in the Petri dishes on the surface of BSM solution containing required amounts of NaCl under constant light (55 W m-2) and room temperature (22-24 oC) conditions for up to 4 days. Chlorophyll fluorescence was measured with a pulse-amplitude modulation portable fluorometer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany) in conjunction with a leaf-clip holder 2030-B with integrated micro-quantum-sensor and temperature sensor (Walz GmbH, Effeltrich, Germany). 20 min before measurements, the light was turned off, and leaf samples were allowed to adapt to the ambient room light (0.8 W m-2). Fv'/Fm' measurements were then taken in the saturation pulse method described in the Mini-PAM manual. Because the

samples were not completely dark-adapted, starting Fv'/Fm' values were around 0.65 (not 0.8 as expected for fully dark-adapted specimens), reflecting the point that not all electrons were drawn from plastoquinone electron acceptor.

Data analysis

The statistical significance of difference between means was determined by the Student's t-test. The peak amplitudes of light-induced K^+ and H^+ flux oscillations were determined by applying the Discrete Fourier Transform (DFT) using SANTIS software (University of Aachen, Germany)(see Shabala et al. 2001 for details). Mean baseline flux values were analysed by the simple data averaging over the appropriate time interval.

Results

Sodium ion concentrations in the apoplast of glycophytes are usually low (< 5 mM) while under salt stress the concentration can increase to over 100 mM (Speer and Kaiser 1991). Here we have considered three possible scenarios: (i) mild salinity stress (20 mM NaCl) found at conditions when plants are capable to control Na^+ delivery to the shoot by efficient exclusion from uptake and/or by control of Na^+ xylem loading, (ii) severe saline stress (100 mM NaCl) mimicking the situation when the above mentioned defence mechanisms fail and the apoplastic content of Na^+ increases dramatically, and (iii) an intermediate scenario (50 mM NaCl).

Increased Na^+ levels in leaf apoplastic space resulted in time- and dose-dependent inhibition of PSII activity, as evident by chlorophyll fluorescence measurements (Fig. 1). Interestingly, even the highest salinity treatment (100 mM NaCl added to leaf mesophyll tissue) did not affect Fv'/Fm' value (maximum photochemical efficiency of PSII) for at least 16 h (Fig. 1). After that, Fv'/Fm' dramatically declined reaching values as low as 0.15 by the end of experiment (~80 h after stress onset). Significant effects of 50 mM NaCl treatment were observed 36 h after stress onset, while lowest (20 mM) treatment inhibited PSII only after 48h (Fig. 1).

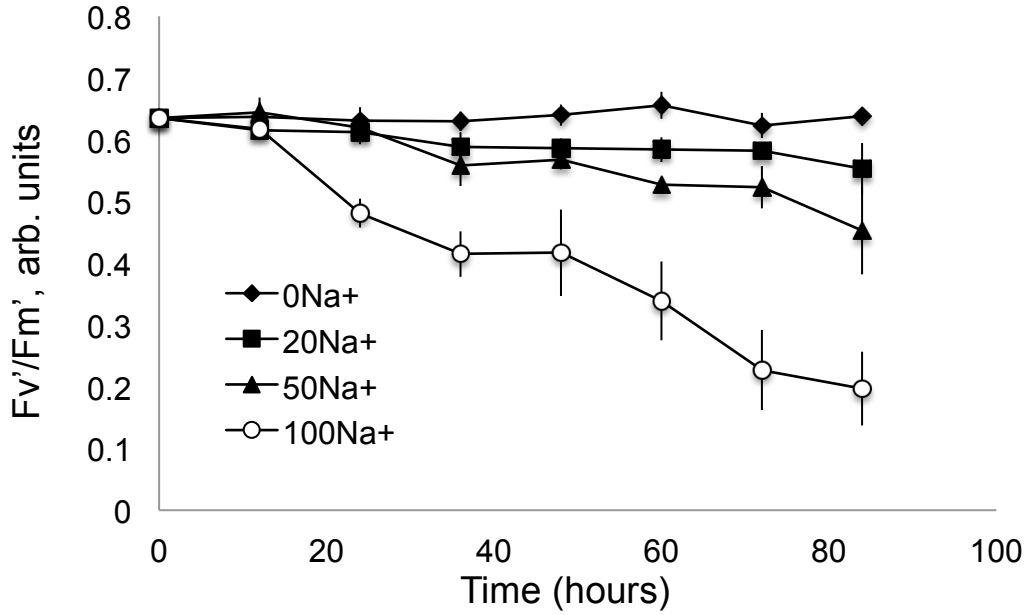


Fig. 1 Maximum photochemical efficiency of PSII (chlorophyll fluorescence Fv'/Fm' values) of bean mesophyll in response to four levels of salinity over 84 h period. Mean ± SE (n=6)

High and low NaCl concentrations were then used to study effects of apoplastic Na^+ on membrane-transport activity in mesophyll cells. The addition of NaCl resulted in an immediate K^+ efflux. For 20 mM NaCl, this efflux was short-lived and ceased after 15 min (Fig. 2a). The addition of 100 mM NaCl led to a more dramatic perturbation to K^+ fluxes, resulting in a sustained K^+ efflux of 120 nmol m^{-2} s^{-1} 50 min after NaCl addition (Fig. 2a). As a result, the total amount of K^+ leaked over 50 min of salt treatment with 100 mM NaCl was ~ 40 times greater than the total amount leaked with 20 mM NaCl treatment (Fig. 2b).

NaCl treatment also induced a pronounced increase in net H^+ efflux from mesophyll tissue (Fig. 2a). The H^+ efflux after the addition of 20 mM NaCl treatment was much less than that of the efflux that occurred after the addition of 100 mM treatment, showing that the H^+ efflux was dose-dependant. For 20 mM treatment there was an initial spike in the H^+ efflux of 140 nmol m^{-2} s^{-1}, this was followed by a decline back

to 50 nmol m^{-2} s^{-1}, with a steady state efflux of 75 nmol m^{-2} s^{-1} achieved 50 min after the treatment (Fig. 2a). The 100 mM treatment had a very different pattern of response. Here, a gradual increase in H^+ efflux to 200 nmol m^{-2} s^{-1} was measured for 20 min after the treatment. After reaching a peak, H^+ efflux then declined to 90 nmol m^{-2} s^{-1} 50 min after addition of the salt (Fig. 2a). The decline in H^+ efflux corresponded with the increase in K^+ efflux (Fig. 2a). For the 100 mM treatment there is a strong linear relationship between H^+ and K^+ flux from 15 min after treatment, with a ratio of approximately 1 to 1 (Suppl Fig. S1a). This relationship is less pronounced under the 20 mM treatment (Suppl Fig. S1b).

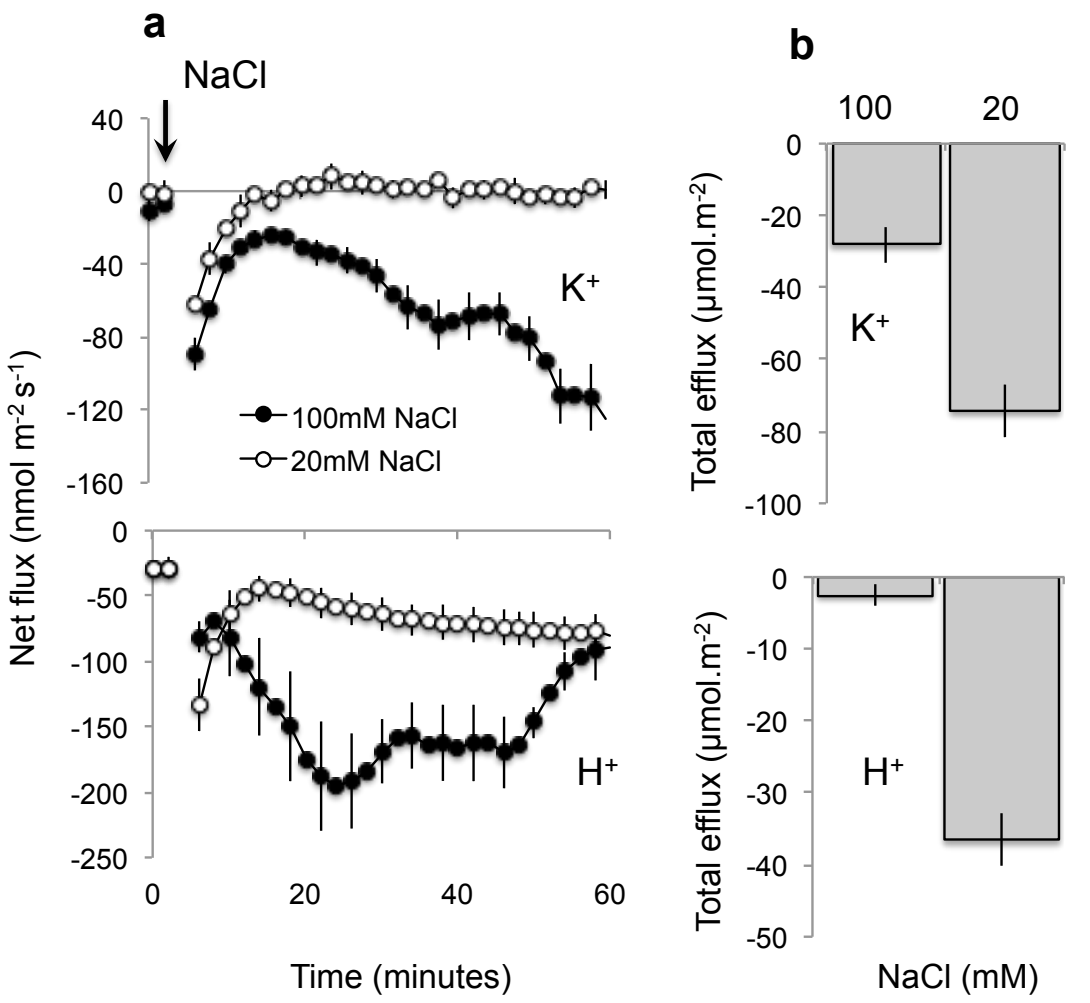
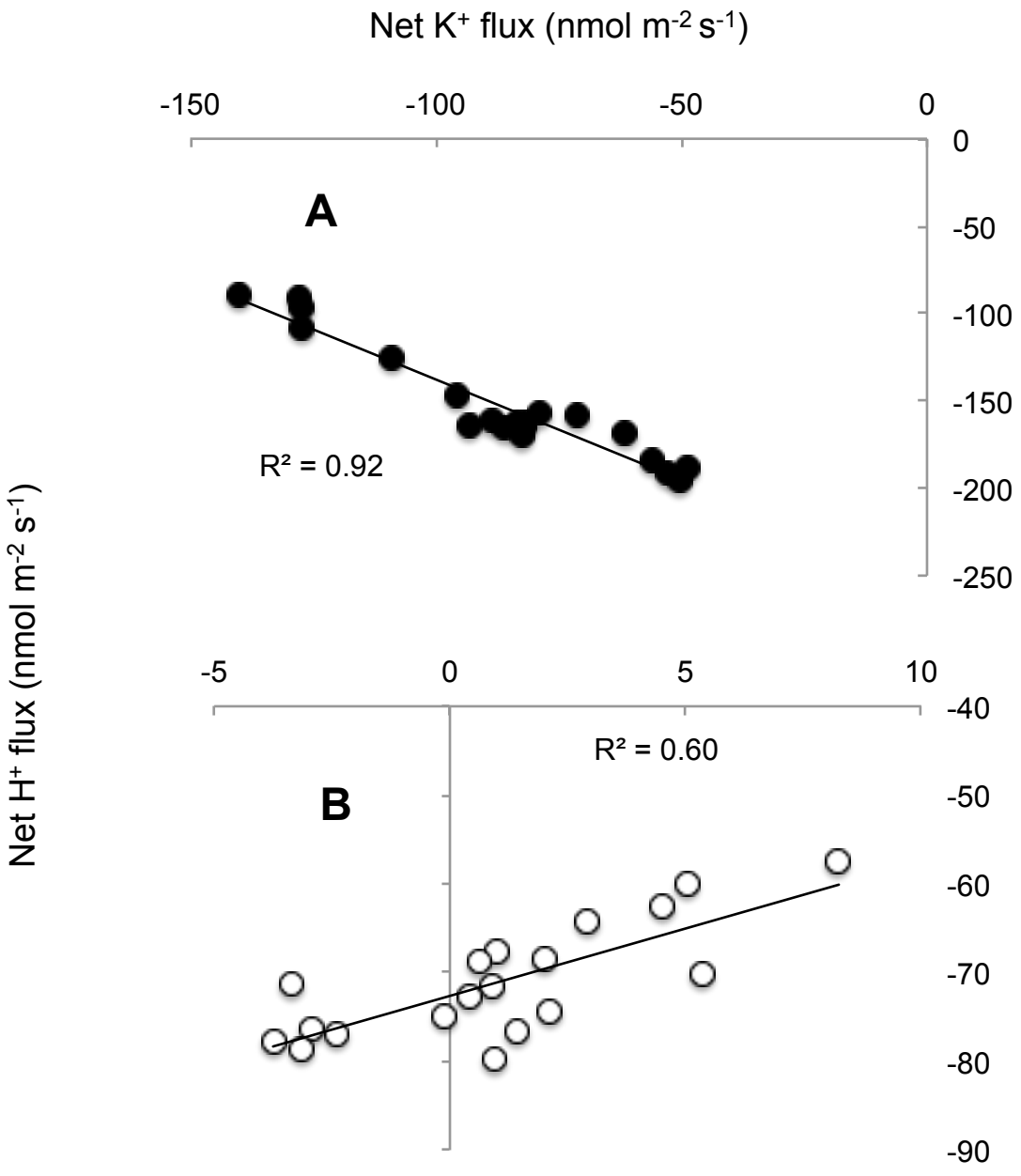


Fig. 2a - transient net K^+ and H^+ flux kinetics measured from mesophyll cells in response to two levels of salt stress. **b** - total K^+ and H^+ flux over the 50 min after Na^+ addition. Mean ± SE (*n*=3-6)



Suppl Fig. S1 a relationship between net H^+ efflux and net K^+ flux measured from leaf mesophyll from 15 min after treatment with NaCl. **a -** 100mM NaCl; **b** - 20mM NaCl. One (of six) representative examples is shown for each treatment.

The identity of specific ionic mechanisms that mediated the above NaCl-induced fluxes from leaf mesophyll was further investigated in a series of pharmacological experiments. 1 mM vanadate (a P-type H^+-ATPase inhibitor) caused a drastic

reduction of the proton efflux induced by 100 mM NaCl treatment, as did 20 mM TEA (K^+ channel blocker) (Fig. 3). 100 µM $GdCl_3$ (NSCC blocker), and 0.03% w/v DMSO (a known ROS scavenger) also reduced H^+ efflux, albeit to a lesser extent (Fig. 3).

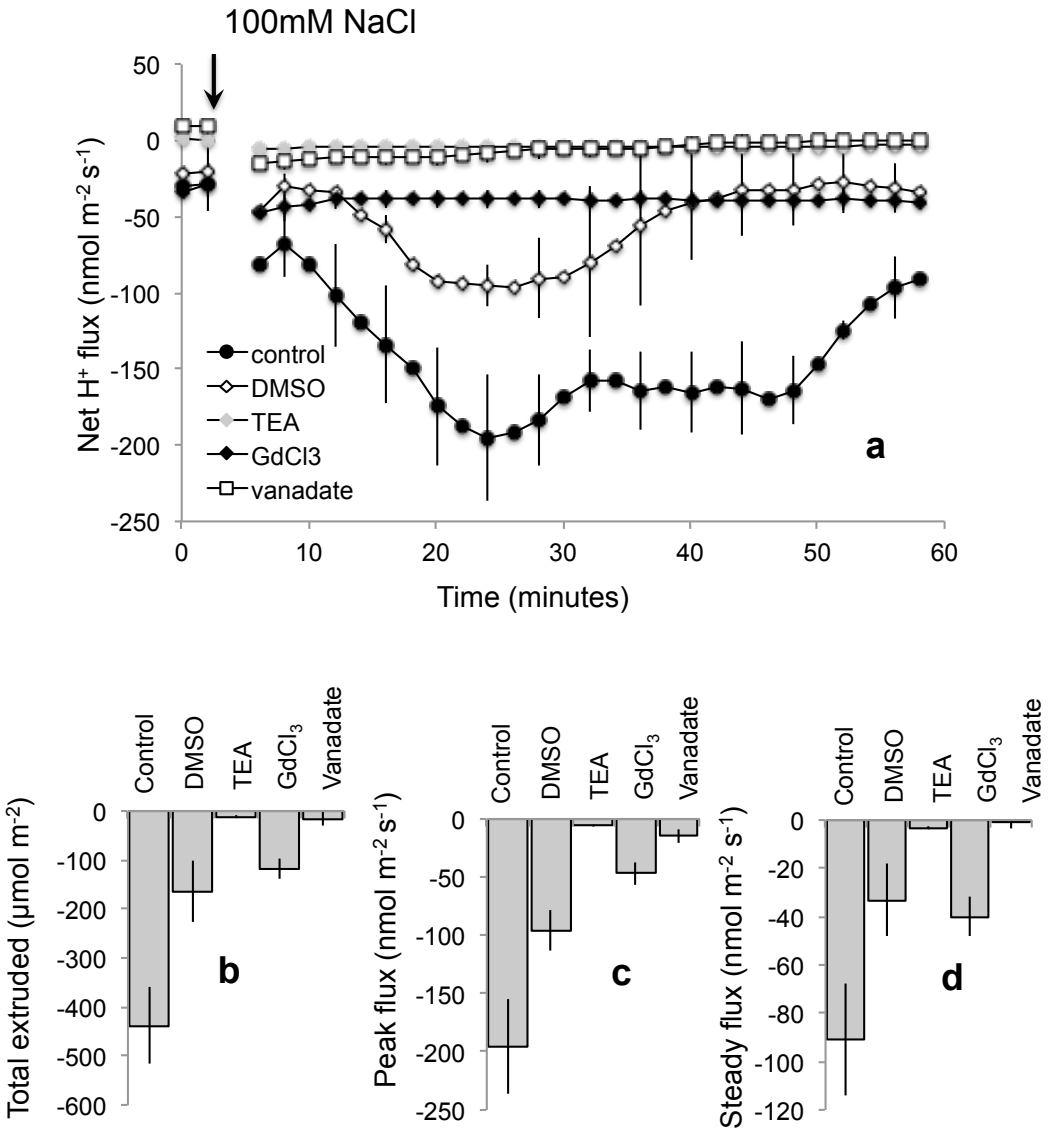


Fig. 3 Effect of pharmacological agents on NaCl induced H^+ flux. **a** - transient net H^+ flux kinetics in response to 100 mM NaCl treatment measured from mesophyll tissue pre-treated for 1 h in a solution containing specific metabolic inhibitor or a channel blocker. **b** - total amount H^+ extruded from mesophyll tissue over 50 min after the addition of Na^+. **c** – peak H^+ flux measured in response to salinity treatment. **d** - steady-state net H^+ flux 50 min after salinity treatment. Mean ± SE (*n*=5-6)

The above pharmacological agents also strongly affected NaCl-induced responses in net K^+ fluxes. TEA and $GdCl_3$ both caused significant reductions in NaCl-induced K^+ efflux (Fig. 4). Vanadate exacerbated the amount of K^+ efflux (Fig. 4). DMSO had no effect on the amount of K^+ efflux until 50 min after treatment where it caused a halving of the efflux. Steady-state fluxes prior to NaCl treatment were also affected (Fig. 4), reflecting metabolic alteration in channel's activity caused by pharmacological agents.

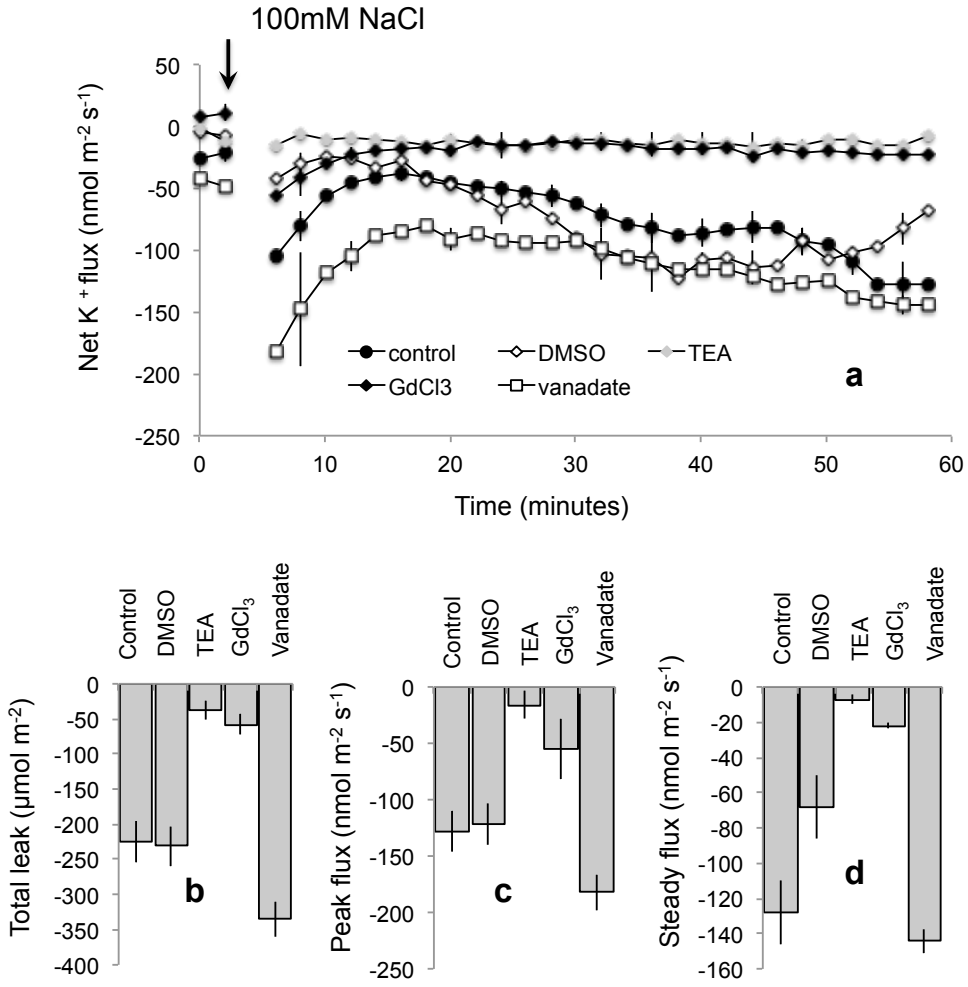


Fig. 4 Effect of pharmacological agents on NaCl induced K^+ flux. **a** - transient net K^+ flux kinetics in response to 100 mM NaCl treatment measured from mesophyll tissue pre-treated for 1 h in a solution containing specific metabolic inhibitor or a channel blocker. **b** - total amount K^+ extruded from mesophyll tissue over 50 min after the addition of Na^+. **c** – peak K^+ flux measured in response to salinity treatment. **d** - steady-state net K^+ flux 50 min after salinity treatment. Mean ± SE (*n*=5-6)

Light/dark induced ion fluxes in leaves are important for the regulation of photosynthesis and plant growth. It has been shown that light induces a K^+ efflux (Shabala and Newman 1999) and activates proton pumping (Shimazaki et al. 1992) in mesophyll tissues. It is known that Na^+ disrupts normal ion homeostasis in mesophyll cells (Shabala 2000) and, as such, could affect the "normal" light/dark cycle-induced changes in K^+ and proton fluxes. Thus, the magnitude of light-induced fluxes could be used to assess the damage done by Na^+ in both the short- and long-terms.

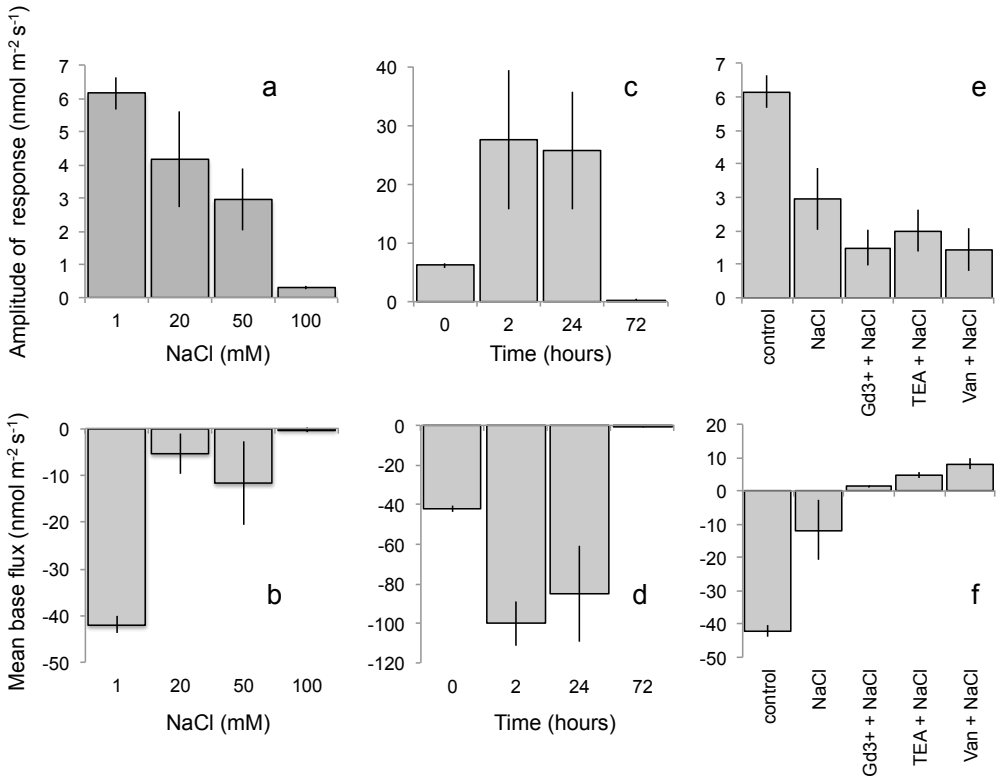
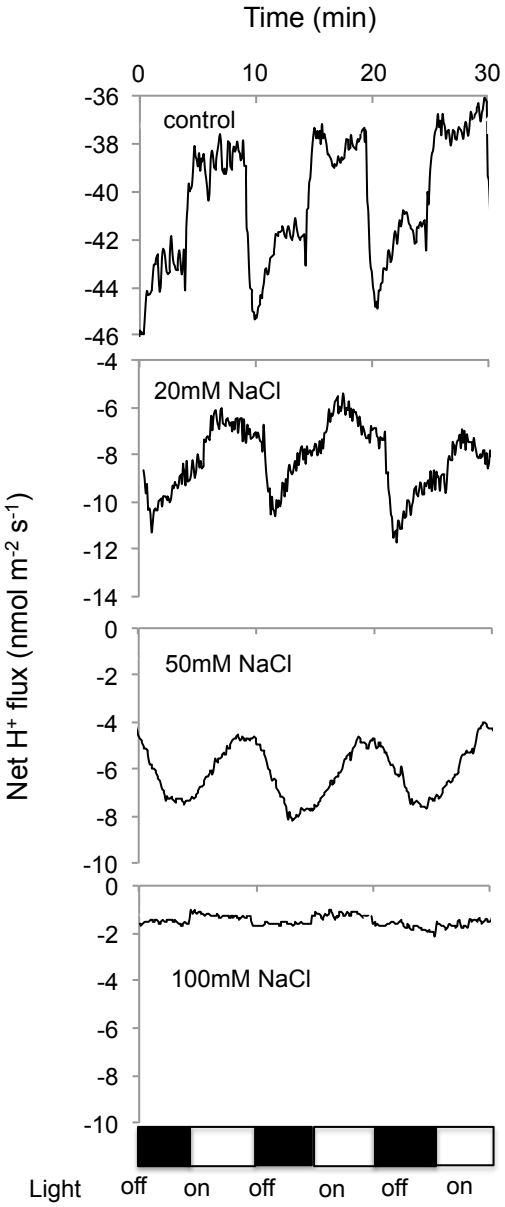


Fig. 5 Dose (**a, b**) - and time (**c, d**) – dependence of the amplitude and mean basal net H^+ flux responses from leaf mesophyll upon light/dark fluctuation. In panels A and B, measurements were taken after 72 h of salinity exposure. In panels **c** and **d**, salinity treatment was 100 mM NaCl. **e**, **f** – amplitude (**e**) and mean basal (**f**) flux responses to light/dark fluctuations measured from mesophyll tissue exposed to 100 mM NaCl and pre-treated for 1 h in a solution containing specific pharmacological agents. Mean ± SE (*n*=5-6)

The ability of leaf mesophyll to respond to light/dark fluctuation by modulating the magnitude of H^+ flux diminished with increasing concentrations of NaCl after 72 h treatment (Fig. 5a and Suppl Fig. S2). The mean basal H^+ flux was also shifted towards reduced net H^+ efflux by 100 mM salt treatment (Fig. 5b and Suppl Fig. S2). $GdCl_3$, TEA, and vanadate all caused similar reductions in light-dark response to about 2/3 that of the 50 mM treatment (Fig. 5e), and have shifted the basal H^+ flux to net influx ($GdCl_3$< TEA< vanadate) (Fig. 5f).



Suppl Fig. S2 Rhythmical net H^+ flux response to light/dark fluctuation measured from mesophyll tissues exposed to a range of salinities for 72 h. One (of 6-8) typical examples is shown.

Effect of salinity on basal H^+ fluxes and light-induced H^+ flux responses showed also a clear time dependency. The amplitude of the light/dark H^+ flux response was increased for the first 24h of 100mM NaCl treatment and then declined to the values below those in control after 72 h of treatment (Fig. 5c). The mean base flux doubled for the first 24 h and then, again was significantly reduced after 72 h (Fig. 5d).

The magnitude of K^+ flux response to light was reduced by 72 h of salinity treatment by 2 to 4 fold (Fig. 6a; Suppl Fig. S3), with an approximate 75% reduction measured for highest (100 mM NaCl) treatment. All three NaCl treatments (20, 50, and 100 mM) have shifted the mean basal flux towards net K^+ efflux (Fig. 6b). Modulation of both K^+ flux responses to light (Fig. 6a) and a shift in basal K^+ fluxes (Fig. 6b) also showed a clearly pronounced time-dependency, with strongest effects observed after 2 h of 100 mM salt treatment. The mean basal K^+ flux changed from an influx of 17 ± 6.8 nmol m^{-2} s^{-1} before 100 mM NaCl treatment to a large -116 ± 14.5 nmol m^{-2} s^{-1} efflux 2 h after treatment; this efflux was then gradually reduced to be essentially non-existent after 72 h of salt treatment (Fig. 6c). The magnitude of light-induced K^+ flux responses was strongly modulated by TEA (Fig. 6e). Basal K^+ flux was shifted towards net K^+ efflux by TEA and towards net K^+ uptake by vanadate (Fig. 6f).

Basal K^+ fluxes in light/dark transient experiments were slightly different from those reported in Figs 2 & 4. This is explained by the fact that in the latter case leaf segments were pealed 10 to 12 h before measurements and salinity treatment was then given for 1 h. In light/dark experiments, salinity exposure was much longer (up to 72 h). Because of this, appropriate controls were also measured 3.5 days after the peeling. Thus, the difference in basal K+ flux most likely reflects the effect difference in mesophyll properties after 12 and 84 h of leaf excision and epidermal peeling.

Membrane potential (MP) of bean mesophyll was -135 mV for 72 h after pealing in controls (Fig. 7). MP was decreased to less than 30% of its original value by 50 mM NaCl treatment within 12 h of treatment and stayed essentially the same for the following 60 h. 20 mM NaCl caused a 56% reduction in MP (to -76 ± 24 mV) 12 h

after the treatment followed by a gradual recovery to -110 ± 12 mV by 24 h and remained stable for the next 48 h (Fig. 7).

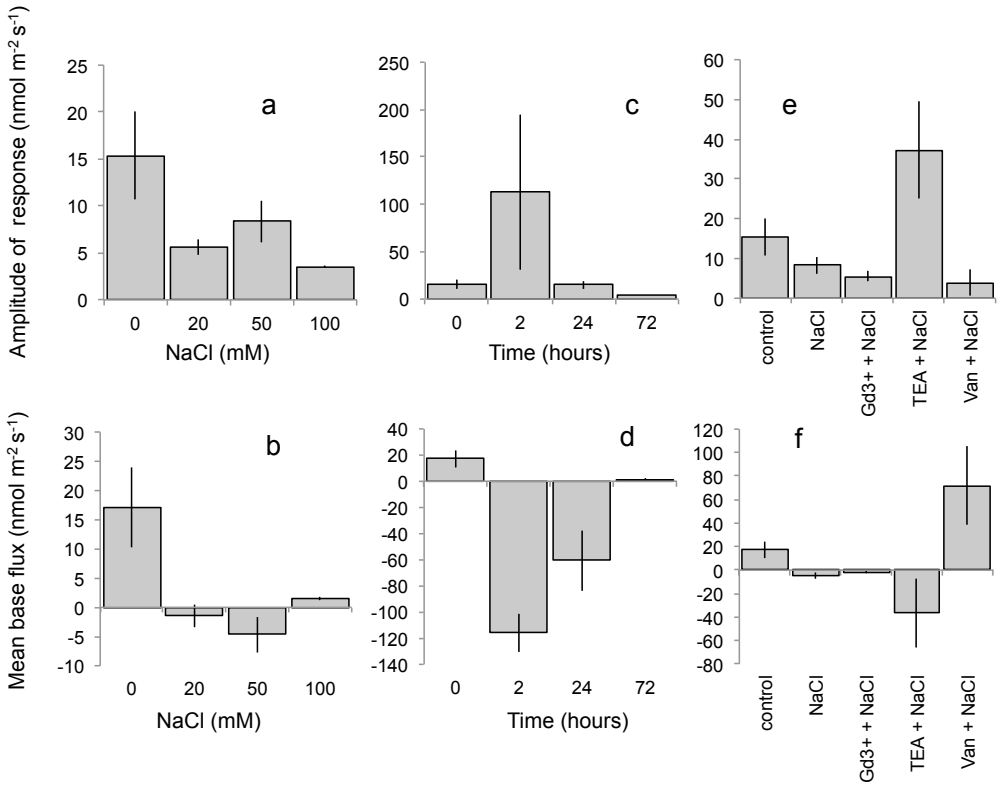


Fig. 6 Dose (**a, b**) - and time (**c, d**) – dependence of the amplitude and mean basal net K^+ flux responses from leaf mesophyll upon light/dark fluctuation. In panels **a** and **b**, measurements were taken after 72 h of salinity exposure. In panels **c** and **d**, salinity treatment was 100 mM NaCl. **e**, **f** – amplitude (**e**) and mean basal (**f**) flux responses to light/dark fluctuations measured from mesophyll tissue exposed to 100 mM NaCl and pre-treated for 1 h in a solution containing specific pharmacological agents. Mean ± SE (*n*=5-6)

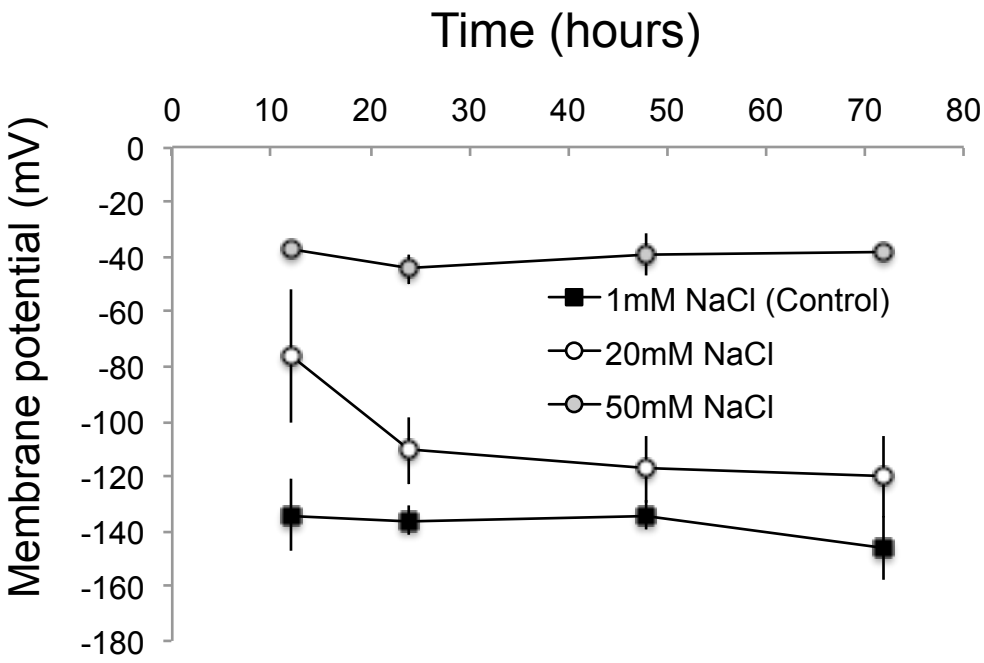
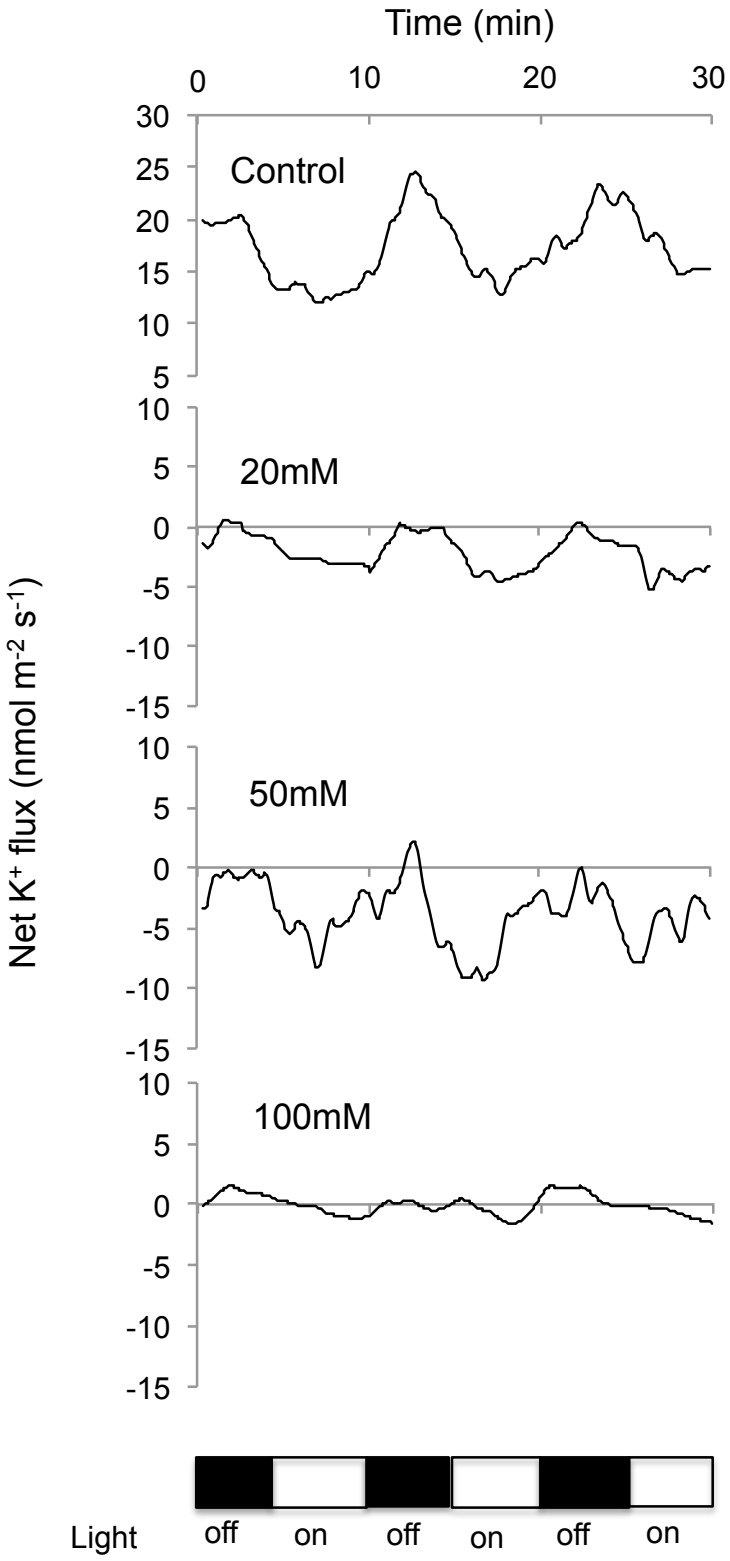


Fig. 7 Dose- and time- dependence of membrane potential in response to salinity treatment. Mean ± SE (*n*=8)



Suppl Fig. S3 Rhythmical net K^+ flux response to light/dark fluctuation measured from mesophyll tissues exposed to a range of salinities for 72 h. One (of 6-8) typical examples is shown.

Discussion

Mesophyll cells have only a limited capacity to prevent detrimental effects of apoplastic Na^+

Salinity causes a dramatic increase in apoplastic Na^+ (Speer and Kaiser 1991) and mesophyll cells have only a limited capacity to deal with this (Blumwald et al. 2000). Under saline conditions the xylem concentrations of Na^+ increase typically to between 5 to 20 mM (Blom-Zandstra et al. 1998). Given the overall volume of the leaf apoplast is rather small (Speer and Kaiser 1991), apoplastic Na^+ concentrations may change dramatically as a function of Na^+ transport in the xylem and leaf transpiration reaching over 100 mM under more severe or longer salt exposures (Speer and Kaiser 1991). The exact mechanisms of Na^+ toxicity are not well understood (Cheeseman 2013), nor are the concentrations at which cytosolic Na^+ causes toxicity. There are only few reports of direct measures of Na^+ concentrations in the cytosol (reviewed in Cheeseman 2013), and the few measurements that do exist vary greatly due to technological challenges and different salt treatments. Flowers and Hajibagheri (2001) reported 245 mM Na^+ in the cytoplasm and 280 mM in the vacuole of barley root cells treated with 200 mM NaCl for 15 days, while Carden et al. (2003) measured 2 to 28 mM Na^+ in the cytosol of the same species under 200 mM salinity treatment for 8 days using multi barrelled microelectrodes. In both these reports the varieties with lower cytoplasmic Na^+ concentrations were determined to be more salt tolerant.

Several possible explanations have been put forward to explain cytosolic Na^+ toxicity. One of the more commonly accepted theories is that elevated cytosolic Na^+ displaces K^+ from activation sites in enzymes, leading to K^+ deficiency. Na^+ has been reported to be only around 20% as efficient as K^+ for enzyme activation (Nitsos and Evans 1969), in other reports Na^+ causes inhibition of protein synthesis, malate dehydrogenase, aspartate transaminase, glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase (Greenway and Osmond 1972). A less explored interaction that Na^+ may have with these enzymes is a possibility that Na^+ and K^+ interact with water. As K^+ and Na^+ form different structures in water (Galamba 2012), these water interactions may have an effect on cytoplasmic structure and ultimately enzyme

structure and functionality (Spitzer and Poolman 2005). Either way elevated cytosolic Na^+ is a major problem in plants affected by salinity stress. Mesophyll maintains a relatively constant Fv'/Fm' (Fig. 1) and is capable to respond to fluctuating light by modulating net ion fluxes under highly saline 100 mM (apoplastic NaCl) conditions for the first 12 h (Fig. 5c and 6c). After 12 h a gradual decline in leaf photochemistry is observed (Fig. 1). Lower concentrations of Na^+ in the apoplast also result in Fv'/Fm' decline, yet at later times (Fig. 1). From this data, it is plausible the observed damage to photosynthetic machinery is caused by the apoplastic Na^+ and is a function of the overall amount ("dose") of Na^+ accumulated within the cell. The latter is a function of the time of exposure and the level of Na^+ in leaf apoplast. Assuming the cell's ability to pump Na^+ into the vacuole and back into the apoplast is constant, then the relative threshold after which a significant effect of salinity on leaf photochemistry becomes noticeable will be a product of exposure time and apoplastic Na^+ concentration, corrected for excluding/sequestering ability (Blumwald et al. 2000) (Table 2).

Table 2 Critical doses of Na^+ accumulated in the cell leading to decline in Fv'/Fm' chlorophyll fluorescence values

Time	$[Na^+]_{apoplast}$	Product (time x $[Na^+]$)
18 h	100 mM	1800
36 h	50 mM	1800
84 h	20 mM	1680

As seen from Table 2, the critical 'dose" leading to the significant decline in leaf Fv'/Fm' characteristic is invariant to applied NaCl levels and is determined by the amount of Na^+ accumulated in cytosol.

The ability of mesophyll to remain undamaged for a short period of time is most likely due to avoidance mechanisms. Avoidance mechanisms include the prevention of Na^+ entry or pumping Na^+ out of the cytoplasm into the apoplast via the SOS1 antiporter (Shi et al. 2000), and into the vacuole via the NHX antiporter (Blumwald et

al. 2000). The decline in PSII activity after this point is likely to be due to the vaculor sequestration failing, resulting in a build up of Na^+ in the cytosol and chloroplast stroma, with major implications for leaf photochemistry.

Another reason for Fv'/Fm' declines under high salt treatment in mesophyll is due to increased ROS production. Salinity elicits an increase in ROS production (Ellouzi et al. 2011). ROS are known to cause damage to DNA, photosynthetic machinery, and cellular membranes (Mittler 2002). Chloroplasts are known as the major sources of ROS production in green leaves (Suzuki et al. 2011). H_2O_2 concentrations in *Arabidopsis thaliana* leaves rise rapidly in the first 4h and continue to rise for 72 h of whole plant 100 mM NaCl treatment, while the concentration of antioxidant enzymes reaches a maximum after 24 h (Ellouzi et al. 2011). DMSO, a known ROS scavenger (Sanmartín-Suárez et al. 2011), reduced the increase in proton efflux under saline conditions within the first hour (Fig. 3). This suggests that ROS is in part responsible for activation of increased proton efflux. DMSO also caused a reduction in the magnitude of K^+ efflux 1 h after 100 mM NaCl treatment (Fig. 4) suggesting that ROS-activated K^+-permeable channels may be responsible for some of the K^+ efflux measured.

Mesophyll cells normally elicit an array of responses to light, including modulation in membrane-transport activity (Blum et al. 1992; Elzenga et al. 1995; Shabala and Newman 1999; Živanović et al. 2005); these responses were shown to be essential for normal plant function. The ability of mesophyll to maintain normal H^+ and K^+ flux response to light is diminished by the addition of Na^+ (Figs. 5a, c and 6a, c). For H^+ flux the amplitude of the response to light is reduced in a dose dependent manner after 72 h of Na^+ treatment. For the first 24 h of 100 mM NaCl treatment there is a large increase in the magnitude of H^+ flux response to light as well as a clear shift in the basal H^+ flux. The reduced ability to respond to light after 72 h salinity treatment is likely to be linked to the damaged photosynthetic apparatus (Fig. 1) and could be explained by the depletion in ATP stores required to fuel H^+-ATPase. It is also possible that K^+ displacement, interaction with Na^+, or reactive oxygen species accumulation, have limited the ability of photoreceptors that have been associated with different fluxes to respond to light (Staal et al. 1994). Both NO and H_2O_2 have been shown to inhibit signal transduction between phototropins and H^+-ATPase

reducing the blue light response in guard cells (Zhang et al. 2007). It is possible therefore that NaCl-induced ROS accumulation could lead to the same outcome in our experiments. Crytochrome photoreceptors are also activated by blue light; the mechanism of activation is a change from a reduced non-radical state to a radical state (Immeln et al. 2007). The latter process is fully reversible and is strongly dependent on oxygen availability (Immeln et al. 2007). It could be suggested that other oxidative agents would also slow the deactivation of cytochromes, leading to a reduced cellular blue light response.

Essentiality of cytosolic K^+ homeostasis in leaf mesophyll

Because K^+ is essential for many cellular and whole plant functions including osmoregulation, phloem loading, and protein synthesis (Cakmak et al. 1994; Anschütz et al. 2014), cytosolic K^+ homeostasis is under strict control. Due to the challenges of measuring cytosolic ion contents, only few papers report direct measurements of cytosolic K^+. Reported values of K^+ concentrations in the cytosol vary depending on the method used and the species of plants grown. X-ray crystallography estimates cytosolic K^+ in healthy barley roots to be between 121 and 132mM (Flowers and Hajibagheri 2001). Such concentrations were found to be in a good agreement with those believed to be optimal for ribosome function (e.g. Greenway and Osmond 1972) including the function of ribulose-1,5-bisphosphate carboxylase activity and CO_2 fixation in general (Jin et al. 2011). At the same time, concentration of K^+ in healthy leaf cell cytosols measured by ion-selective microelectrodes was estimated between 68 and 79 mM (Cuin et al. 2003). The discrepancies between these *in vitro* measurements and what was measured in plants could be due to the technological challenges associated with measuring ion concentrations in the cytosol or due to the cytoplasm being highly ordered (Spitzer and Poolman 2005) and localisations of K^+ concentrations within the cytoplasm (Cheeseman 2013). At the same time, both these methods detect a drop in cytosolic K^+ content under salinity stress, to 60 mM (Flowers and Hajibagheri 2001) and 64 mM (Cuin et al. 2008).

Previous reports showed a strong correlation between K^+ retention ability in plant roots (Chen et al. 2005; Cuin et al. 2008) and leaves (Chen et al. 2005; Cuin et al. 2008; Wu et al. 2013) in cereal species and the extent of their salinity stress tolerance.

Here we show that decline in bean leaf photochemistry is also strongly dependent on mesophyll cells' ability to retain K^+ (Table 3). Assuming bean leaf thickness being 150 μm, then the overall volume of all leaf cells for $1m^2$ surface area will be 150 ml. Taking average values of NaCl-induced K^+ efflux for each particular time (Fig. 6d), and assuming intracellular K^+ content in control being 150 mM, one can then calculate the resultant changes in intracellular K^+ as a function of time. As shown in Table 3, 72 h of exposure to 100 mM NaCl will result in 60% decline in intracellular K^+ content (from 150 to 59 mM; Table 3).

Table 3 Time-dependent changes in intracellular leaf K^+ content estimated based on the rate of measured K^+ efflux (as in Figs. 6B & 2) and associated decline in leaf photochemical efficiency

Time hours	Mean K^+ efflux, nmol $m^{-2}s^{-1}$	Amount K^+ lost, mmol	Estimated $[K^+]$ concentration, mM	Fv'/Fm'
0	-	-	150	0.635
1	66.7	0.24	148	-
2	120	0.43	146	-
12	-	-	-	0.617
24	88.35	7	93	0.481
72	29.6	5.1	59	0.197

Potassium leak has previously been linked to higher respiration rates (Bottrill et al. 1970). Here, a major decline in Fv'/Fm' value occurs when intracellular K^+ drops below 100 mM level (Table 3). Previous reports have shown that under salinity stress barley mesophyll cells relocate K^+ from the vacuole into the cytosol (Cuin et al. 2003). This is also likely the case for beans, as the mesophyll maintained its steady Fv'/Fm'

values for the first 12 h despite an overall reduction in mesophyll K^+ concentration. However, there is a certain limit on the ability of the vacuole to replenish K^+ lost from the cytosol. Once the capacity of vacuolar K^+ pool is exhausted, PSII is affected, and a sharp drop in Fv'/Fm' occurs.

A reduction in the concentration of cytosolic K^+ can also lead to an increase in ROS production (Hafsi et al. 2010) and eventually PCD (Shabala 2009; Demidchik et al. 2010). High cytosolic K^+ levels are essential for suppressing activities of caspase-like proteases and endonucleases, both in mammalian (Lam and del Pozo 2000) and plant (Shabala et al. 2007; Shabala 2009; Demidchik et al 2010) systems. Thus, salinity-induced increase in ROS production and K^+ leak-induced activation of caspase-like enzymes maybe suggested as two factors responsible for the damage to PSII and observed reduction in Fv'/Fm' (Fig. 1). Further evidence that this maybe the case is that the application of chemicals that reduce Na^+ induced K^+ efflux improve the Fv'/Fm' of salinity treated chloroplasts (unpublished data).

Both KOR and NSCC channels mediate NaCl-induced K^+ leak from leaf mesophyll

There are numerous different trans-membrane proteins involved in K^+ transport across the plasma membrane of mesophyll cells. In Arabidopsis, these are encoded by at least 75 genes grouped in 7 different families. These include (1) Shaker-type channels; (2) "two-pore" K^+ channels; (3) cyclic nucleotide-gated K^+-permeable non-selective cation channels; (4) glutamate receptors; and (5-7) KUP/HAK/KT, HKT and K^+/H^+ transporters (Very and Sentenac 2003). In their turn, Shaker-type channels are divided further into hyperpolarisation activated inward rectifying channels (KIR), weakly inward rectifying channels and depolarisation activated outward rectifying channels (KOR) (Shabala and Cuin 2008). All these are activated in different ways. The channels can only facilitate the movement of ions along the electrochemical gradient, while the transporters move ions either by coupling the movement with another ion in the same direction or the opposite direction. Channels have many times the conductance of transporters, however both are important for normal plant growth and function.

A strong ($R^2 = 0.92$) relationship between NaCl-induced changes in H^+ and K^+ fluxes (Suppl Fig. S1), and dose-dependent changes in MP (Fig. 7) suggests K^+ efflux from bean mesophyll is most likely mediated by the voltage-gated transport system. Depolarization-activated outward-rectifying KOR channels may be one of these. KOR channels are blocked effectively with TEA (Hedrich and Schroeder 1989), and in our experiments TEA reduced the amount of K^+ leak from mesophyll tissue in response to salt stress (Fig. 4). Previously KOR and NSCC have been implicated in K^+ leak from roots under saline conditions (Shabala and Cuin 2007). Shabala (2000) has also suggested that voltage gated KOR were involved in K^+ leak from bean mesophyll under saline conditions, however this was not directly proven with the use of pharmacological agents. From our data, about 84% the initial (first hour) leak of K^+ occurs though KOR (Fig. 4). The remaining 16% maybe attributed to NSCC.

NSCC are blocked by Gd^{3+} ions (Demidchik and Maathuis 2007). The effectiveness of Gd^{3+} in reducing K^+ leak by 74% (Fig. 4) is most likely caused by a combination of reducing K^+ leak through NSCC and preventing Na^+ entry into the cell, thus reducing the extent of membrane depolarisation (and accompanying K^+ leak via KOR).

NSCC are a diverse group of cation channels that have little if any selectivity between different cations but are highly selective in preventing anion flow (Demidchik and Maathuis 2007). NSCC have been found in many plant systems (Demidchik et al. 2002). Hydroxyl radical-activated non-selective cation channels have been implicated in the stress response that occurs in PCD (Demidchik et al. 2010). Consistent with this notion, mesophyll pre-treatment with DMSO reduced the steady efflux of K^+ after the initial depolarisation-induced efflux (Fig. 4). This may be taken as the evidence that the hydroxyl radical activated NSCC are in part responsible for some of the observed K^+ leakage. It also seems plausible that DMSO could reduce Na^+ influx through NSCC due to reduced ROS activation, thus reducing the extent of membrane depolarisation and K^+ efflux through KOR (Fig. 4).

Essentiality of NaCl-induced H^+-ATPase pump activation

Salinity stress caused a H^+ efflux from mesophyll cells (Fig. 3). As this efflux was suppressed by vanadate, a known inhibitor of the H^+-ATPase (Fig. 3), it may be suggested that it originated from NaCl-induced activation of the H^+ pump (e.g. plasma

membrane H^+-ATPase). Such NaCl-induced H^+ pump activation under saline conditions has been reported in direct experiments on plants (Ayala et al. 1996; Yang et al. 2007).

Salinity treatment causes significant membrane depolarisation (Fig. 7). At the same time, maintaining a negative membrane potential is essential for the normal transport of ions in and out of the cell (Palmgren 2001). Plasma membrane H^+-ATPase is known to be central to maintaining membrane potential (Sze et al. 1999) and restoring it under salinity stress. Indeed, Ayala et al. (1996) showed increased H^+-ATPase activation in both the plasma and vacuolar membranes of the halophyte *Salicornia bigelovii* Torr. Increased H^+-ATPase activation has also been shown in the plasma membranes of the glycophytes *Populus euphratica* callus and *Medicago* species (Yang et al. 2007). In our experiments salinity induced H^+ efflux occurs for the first 24 h (Fig. 5d), with a fairly steady rate of H^+ pumping between 2 and 24 h after salinity treatment. Net H^+ efflux decreased significantly after 24 h under high levels of salinity stress. This reduction can be attributed to the H^+ pumping being an energy dependant process requiring ATP. Eventually the cell would no longer be able to pump H^+ at the same rate as the cells run out of energy due to depletion of energy as a result of inhibition of mitochondria (Jacoby et al. 2011) and chloroplasts (Hernandez et al. 1995) under saline conditions. Additional to this, at some point the cell would also become alkaline if the pumps continue to pump out H^+, so H^+ pumping would become self-limiting. A lower rate of H^+ pumping is required under low levels of salinity stress (20 mM), resulting in a nearly fully recovery of MP (Fig. 7). This restoring of MP has beneficial carry-over effects for reducing potassium leak from the cell via depolarisation-activated KOR channels (Suppl Fig. S1).

Proton pumping is not only important for the maintenance of membrane potential but also for removing Na^+ from the cytosol to both apoplastic space and into vacuoles. Na^+ is pumped back into the apoplast by a H^+/Na^+ antiporter encoded (in Arabidopsis) by the SOS1 gene (Shi et al. 2000). Higher rate of the plasma membrane H^+-ATPase activity is also essential to enable K^+ uptake into leaf mesophyll via the HUK/KUP symporter which couples H^+ and K^+ transport in the same direction (Banuelos et al. 2002). These two transport mechanisms rely on the cytosol being more basic than the apoplast. In addition to the mechanisms on the plasma membrane, the tonoplast

membrane also has the NHX Na^+/H^+ antiporter (Apse et al. 1999) as well as the HUK symporter which couples K^+ and H^+ transport (Banuelos et al. 2002). This means in addition to the H^+ gradient needed across the plasma membrane, a gradient is needed across the tonoplast. The maintenance of this H^+ gradient is essential so that the cytosolic K^+/Na^+ ratio can be maintained (Shabala 2013). In the more severe Na^+ treatment an increase in H^+ efflux was correlated ($R^2 = 0.92$) at approximately a 1 to 1 ratio with a decrease in K^+ efflux (Suppl Fig. S1). There are two possible explanations for this strong correlation, one is that the increased H^+ efflux resulted in membrane repolarisation, therefore reducing the efflux of K^+ through depolarisation-activated KOR. The other explanation is that the H^+ efflux resulted in acidification of the media outside of the cells and activation of the HUP/KUP symporter, effectively pumping K^+ back into the cell. It is possible that both of these mechanisms are true and work synergistically.

We also showed that the addition of vanadate caused an increase in K^+ leak (Fig. 4). This increase is most likely caused by the two mechanisms mentioned above, reduced membrane potential and the inability to use H^+ gradients for returning K^+ back into the cell via HUP/KUP. Suppression of H^+-ATPase activity by vanadate is also expected to reduce the efficiency of cytosolic Na^+ removal by SOS1 and NHX, causing further membrane depolarisation and greater K^+ leak through depolarisation-activated KOR.

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Chapter 3: Salinity effects on chloroplast photosynthetic performance in glycophytes and halophytes

William J. Percey[1], Andrew McMinn[2], Jayakumar Bose[1], Michael C. Breadmore[3], Rosanne M. Guijt[4], Sergey Shabala[1]

[1]School of Land and Food, University of Tasmania, Hobart, Tasmania 7001, [2]Institute for Marine and Antarctic Studies, University of Tasmania, Hobart, Tasmania, [3]Australian Centre for Research on Separation Science (ACROSS), School of Chemistry, University of Tasmania 7001 and [4]School of Pharmacy, University of Tasmania, Hobart, Tasmania 7001

Abstract

The effects of NaCl stress and K^+ nutrition on photosynthetic parameters of isolated chloroplasts were investigated using PAM fluorescence. Intact mesophyll cells were able to maintain optimal photosynthetic performance when exposed to salinity for more than 24 hours while isolated chloroplasts showed declines in both the relative electron transport rate (rETR) and the maximal photochemical efficiency of photosystem II (F_V/F_M) within the first hour of treatment. The rETR was much more sensitive to salt stress compared with F_V/F_M, with 40% inhibition of rETR observed at apoplastic NaCl concentration as low as 20 mM. In isolated chloroplasts, absolute K^+ concentrations were more essential for the maintenance of the optimal photochemical performance (Fv/Fm values) rather than sodium concentrations *per se*. Chloroplasts from halophyte species of quinoa showed an overall 18% decline in F_V/F_M under salinity, while the Fv/Fm decline in chloroplasts from glycophyte pea and bean species was much stronger (31% and 47%, respectively). Vanadate (a P-type ATPase inhibitor) significantly reduced F_V/F_M in both control and salinity treated chloroplasts (by 7% and 25%, respectively), while no significant effects of gadolinium (a known

blocker of non-selective cation channels) were observed in salt-treated chloroplasts. Tetraethyl ammonium (TEA) (a K^+ channel inhibitor) and amiloride (an inhibitor of the Na^+/H^+ antiporter) increased the F_V/F_M of salinity treated chloroplasts by 16% and 17% respectively. These results suggest that a chloroplasts's ability to regulate ion transport across the envelope and thylakoid membrane play a critical role in leaf photosynthetic performance under salinity.

Keywords: PAM, Sodium, Potassium, Ion channels, Envelope, Thylakoid

Submitted to *Photosynthesis Research*

Introduction

Salinity is an increasing problem worldwide, causing multibillion dollar losses in agricultural production (Flowers 2004; Rengasamy 2006). To combat this problem, a better understanding of the key physiological mechanisms conferring salinity tolerance in crops is required. Salinity affects plant growth by imposing both osmotic and ionic stresses. Osmotic stress occurs immediately after stress onset and causes reduction in cell turgor and associated turgor-driven cell expansion, resulting in retarded development at the whole-plant level (Munns and Tester 2008). It is believed osmotic stress dominates over the first days or weeks of salinity stress (Munns 2002), although turgor is regained within the plant within hours. The longer-term component of salinity stress is the ionic effect (Munns and Tester 2008); this component can be broken into two distinct parts, namely disturbed nutrition and ion toxicity. Salinity interferes with the uptake and retention of other mineral nutrients (Cakmak 2005; Shabala and Pottosin 2014). In particular Na^+ can directly interact with some enzymes and proteins, thus attempting to replace K^+ in key enzymatic reactions (Wakeel et al. 2011). The efficiency of the above replacement is rather low, as most enzymes are K^+ but not Na^+ -specific (Nitsos and Evans 1969; Wakeel et al. 2011). For example, Na^+ is only 20% as efficient as equal concentrations of K^+ at activating sucrose synthase (Nitsos and Evans 1969). Na^+ binding can also cause changes in water structure leading to changes in enzyme conformation and function (Kronzucker et al. 2013).

In most crop plants, an increase in salinity causes a major decline in photosynthesis, with resulting detrimental effects including reduced carbon fixation rate (Seemann and Critchley 1985; Belkhodja et al. 1999), decreased stomatal conductance (Seemann and Critchley 1985; Brugnoli and Lauteri 1991), changes in the ratio between chlorophyll *a* and *b*, reduced efficiency of photosynthetic apparatus including PSII and the electron transport chain (Parida et al. 2003; Wang et al. 2007), and disorganisation of thylakoid membranes (Parida et al. 2003). A marked increase in non-photochemical quenching under saline conditions (Wang et al. 2007) also reduces the overall photosynthetic efficiency.

The limitations salinity imposes on the photosynthetic processes are usually divided into two components, the stomatal and non-stomatal limitations (Brugnoli and Björkman 1992; Munns 2006). Stomatal limitations are caused by reduced stomatal aperture limiting gas exchange into and out of the leaf. Reduced gas exchange results in reduced CO_2 uptake and increased photorespiration. Non-stomatal limitations are limitations that are imposed through ion imbalances in the leaf and, specifically, accumulation of Na^+ and Cl^- and decreases in leaf K^+ (Munns and Tester 2008). To date, most research has concentrated around stomatal limitation to photosynthesis (Munns and Tester 2008; Sirault et al. 2009; Rajendran et al. 2009), with non-stomatal limitations receiving much less attention.

Non-stomatal limitations to photosynthesis arise from ion imbalances, with Na^+ inhibiting many key metabolic processes such as protein synthesis (Hall and Flowers 1973) and oxidative phosphorylation (Flowers 1974). Salinity stress-induced increases in the amount of reactive oxygen species (ROS) may be another significant component of such inhibition. Most ROS production in the chloroplasts is from photosystem one (PSI) (Asada 2006; Pospíšil 2009). Under stress conditions PSI increases the amount of ROS produced via the Mehler reaction (Asada 2006). PSII produces ROS in the thylakoid membrane when there is a limitation to the electron transport chain between photosystems. When the electron transport chain is limited, photoreduction of molecular oxygen produces superoxide on the reducing side of PSII (Pospíšil 2009). The electron donor side of PSII can also produce hydrogen peroxide through the incomplete oxidation of water due to the inhibition of the water splitting manganese complex (Pospíšil 2009).

It has been demonstrated the sequestration of Na^+ into the vacuole can increase plant salt tolerance (Apse et al. 1999; Zhang and Blumwald 2001). The sequestration of Na^+ in vacuoles has two positive effects under salinity stress, taking Na^+ away from metabolically active areas in the cell as well as providing cheap osmoticum to overcome the osmotic stress associated with salinity (Wakeel et al. 2011). The other benefit of Na^+ sequestration in the vacuole is that cells can then relocate K^+ from the vacuole into the cytosol (Wakeel et al. 2011), to compensate for salinity-induced K^+ leak from mesophyll tissue (Shabala 2000; Wu et al 2013) and ultimately the chloroplast so K^+ can then fulfil vital metabolic roles.

Superior ROS protection is another potentially important mechanism for overcoming detrimental effects of salinity on leaf photochemistry. Plants have evolved complex antioxidant defence mechanisms consisting of enzymatic and non-enzymatic pathways to detoxify ROS preventing excessive ROS accumulation while maintaining optimum level of ROS for signalling (Baxter et al. 2014; Bose et al. 2014). Salinity stress-induced an increase in antioxidant activity in leaf mesophyll tissue is a widely reported phenomenon (Qiu-Fang et al. 2005; de Azevedo Neto et al. 2006; Gill and Tuteja 2010), and genetic modifications resulting in the increased accumulation of these antioxidants has increased salinity tolerance in various species (rice - Tanaka et al. 1999; tobacco - Hamid Badawi et al. 2004; cabbage -Tseng et al. 2007).

Plant species vary widely in their ability to grow in saline soils (Munns and Tester 2008), and some of them (classified as halophytes) are actually benefiting from the presence of the moderate – 50 to 150 mM – (Flowers et al. 1977; Flowers and Colmer 2008; Shabala et al. 2012) amounts of NaCl in the growth media. While most glycophytes have severely reduced photosynthetic characteristics under these conditions (Brugnoli and Lauteri 1991; Belkhodja et al. 1999; Parida et al. 2003; Wang et al. 2007; Abbasi et al. 2014), in halophytes these are either unaffected or even increased (Qiu-Fang et al. 2005; Cai Hong et al. 2005). Thus, halophytes represent an ideal model to understand complex physiological and genetic mechanisms of salinity stress tolerance, especially in a comparison with glycophytes (Bose et al. 2013). The hallmark of halophyte species is their ability to use inorganic ions (Na^+, K^+ & Cl^-) for osmotic adjustment under saline conditions (Flowers et al. 1977; Hariadi et al. 2010). Their antioxidant pool profile also seems to differ from

glycophytes (Bose et al. 2013), allowing them to handle some of the most toxic ROS species including the hydroxyl radical.

The classical view is that enzymatic reactions in both glycophytes and halophytes are equally sensitive to Na^+ (Greenway and Osmond 1972). However, the direct effects of elevated Na^+ content on photosynthesis in the cytosol are not well understood (Cheeseman 2013; Kronzucker et al. 2013). Moreover, most of reported evidence for this are from *in vitro* experiments, and the concentration at which Na^+ becomes toxic *in planta* is also not known (Wakeel et al. 2011; Cheeseman 2013; Kronzucker et al. 2013). Despite this, numerous papers have pointed out the essentiality of maintaining an optimal cytosolic K^+ to Na^+ ratio for plant performance under saline conditions (Blumwald et al. 2000; Munns and Tester 2008). Neither the ratio nor threshold of the ratio has been properly quantified in direct *in situ* experiments.

Chlorophyll fluorescence is a very powerful non-invasive tool for assessing the "health" of the photosynthetic apparatus (Maxwell and Johnson 2000). In this study chlorophyll fluorescence measurements were used to assess whether Na^+: K^+ ratios affect photosynthesis in isolated chloroplasts. The difference in response of halophyte chloroplasts to glycophyte chloroplasts to salinity stress was also assessed. The effect on photosynthetic parameters of blocking specific ion channels and transporters in chloroplasts under salinity stress was studied. The concentration of K^+ was found to be more important for maintaining optimal photosynthetic performance than either the concentration of Na^+ *per se*, or the ratio between Na^+ and K^+. Chlorophyll fluorescence characteristics in chloroplasts isolated from halophyte quinoa species were less affected by salinity than those isolated from glycophyte bean and pea species. Pharmacological studies have suggested that cell's ability to regulate ion transport across the envelope and thylakoid membrane play a critical role in leaf photosynthetic performance under salinity.

Materials and methods

Plant material

Broad beans (*Vicia faba* L cv Oswald; Hollander Imports, Hobart, Australia), green peas (*Pisum sativum*) and quinoa (*Chenopodium quinoa* cv 19) were grown from seed in 0.51 L plastic pots under controlled greenhouse conditions (temperature between 19 ^{o}C and 26 ^{o}C; day length 12-14 h; average humidity ~65%) at the University of Tasmania greenhouse. The potting mixture included 70% composted pine bark, 20% course sand, 10% sphagnum peat (pH6.0) which was fertilised with 1.8 kg m^{-3} Limil, 1.8 kg m^{-3} dolomite, 6.0 kg m^{-3} osmocote plus and 0.5 kg m^{-3} ferrous sulphate. Plants were irrigated twice daily with tap water to maintain potting mix at full field capacity. Plants were grown for four to six weeks. The newest fully expanded leaves were used for all measurements.

Chlorophyll fluorescence measurements from leaf mesophyll

Mesophyll tissue measurements were undertaken as described by Percey *et al.* (2014). The first fully expanded leaf was removed from the plant and brought into laboratory in a sealed plastic bag. The abaxial epidermis was peeled off using a fine forceps. Pealed leaves were immediately cut into 5x7 mm segments and placed pealed side down floating on a shallow layer of basic salt media solution (1 mM NaCl; 0.5 mM KCl; 0.1 mM $CaCl_2$; pH 5.7 non-buffered) in 35 mm Petri dishes. Samples were left in the dark for 12 hr after peeling to recover from the peeling process before treatment. Treated leaf segments were placed on a glass microscope slide and suspended in the study solutions. A cover slip was placed over the specimen, and mounted in the Microscopy-PAM (Walz, Effeltrich, Germany) then focused under dim green light. Once the specimen was in focus, the microscope light was turned off, a dark curtain was pulled around the microscope to allow for 5 min dark adaption. After dark adaption fluorescence measurements were taken.

Chloroplast isolation

Chloroplasts were isolated using a method modified from that described by Wignarajah & Baker (1981). In brief, three newly expanded leaves were placed in 60 mL isolation media. The isolation media varied depending on treatment. For control conditions it contained 150 mM KCl, 1 mM ATP and made up to 570 mmol kg^{-1} with sucrose, and adjusted to pH 7.1 using saturated KOH solution. For non-control treatments, the chloroplasts were isolated in the treatment media described in figure legends. Treatment media were pH and osmolality adjusted to be the same as the control isolation media. The leaf material was blended on a high setting in a kitchen blender (Breville Power Max 550 w model BBL200) for 30 s until all leaf material was broken up. The solution was then filtered through four layers of kitchen cloth (CHUX® Superwipes® original) to remove debris; the resulting solution had an average concentration of 6500 chloroplasts per mL. This solution was then diluted 10 times in the same media to give desired concentration for PAM measurements.

Chloroplast PAM fluorescence measurements

For fluorescence measurements, half hour dark-adapted 2.5 mL samples of suspended chloroplasts were placed in the cuvette of a Water-PAM (Walz, Effeltrich, Germany), rapid light curves (RLC) were obtained by exposing samples to 10 s of blue radiation at eight incremental steps of irradiance ranging from 0 to 242 mol photons $m^{-2}s^{-1}$. Relative electron transport rate (rETR) was determined according to the following formula:

$$rETR = \Phi_{PSII} \times PAR$$

Φ_{PSII}: light adapted maximum photochemical efficiency, PAR: photosynthetically active radiation

The following photosynthetic parameters were derived from the rapid light curves; photosynthetic efficiency (initial slope of the curve), irradiance of onset of maximum photosynthesis (E_k), and maximum relative electron transport rate (rETRmax). The data was exported from WinControl (Walz, v3.1), into Excel and SPSS regression

models were fitted to the rapid light curve data in the method described by Ryan et al. (2004).

Fluorescence transients of chloroplast suspensions were measured in a Water-PAM (Walz, Effeltrich, Germany). First 2.5 mL of dark adapted chloroplast solution was pipetted into the cuvette, the F_o was determined and normalized for all samples then a saturating actinic light of ~8000 µmol photons $m^{-2}s^{-1}$ was applied, causing a sharp increase in the chlorophyll fluorescence, this peak fluorescence was normalized 180 s after the application of the actinic light. Treatment was applied to the sample 230 s after actinic light and the chlorophyll fluorescence response (F_t) was recorded. This method of measuring changes in F_t has been used in the past to determine limiting nutrients for single cellular algae (Holland et al. 2004) but have not been used for similar studies in higher plants. The changes in F_t are likely related to changes in charge balancing between PSII and PSI (Holland et al. 2004; Petrou et al. 2008).

Pharmacology

Pharmacological agents were added to the media prior to blending the chloroplast for measurement of F_V/F_M. Specific details on the names and working concentrations of pharmacological agents used in this work, and their potential targets are given in Table 1. All pharmacological agents were added before pH and osmolality adjustment of solutions.

Pharmacological agent	*Concentration used*	*Targeted transport system*
Vanadate (Na_3VO_4)	1mM	Ca^{2+}ATPase and H^+ATPase
Gadolinium ($GdCl_3$)	100uM	Non-selective cation channels
TEA (tetraethylammonium)	20mM	Potassium-selective channels
Amiloride	100uM	Cation/H^+ exchangers

Table 1 pharmacological agents, concentration used and pharmacological targets.

Results

Effects of salinity on mesophyll

The effect of salinity on maximum photochemical efficiency of PSII (F_V/F_M) was studied over time intact leaf mesophyll tissue, with the relative electron transport rate (rETR) recorded at 72 hr, with results presented in Figure 1 a and b, respectively. No significant ($P<0.05$) effect of the salinity treatment on F_V/F_M was detected for treatments as high as 100 mM NaCl for up to 24 h (Fig. 1). More prolonged treatment (72 h) resulted in a significant ($P< 0.05$) decline in F_V/F_M for the 100 mM NaCl treatment, with no significant decline observed for 20 and 50 mM relative to the control. After 72 h of salt stress, the rETR did show an obvious dose-dependent decline (Fig. 1b) with 40% inhibition of rETR observed at apoplastic NaCl concentrations as low as 20 mM (Fig. 1b) at 72 h.

Effect of salinity stress on chloroplasts

To avoid the confounding effects of the plasma membrane and tonoplast transporters removing toxic Na^+ from cytosol, fluorescent measurements were also conducted on isolated chloroplasts. Before salinity effects were studied, the isolation media was optimised to ensure chloroplasts viability. This was done using F_V/F_M as a quick and convenient method for assessing chloroplast health. F_V/F_M values gradually declined with time (Fig. 2); however, the rate of decline was strongly influenced by the media composition. The optimal concentration of KCl was determined to be 150 mM (Fig. 2, 3). 1 mM ATP was beneficial as were 1 and 5 mM phosphate with no significant difference ($P<0.05$) between the two treatments (Fig. 3). A concentration of 5 mM phosphate was chosen to allow phosphate to effectively buffer the pH of the isolation media. Magnesium had no significant ($P< 0.05$) impact on F_V/F_M (Fig. 3). The final media chosen was 150 mM KCl, 1 mM ATP, and 5 mM PO_4^{3-} adjusted to an osmolality of 570 mOsm kg^{-1} with sucrose and pH adjusted to 7.1 using saturated KOH solution.

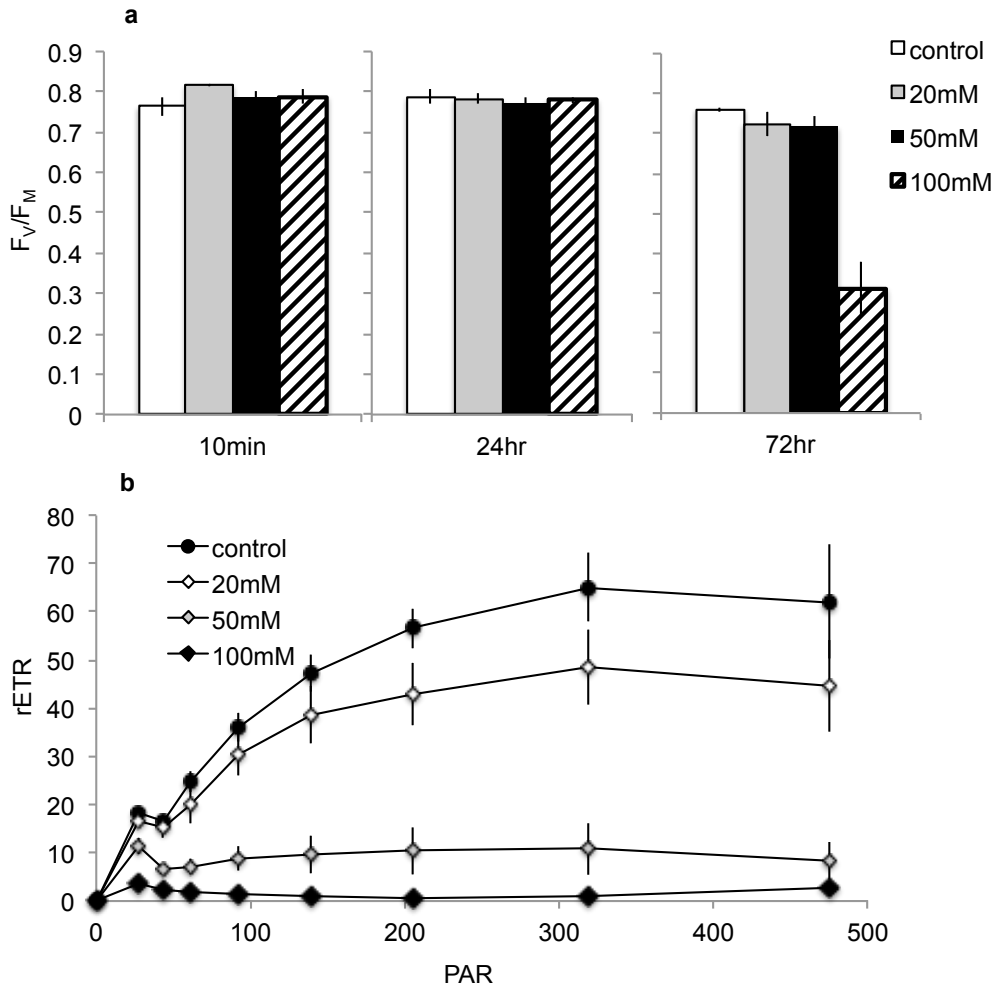


Fig. 1 Effects of NaCl on bean mesophyll tissue. a; dose and time dependency of NaCl treatment on mesophyll F_V/F_M. b; effect of different concentrations of NaCl on rETR after 72hr treatment. Mean ± SE (*n*=8)

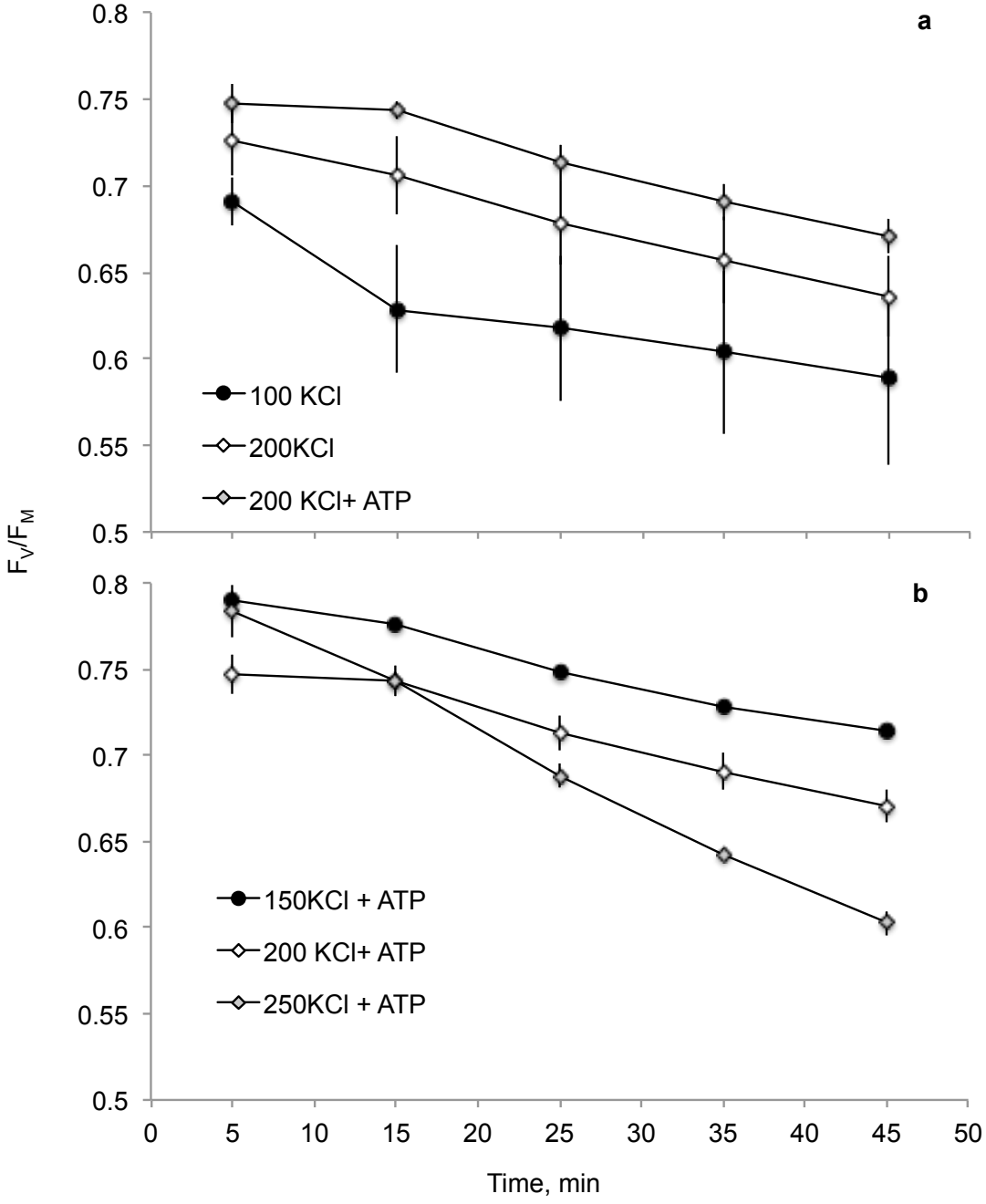
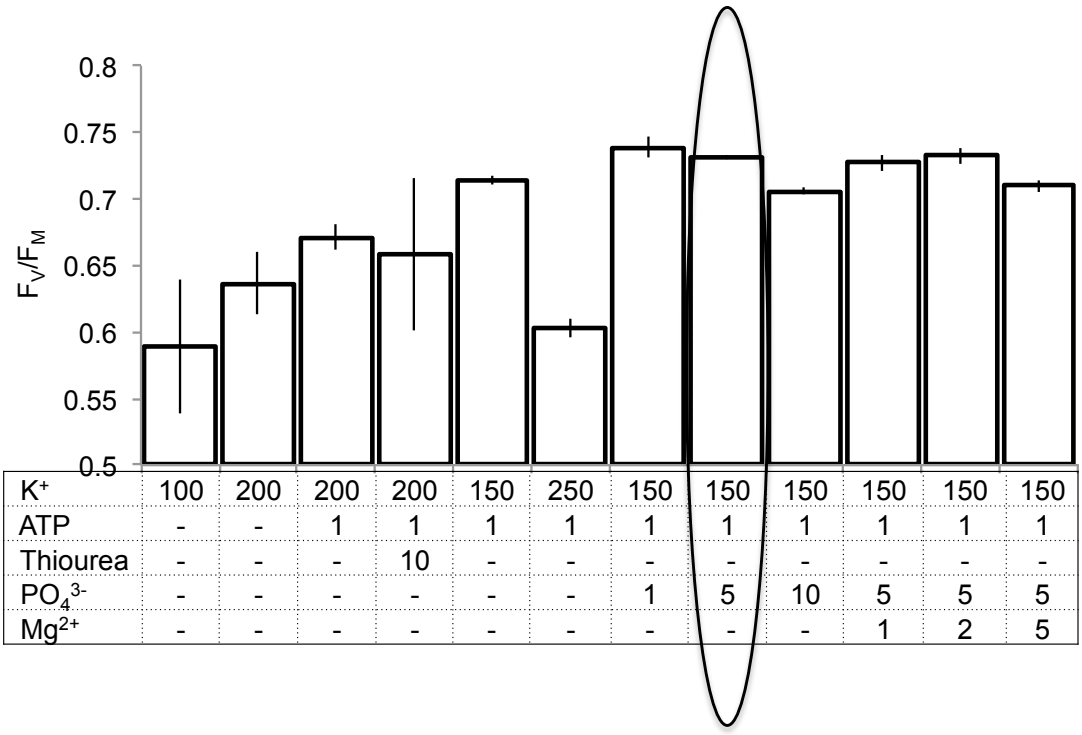


Fig. 2 Optimisation of isolation media for chloroplasts. a; optimisation of KCl and ATP content. b; optimisation of K^+ in the presence of ATP. All solutions were prepared to an osmolality of 570 mmol.kg^{-1} with sucrose and adjusted to pH 7.1 using KOH. Mean ± SE (*n*=3 batches each containing leaves from 3 individual plants)



K^+	100	200	200	200	150	250	150	150	150	150	150	150
ATP	-	-	1	1	1	1	1	1	1	1	1	1
Thiourea	-	-	-	10	-	-	-	-	-	-	-	-
PO_4^{3-}	-	-	-	-	-	-	1	5	10	5	5	5
Mg^{2+}	-	-	-	-	-	-	-	-	-	1	2	5

Fig. 3 Summary for the optimisation of the chloroplast isolation media. All solutions were adjusted to an osmolality of 570 mmol.kg^{-1} and to pH 7 using sucrose and KOH respectively. Mean ± SE (*n*=3 batches each containing leaves from 3 individual plants)

To emulate the effect of salinity stress at a whole plant level 50 mM KCl and 200 mM NaCl were used as the salinity treatment as these concentrations reflect those measured from the cytosols of salinity-treated plants (Flowers and Hajibagheri 2001; Cuin et al. 2003; James et al. 2006; Kronzucker et al. 2006). Salinity stress caused significant reductions ($P<0.05$) in both F_V/F_M and rETR after 45 min (Fig. 4ab), which is much faster than in mesophyll tissue where 72 h (3 days) was required to observe the same effect (Fig. 1). Reductions in rETR were accompanied by reductions in the amount of light required to saturate the photosystems as well as reductions in photosynthetic efficiency, alpha (Fig. 4bc).

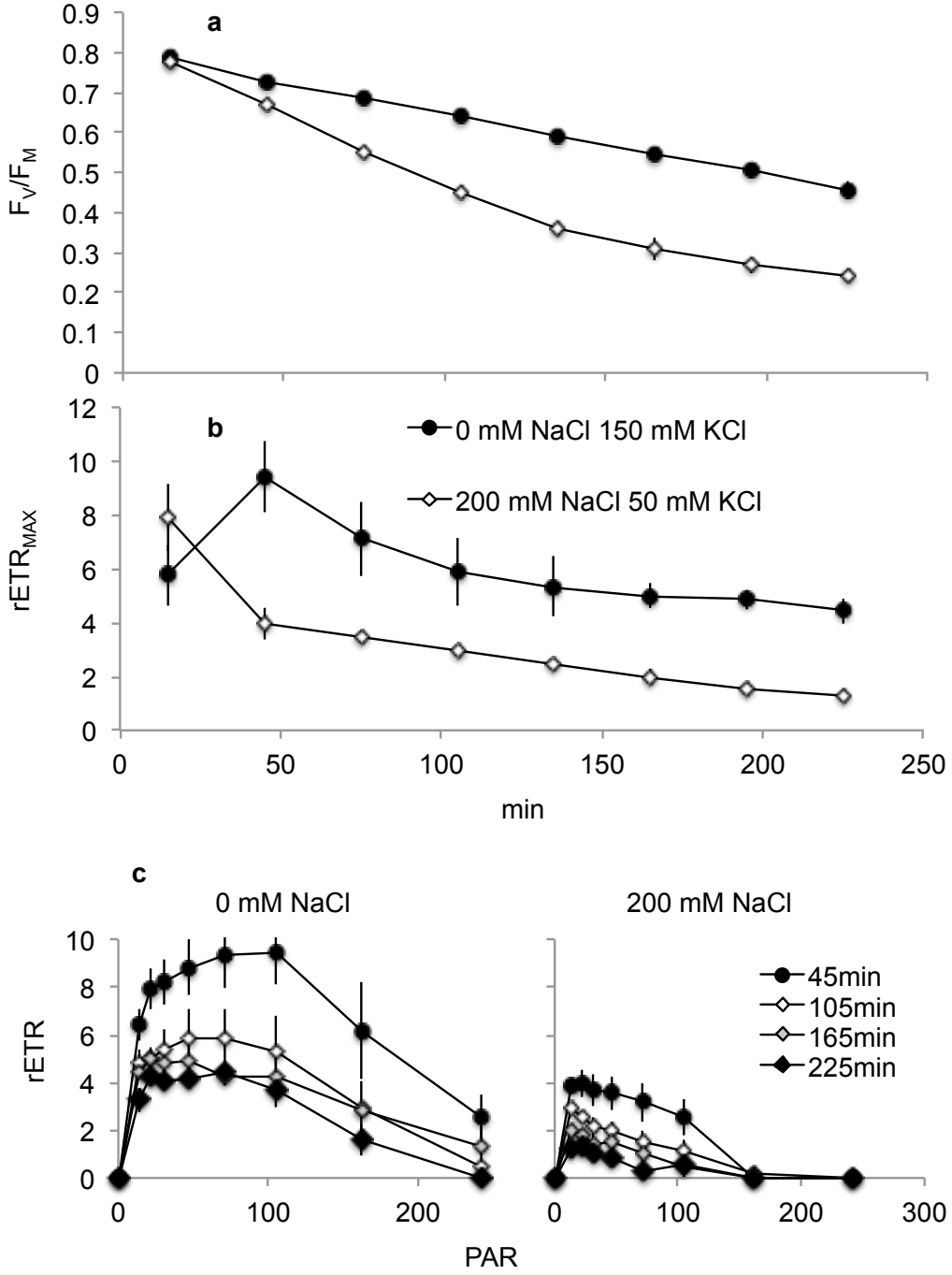


Fig. 4 Salinity damage to isolated chloroplasts over time. a; change in F_V/F_M over time. b; change in $rETR_{MAX}$. C; light curves at different times after isolation, for both salinity treated and control chloroplasts. Chloroplasts were isolated in a media containing 200 mM NaCl, 50 mM KCl, 1 mM H_2KPO_4, 1 mM ATP for salinity treatment and 150 mM KCl 1 mM H_2KPO_4, 1 mM ATP for control. Both solutions were adjusted to pH 7.10 using KOH and an osmolality of 570 $mmol.kg^{-1}$ using sucrose Mean ± SE (*n*=3 batches each containing leaves from 3 individual plants)

To separate between the effects of ionic and hyperosmotic components of salt stress, experiments were conducted by incubating chloroplasts in the bath with (1) various amounts of Na^+ added in addition to baseline osmolality, inducing both ionic and osmotic stress; or (2) in the bath at constant osmolality (570 mOsm kg^{-1}) but with variable Na^+ and K^+ content (Fig. 5). Additional osmotic stress had no significant ($P < 0.05$) effect of chloroplast performance, compared with ionic effects only. The effect of K^+ availability was more important than the presence of Na^+; this can be seen in the F_V/F_M response of chloroplasts incubated in optimal 150 mM KCl solution, which remained constant or even slightly increased with NaCl treatments (20 to 200 mM range) (Fig 5b). However, low-K^+-treated chloroplasts (10 mM treatment) performed very poorly both in low and high NaCl treatments (Fig. 5b). Interestingly, the impaired F_V/F_M performance of the low-K^+ chloroplasts was significantly ($P < 0.05$) improved by moderate (50 to 100 mM) NaCl concentrations, suggesting that Na^+ can indeed replace K^+ in some physiological processes. In K^+-sufficient chloroplasts, concentrations of Na^+ up to 100 mM were beneficial, suggesting Na^+ itself at low concentrations is not toxic to chloroplasts over a short period (45 minutes) (Fig. 5).

Halophyte chloroplasts and salinity stress

A further study of detrimental effects of salinity on chloroplast functioning was undertaken by comparing species contrasting in the salinity tolerance. This study included a halophyte (naturally salt-loving) species quinoa and two glycophyte (salt-sensitive) species of pea and broad bean. Quinoa had the lowest F_V/F_M under control conditions, but suffered significantly ($p<0.05$) less reductions in F_V/F_M under salinity treatment than beans and peas (Fig. 6). Indeed, F_V/F_M characteristics of salt-treated quinoa chloroplasts remained unchanged for the entire duration of the experiment (3 h; Fig. 6b), while in peas and beans, F_V/F_M values dropped by ~ 20% and 40%, respectively, over the 3 h of salinity treatment (Fig. 6b).

The capacity of quinoa to maintain optimal photosynthetic processes in chloroplasts in the presence of Na^+ was further demonstrated in experiments using transient fluorescence curves (Fig. 7). Quinoa chloroplasts had the lowest difference between the control and the 100 mM NaCl-treated chloroplasts ($P<0.05$); there were no significant differences between pea and broad bean.

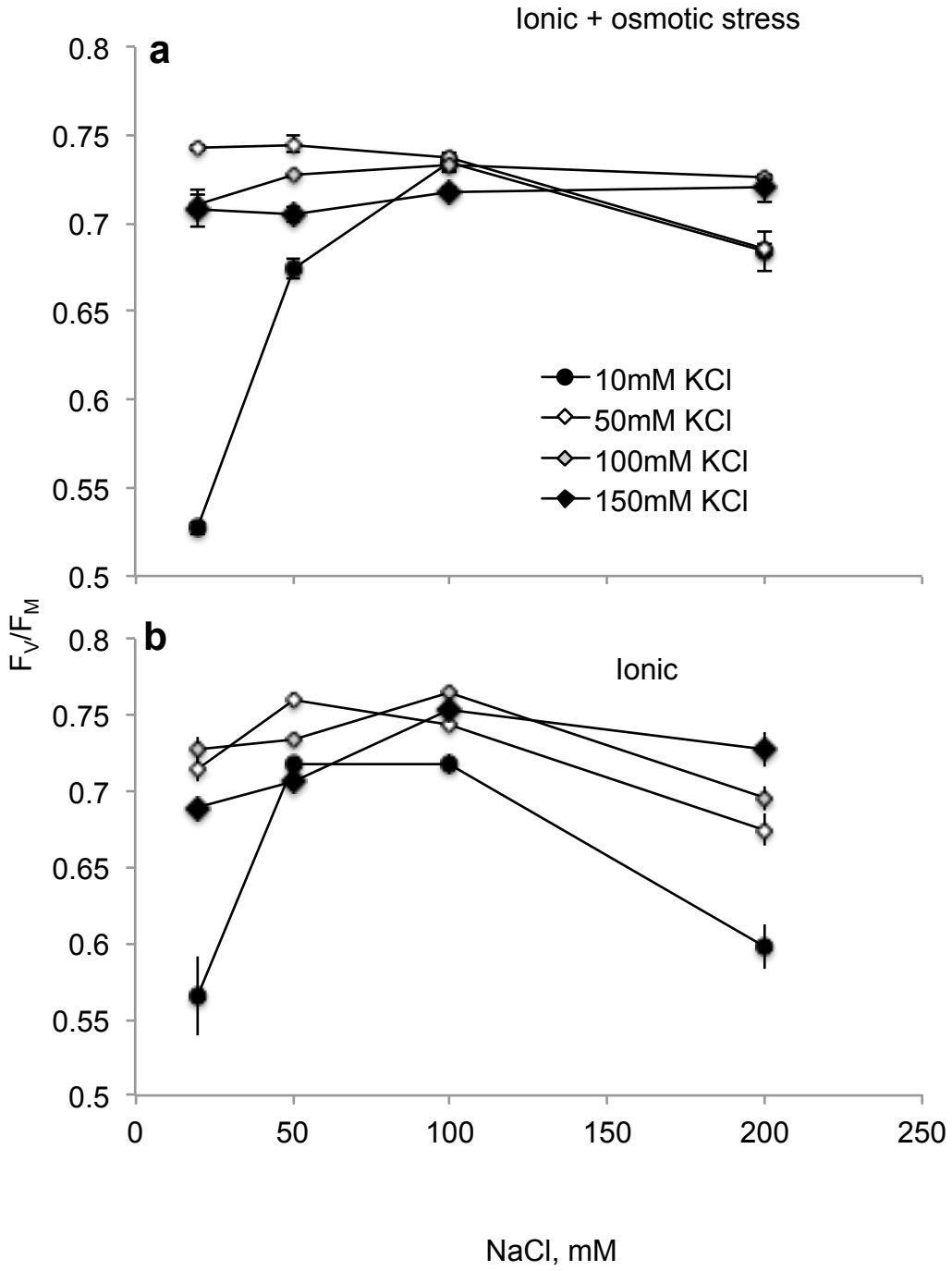


Fig. 5 Effects of K^+ and Na^+ the F_V/F_M of isolated bean chloroplasts 45 min after treatment. a; ionic + osmotic stress, K^+ solutions were prepared to 570 mmol.kg^{-1} with sucrose, then the required amount of Na^+ was added, creating hyperosmotic solutions. b; ionic solutions of Na^+ and K^+ were prepared 570 mmol.kg^{-1} with sucrose. All solutions contained 1 mM H_2KPO_4, 1 mM ATP and were adjusted to pH 7.10 using KOH. Mean ± SE (*n*=3 batches each containing leaves from 3 individual plants)

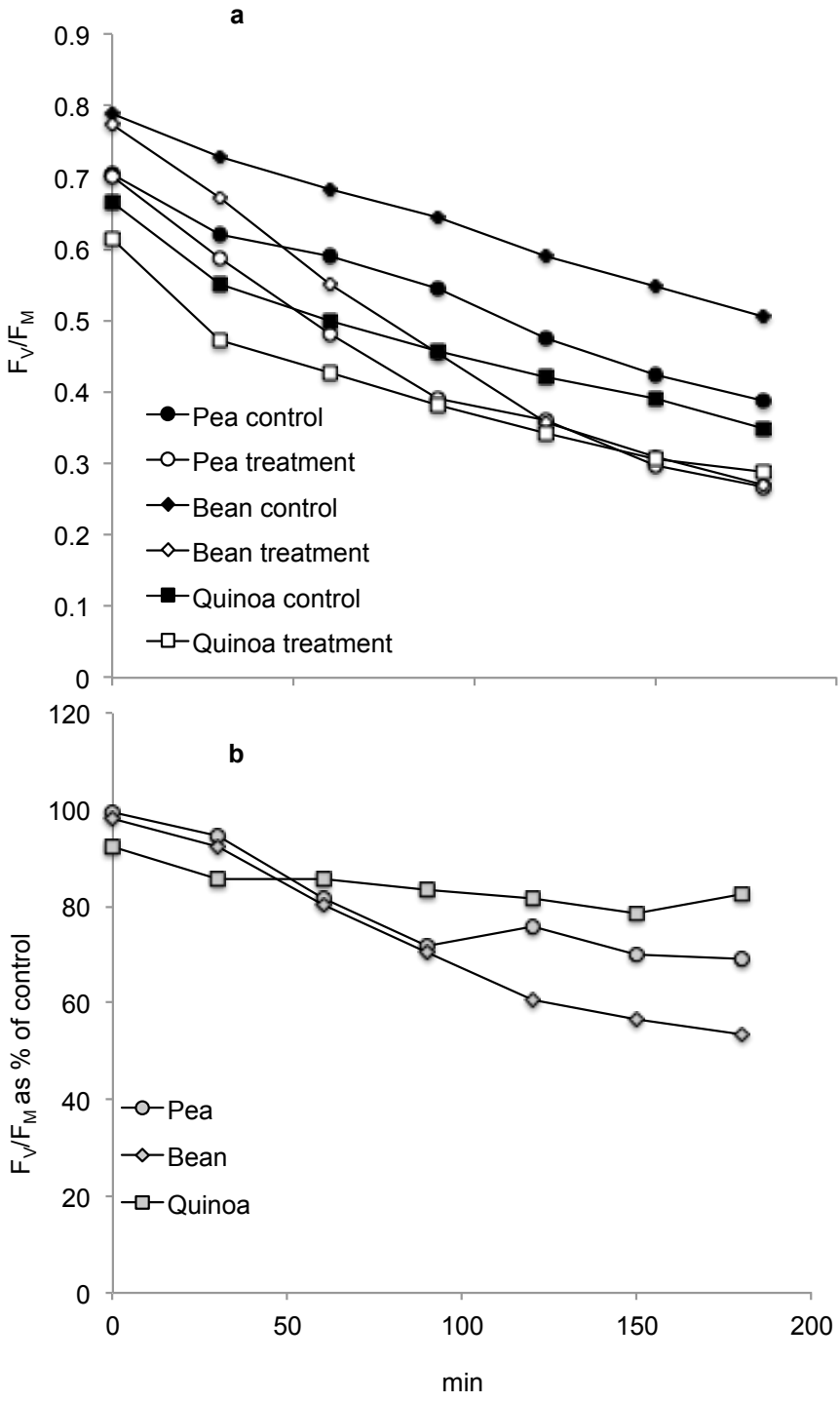


Fig. 6 F_V/F_M for chloroplast suspensions from 3 different species (pea, broad bean and quinoa) measured with and without 100 mM NaCl in the bath media. Both absolute (a) and relative (b; % control) values are shown.

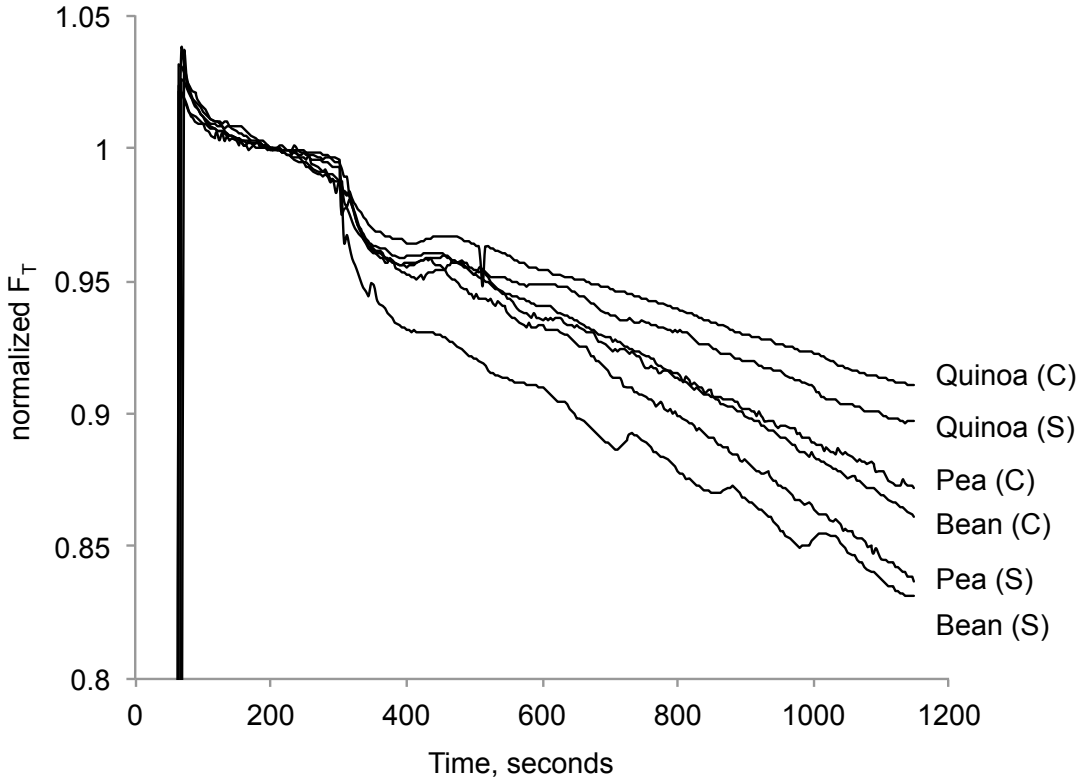


Fig. 7 Normalised fluorescence transients (F_T) of chloroplast suspensions from 3 different species (pea, broad bean and quinoa). Actinic light was turned on at 60 s. 200 µL of either 4 mol L^{-1} NaCl or BSM was added at 300 s. F_T values were normalised over the 5 s interval between 50 and 55 s. Three biological replicates were averaged for each treatment/species combination.

Ion transport and salinity stress on chloroplasts

Vanadate (a P-type ATPase inhibitor) significantly reduced F_V/F_M in both the control and salinity-treated chloroplasts (by 7% and 25%, respectively) after 100 min treatment. Gadolinium, a known blocker of non-selective cation channels (Kronzucker and Britto 2011), caused a significant decline in F_V/F_M in Na^+-free media, however, no significant difference was observed in salt-treated chloroplasts. Tetra ethyl ammonium (TEA) a blocker of potassium channels had no significant effect on non-Na^+ treated chloroplasts but significantly increased F_V/F_M in salinity-treated chloroplasts (by 19% compared with saline controls; Fig. 8). A similar effect was observed for amiloride, a known blocker of cation/H^+ antiporters (Blumwald and Poole 1985; Kleyman and Cragoe 1988; Cuin et al. 2011).

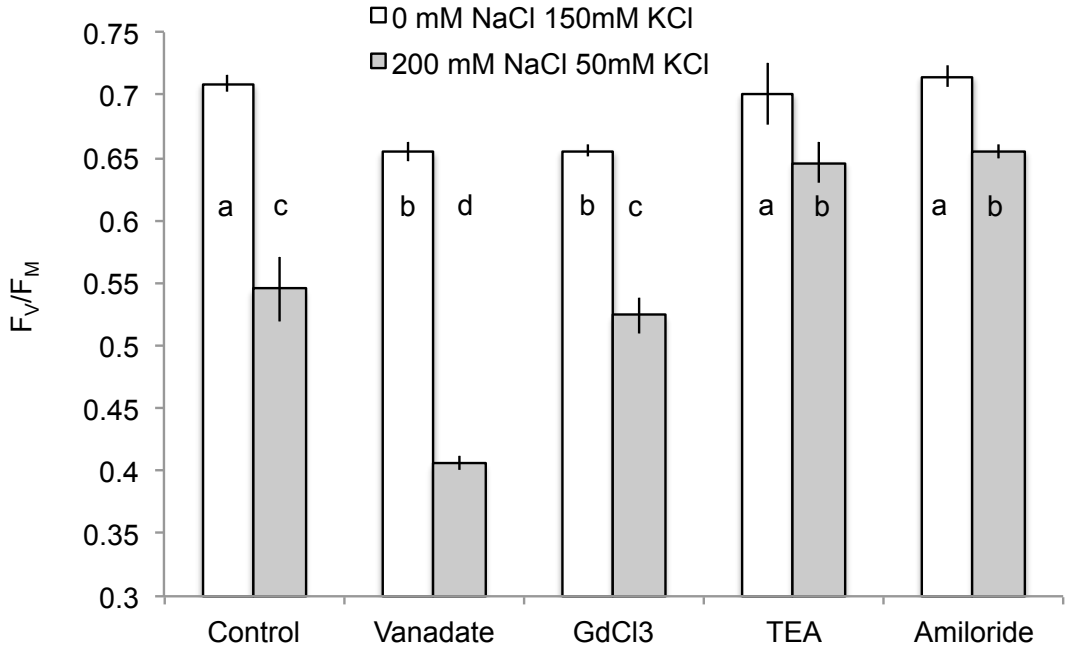


Fig. 8 F_V/F_M of suspended bean chloroplasts treated with a range of pharmacological agents. These measurements were taken 100min after treatment with pharmacological agents in the presence or absence of salt. Solutions used were: control - 150 mM KCl, 1mM H_2KPO_4, and 1 mM ATP; treatment - 200 mM NaCl, 50 mM KCl, 1 mM H_2KPO_4, and 1 mM ATP. Both solutions were adjusted to pH 7.10 using KOH and had osmolality of 570 mmol kg^{-1} (sucrose). Letters denote significance levels at <0.05, mean ± SE (*n*=3 batches each containing leaves from 3 individual plants

Discussion

Effect of NaCl on intact leaf mesophyll

There are a few different mechanisms that bean mesophyll can employ to delay the impact of exposure to increased salinity, including limiting Na^+ uptake, sequestering Na^+ in the vacuole (Apse et al. 1999; Munns and Tester 2008) and relocating potassium from the vacuole into the cytosol (Walker et al. 1996; Leigh 2001) , but once these mechanisms are exhausted F_V/F_M and rETR are affected. These mechanisms explain why increasing apoplastic salinity using 100 mM NaCl only

caused a significant decline in rETR and F_V/F_M in intact leaf mesophyll tissue after 72 hrs (Fig. 1a). Removing aforementioned protection mechanisms by isolating chloroplasts from mesophyll tissue resulted in a rapid decline in F_V/F_M and rETR values within an hour of NaCl exposure (Fig. 4).

Essentiality of potassium for chloroplast function

Stress causes a massive K^+ efflux from mesophyll tissues (Chen et al. 2005; Cuin et al. 2008). This results in significant reductions in the concentration of K^+ in the cytosol, to levels below 20 mM, as shown in direct measurements using K^+-selective impaled microelectrodes (Cuin et al. 2003). In leaves, K^+ deficiency results in reduced photosynthetic rate due to lowered chlorophyll content, changes in chloroplast ultrastructure and restricted sugar translocation (Cakmak et al. 1994; Zhao et al. 2001; Cakmak 2005). K^+ is one of the dominant cations involved in charge balancing across the thylakoid membrane and, thus, mediates the light induced changes in H^+ flux in chloroplasts (Hind et al. 1974; Chow et al. 1976; Krause 1977). Potassium is also important for normal functioning of the electron transport chain, reduced K^+ concentrations lead to greater superoxide production, causing further damage (Cakmak et al. 1994).

The direct measurement of cytosolic K^+ is technically challenging and therefore there are very few actual values (cited by Britto and Kronzucker 2008). Reported values vary between 25 and 320 mM, which is not consistent with the concept of cytosolic K^+ homeostasis (Wakeel et al. 2011; Anschütz et al. 2014). However, the most extreme values reported are not direct measures but rather estimates derived from radiotracer decay kinetics (Behl and Wolf 1982; Szczerba et al. 2006; Britto and Kronzucker 2008).

Determining the lower K^+ threshold for chloroplast function is not a trivial task *in vivo*, as other non-stomatal effects on photosynthesis are occurring simultaneously. *In vitro* measurements of isolated chloroplasts, reported herein, suggest the optimal K^+ concentration in the cytosol is around 150 mM, as judged by the highest F_V/F_M values (Figs. 1 & 5). This value is consistent with measures of cytosolic K^+ made by a variety of different techniques including efflux analysis, longitudinal ion profiling, x-ray micro analysis, fluorescent dies, K^+ selective micro electrodes and cell

fractionation under many different experimental conditions (as reviewed in Britto and Kronzucker 2008). It is also consistent with optimal K^+ concentrations for enzyme activation (Miller 1993; Jin et al. 2011) and for suppression of PCD enzymes (Hughes et al. 1997; Lam and del Pozo 2000; Demidchik et al. 2010). Both higher and lower concentrations caused a pronounced decline in PSII efficiency (Figs. 2 & 5).

Suboptimal K^+ concentrations lead to reduced enzyme functioning, such as Rubisco (Jin et al. 2011) or 2,2-dialkylglycine decarboxylase (Miller 1993). This has an impact on processes such as oxidative phosphorylation (Flowers 1974) and biosynthesis of NADPH (Pflüger and Mengel 1972). Potassium depletion may also reduce the ability of chloroplasts to carry out charge balance; a feature considered to be essential for photosynthesis. By changing the chaotropic and lyotropic properties of the aqueous environment as well as the opportunities for ionic interactions, (Mancinelli et al. 2007; Galamba 2012), changes in K^+ concentration affect the 3D structure of proteins and other biomolecules. For example, PSII is a highly complex structure imbedded into lipid environments and is associated with over 1300 water molecules (Nelson and Yocum 2006; Umena et al. 2011). The structural complexity of PSII makes it vulnerable to changes in the ionic environment (Vander Meulen et al. 2002; Demetriou et al. 2007). Thus, the reductions in F_V/F_M observed under low or high K^+ (Figs. 2 & 5) could be explained by the ionic perturbation of the proteins and the light-harvesting complex.

Effects of NaCl on chloroplasts

Increased Na^+ had less effect on F_V/F_M when there was a high K^+ concentration in the media (Fig. 5). This suggests that it is the depletion of K^+ rather than concentration of Na^+ that causes the damage to chloroplasts. While similar conclusions have been published previously (Kronzucker et al. 2013), this is the first time this relationship is confirmed in direct relationship to leaf photochemistry. Previous reports where increased K^+ concentrations, up to 100 mM, were shown to enhance photochemical parameters (Bulychev and Vredenberg 1976; Demmig and Gimmler 1983) failed to investigate the potential negative effects of Na^+. Kronzucker et al. (2013) suggested the Na^+ concentration in the cytosol *in planta* does not reach sufficient levels to cause enzyme inhibition. While this may be true, there remains uncertainty around the

actual Na^+ concentrations in the cell cytosol, with reports ranging from 5–40 mM (Schubert and Lauchli 1990; Carden et al. 2003) to over 200 mM (Flowers and Hajibagheri 2001; James et al. 2006; Kronzucker et al. 2006) depending on the technique and salt treatment used. This 10-fold uncertainty surrounding the cytosolic Na^+ concentration makes it difficult to draw an unambiguous conclusion about the extent of Na^+ damage to chloroplasts. In our study, we controlled the bath Na^+ concentrations, mimicking cytosolic Na^+ content. In fact, 100 mM of Na^+ in the "cytosol" may even be beneficial when chloroplasts are exposed to low K^+ media (mimicking stress-induced K^+ exodus from the cytosol)(Fig. 5b). Furthermore, no detrimental effects of Na^+ on F_V/F_M was found in plants with optimal "cytosolic" K^+ content (100 - 150 mM range) in a salinity range mimicking up to 100 mM Na^+ in the cytosol (Fig. 5a). These results strongly suggest that it is not the excess of Na^+ but the lack of K^+ in the cytosol that impairs chloroplast functioning under saline conditions. Kronzucker et al.'s (2013) suggestion that Na^+ is able to replace K^+ functionally in some enzymes and even enhance the activity of some enzymes when K^+ is present is consistent with this finding.

Osmotic stress at the whole plant level causes a reduced transpiration rate and reduced stomatal conductance (Munns 2002), leading to stomatal limitation of photosynthesis (Munns and Sharp 1993). These stomatal limitations lead to photorespiration and oxidative damage. At an intra-cellular level, the effects of increased cytosolic osmotic potential do not inhibit PSII more than Na^+ toxicity itself (Fig. 5). Increased cytosolic osmotic potential would be expected to cause hypertonic stress removing water from the stroma and lumen of the chloroplasts, which in turn would lead to a reduction in photosynthetic efficiency. Wignarajah & Baker (1981) observed that increases in osmotic pressure can lower PSI activity without effecting PSII in *Pisum sativum* and *Aster tripolium*. F_V/F_M is a very quick measure of the intactness of PSII but not of overall photosynthesis. In this context, it does not measure the electron transport chain or other membrane bound structures. The shrinking and swelling of the chloroplast would be expected to change membrane configurations in the thylakoid membrane and cause perturbations in the latter parts of photosynthesis. This would explain the lack of ionic effect on F_V/F_M.

Halophytes and glycophytes

Despite the superior ability of halophytes to grow optimally in saline environments (Flowers et al. 1977; Cheeseman 2013), it is assumed that halophytes do not have higher enzymatic tolerance to high salinity compared with glycophytes (Greenway and Osmond 1972). Instead, they are suggested to possess mechanisms to maintain optimal cytosolic ion homeostasis by efficient pumping of cytotoxic Na^+ into vacuoles (Glenn et al 1999; Flowers and Colmer 2008). Quinoa is a halophyte species that relies heavily on vacuolar Na^+ sequestration. It has been calculated that 93% of the total osmotic adjustment in mature quinoa leaves is achieved by means of accumulation of inorganic ions (Shabala et al. 2012), while in glycophyte crops these values range between 47 % and 93 % (Pérez Alfocea et al. 1993; Meloni et al. 2001; Morant-Manceau et al. 2004). Quinoa also possesses some other important mechanisms of salinity tolerance in leaf mesophyll, such as (i) a higher rate of Na^+ exclusion from leaf mesophyll; (ii) maintenance of low cytosolic Na^+ levels; (iii) better K^+ retention in the leaf mesophyll; (iv) a high rate of H^+ pumping, which increases the ability of mesophyll cells to restore their membrane potential; and (v) the ability to reduce the activity of slow activating (SV) and fast-activating (FV) channels under saline conditions and thus prevent a leaking of cytotoxic Na^+ back to cytosol (Bonales-Alatorre et al. 2013a; 2013b). Here it is demonstrated that quinoa chloroplasts also possess an enhanced ability of to maintain optimal performance under elevated (mimicking cytosolic) NaCl concentrations, as compared with glycophyte species (Fig 6, 7). Several possible explanations can be given. *In vitro* reports of the equal sensitivity of metabolic enzymes of halophytes to Na^+ (Greenway and Osmond 1972; Flowers and Colmer 2008) are not accurate and cannot be extrapolated to *in planta* systems. Besides, halophytes chloroplasts may possibly have intrinsically higher levels of oxidative protection, more stable photosynthetic apparatus, and/or better control over chloroplast membrane transport.

Chloroplasts are the main cite of reactive oxygen species (ROS) production in photosynthesising green tissues (Asada 2006; Pospíšil 2009; Kozuleva et al. 2011; Bose et al. 2014), and salt-stress induced ROS accumulation has been reported in leaf mesophyll in both halophyte (Benzarti et al. 2012) and glycophyte (Hernandez et al. 1995; de Azevedo Neto et al. 2006) species. The main ROS species produced are

hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\cdot-}$) and singlet oxygen ($^1O_2$) (Bose et al. 2014).To combat salinity-induced oxidative stress, plants increase the amount of antioxidants in their chloroplasts; such increases have been reported for both peas (Hernandez et al. 1995) and quinoa (Qiu-Fang et al. 2005; Cai Hong et al. 2005). Amor *et al.* (2006) showed that differences in antioxidant enzyme activity and reactive oxygen species concentrations in chloroplasts were responsible for differences in salt tolerance between two varieties of the halophyte species *Cakile maritime*. Plants displaying higher inducible antioxidant enzyme activities (including: superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and glutathione reductase (GR)) in their leaves are generally more salt tolerant (Gossett et al. 1994; Hernandez et al. 1995; 2000; Shalata and Tal 1998; de Azevedo Neto et al. 2006). Many halophytes have constitutively higher antioxidant enzyme activities (as reviewed by Bose et al. 2014). Oxidative studies using ozone in *Populus* leaves indicate that it takes between 15 and 30 minutes for glutathione concentrations to increase; in this time much of the existing glutathione had been oxidised (Gupta et al. 1991). In our experiments differences in charge balancing between the two photosystems (F_t) were observed within 1 minute (Fig. 7). Based on this timeframe, the differences seen between quinoa chloroplasts and those from the glycophyte plants are likely to be caused by differences in the constitutive rather than inducible antioxidative mechanisms, as the isolated chloroplasts would not have time to produce new antioxidant defences. Furthermore, SOD isoforms are all encoded in the nuclear DNA, and as such would not be able to be up regulated in isolated chloroplasts (Pilon et al. 2011).

Differences between the three species could also be related to differences in their ion transport activity and ionic homeostasis within chloroplasts. In the halophyte, *Mesembryanthemum crystallinum* chloroplasts, the amount of K^+ declines from 133 to 45 mM as the amount of Na^+ increases from 1.5 to 195 mM when the salinity increases from no added NaCl to 400 mM NaCl; these changes were much less than the changes that occurred in the total leaf sap (Demmig and Winter 1986). While in another halophyte species, *Suaeda australis,* the concentration of Na^+ in the chloroplast is more tightly controlled despite similar increases in ion content in the leaf, even with reduced Na^+ contents in plants from the most saline treatment (Robinson and Downton 1985). In peas, 100 mM treatment for 10 days resulted in a

drop in K^+ concentration in the chloroplasts from 53 to 29 mM (Speer and Kaiser 1991). At the same time, the vaculor content decreased from 104 to 42 mM, and Na^+ concentration increased from 19 to 60 mM in the chloroplast and from 3 to 114 mM in vacuoles (Speer and Kaiser 1991). Although the differences between the reported chloroplast concentrations of Na^+ and K^+ of halophytes and glycophytes are similar, the NaCl concentrations to which the halophytes had been exposed are much higher, showing their superior ability to maintain ion homeostasis. This ability to control ion transport is important for the maintenance of metabolic pathways in the chloroplast including the activity of two key antioxidative mechanisms SOD and peroxidase (Wang et al. 2007). Overall, these results suggest that there are differences in the abilities of chloroplasts from different species to control ion transport into and out of the chloroplast. The pharmacological studies below were conducted to gain some insights into the possible transport systems involved.

Membrane transport systems mediated ionic homeostasis in chloroplasts

Ion channels in chloroplasts regulate photosynthesis through modulation of the proton motive force (Checchetto et al. 2013) and the ionic composition of the chloroplast. In addition to the direct regulation of photosynthesis, ion channels are also responsible for the entry and exit of all ions in and out of the chloroplast and hence the ionic environment in which chloroplasts operate. Photosynthesis causes light induced proton movement into the lumen from the stroma via cytochrome *b6f*; this in turn creates a proton gradient which is used for ATP synthesis, with the release of H^+ back into the stroma via ATP synthase (Checchetto et al. 2013). The increase in H^+ in the lumen leads to acidification and a build-up of a pH gradient across the thylakoid membrane and a charge gradient is generated. If this charge gradient is not dissipated, a negative feedback on photosynthesis occurs (Rott et al. 2011). Ion channels are essential for the charge balancing during photosynthesis (Pottosin and Schönknecht 1996), and this balancing occurs through the efflux of K^+, Mg^{2+} from the lumen and an influx of Cl^- (Hind et al. 1974; Chow et al. 1976; Krause 1977).

Specific ion channels in the chloroplast inner envelope and the thylakoid membranes can be blocked using pharmaceutical agents, providing insights into the mechanisms

of ion homeostasis. When F_V/F_M is used to measure PSII activity, the direct effects of changes in ionic composition are measured by applying a very short pulse of light to the photosystem not to obstruct the charge balancing capacity. Chloroplasts have three membrane structures, namely the outer envelope, the inner envelope and the thylakoid. The outer envelope has three relatively non-selective porins *OEP24*, *OEP26* and *OEP21*, which means the outer membrane has little if any influence on the ion homeostasis within the chloroplast (Flügge 2000). The other two membranes, the inner envelope and the thylakoid, both host an array of different and often highly selective transporters that regulate the concentration of various ions in the lumen and stroma.

The two known non-selective cation channels in chloroplasts are the thylakoid voltage gated cation channel (Pottosin and Schönknecht 1996) and the inner envelope membrane fast activated cation channel (FACC; Pottosin et al. 2005). Both these channels are permeable to Na^+ and can be blocked by $GdCl_3$. The FACC is likely to be the channel by which Na^+ enters the chloroplast. However, blocking it with $GdCl_3$ did not affect F_V/F_M (Fig. 8), suggesting restricts Na^+ entry into the chloroplast is not important in conferring chloroplast tolerance to salinity. This is consistent with our observation that increased Na^+ concentration applied to the chloroplasts did not cause reductions in F_V/F_M unless the chloroplasts were incubated in the low-K^+ media (Fig. 5).

It has been shown that TEA is capable of reducing rETR, through the blockage of *TPK3* (a two pore K^+ selective channel) expressed at the thylakoid membrane (Fang et al. 1995; Carraretto et al. 2013), by causing a high charge gradient and a negative feedback on cyt *b6f* (Checchetto et al. 2013; Carraretto et al. 2013). However, TEA had no effect on F_V/F_M in isolated chloroplasts exposed to normal non-saline conditions, but TEA caused a significantly reduction in the salinity-induced decrease in F_V/F_M (Fig. 8). It is possible that this beneficial effect may be due to TEA blockage of K^+ leakage from the chloroplast that would otherwise have been induced by high external salinity. At a cellular level, the NaCl-induced K^+ leakage is usually mediated by depolarization-activated outward-rectifying K^+ channels (GORK in Arabidopsis) (Shabala and Cuin 2007; Shabala and Pottosin 2014). In the chloroplast inner envelope, the most likely candidate is a voltage independent K^+ channel. These

channels are typically blocked by TEA, Mg^{2+} and Ba^{2+} and show no voltage gating. The K^+ selectivity was not measured directly but it is likely due to sequence homology with other K^+ selective channels (Mi et al. 1994); this sequence homology also makes blockage by TEA likely.

Vanadate has been shown to inhibit ion transport in chloroplasts through direct inhibition of the inner envelope Ca^{2+}-ATPase (Hochman et al. 1993) and the H^+-ATPase (Carmeli et al. 1992). Vanadate caused a dramatic decline in F_V/F_M in both salt treated and control chloroplasts (Fig. 8), with a more pronounced effect salt-treated chloroplasts. These results indicate that ATPase-pump activity is essential for salinity tolerance in chloroplasts. H^+-ATPase is also essential for pH homeostasis in the stroma of chloroplasts, this in turn is needed for ATPsynthase as it relies on a pH gradient across the thylakoid membrane (Checchetto et al. 2013). Ca^{2+}-ATPase has been shown to play a key role in restoring the basal level of cytosolic Ca^{2+} in response to a range of abiotic and biotic stresses, once the signalling has been completed (Bose et al. 2011). A similar role is possible for stroma. Vanadate also directly inhibits ATP synthase (Rexroth et al. 2004), the inhibition of ATP synthase would have an effect on longer term measurements than the measurement of F_V/F_M used here.

There are a few known monovalent cation (K^+ and Na^+) antiporters in the inner envelope of the chloroplast, including *CHX23* (Song et al. 2004; Evans et al. 2012) and K^+ efflux antiporters (*KEA1* and *KEA2*) (Pfeil et al. 2013). These transporters are believed to be involved in the regulation of the stroma pH through the electro neutral exchange of H^+ with monovalent cations from the stroma into the cytoplasm (Huber and Maury 1980; Maury et al. 1981; Song et al. 2004; Pardo et al. 2006). In mammalian systems, $Na^+(K^+)/H^+$ exchangers are blocked by amiloride (Kleyman and Cragoe 1988); similar reports were presented for *SOS1* plasma membrane Na^+/H^+ antiporters (Cuin et al. 2011) and a tonoplast Na^+/H^+ antiporter (Blumwald and Poole 1985). Unlike at the plasma membrane the chloroplast antiporters are responsible for K^+ loss from the stroma (Huber and Maury 1980; Maury et al. 1981). Blockage of these inner envelope antiporters with amiloride would result in reduced K^+ loss from the stroma under saline conditions. This reduced K^+ efflux from the stroma could be the reason for the positive effects of amiloride on F_V/F_M under saline conditions. In addition, the positive effect could also be caused by blocking the Ca^{2+} antiporter

(*CAX*) on the thylakoid membrane (Ettinger et al. 1999), as Ca^{2+} ions are essential to the correct functioning and structure of the oxygen evolving complex (Brand and Becker 1984; Vander Meulen et al. 2002). Further studies would be required to distinguish between the pharmacological activities of amiloride.

Conclusion

Halophyte (quinoa) chloroplasts were found to be less sensitive to changes in K^+ and Na^+ concentrations in the cytosol than chloroplasts from glycophytes (peas and beans). For salt tolerance, the capacity to maintain high K^+ concentrations in the cytosol appears to be more important than the capacity to reduce Na^+ concentrations. It was also found that the ability of chloroplasts to regulate their ion uptake under saline conditions contributes to their ability to prevent salinity-induced damage to the photosynthetic apparatus as TEA and amiloride both increased the F_V/F_M of low K^+ high Na^+ treated chloroplasts.

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Chapter 4: Amelioration of the effects of salinity on chloroplasts

William J. Percey[1], Andrew McMinn[2], Jayakumar Bose[1], Michael C. Breadmore[3], Rosanne M. Guijt[4], Sergey Shabala[1]

[1]School of Land and Food, University of Tasmania, Hobart, Tasmania 7001, [2]Institute for Marine and Antarctic Studies, University of Tasmania, Hobart, Tasmania, [3]Australian Centre for Research on Separation Science (ACROSS), School of Chemistry, University of Tasmania 7001 and [4]School of Pharmacy, University of Tasmania, Hobart, Tasmania 7001

Abstract

The remediation of the effects of salinity on the photosynthetic properties of isolated chloroplasts and mesophyll tissue was investigated for a number of compounds using PAM chlorophyll fluorescence techniques. The antioxidant dimethyl sulfoxide (DMSO) and the compatible solute glycine betaine were able to ameliorate the effects of salinity stress on mesophyll tissue, increasing both the maximum photochemical efficiency of photosystem II (F_V/F_M) and the relative electron transport rate (rETR). The polyamines putrescine and spermine were found to increase the F_V/F_M of salinity treated chloroplasts by 15 and 22% respectively. Proline was the most effective compatible solute at remediating the effects of salinity stress on the F_V/F_M of isolated chloroplasts causing 100% remediation. The compatible solutes trehalose, mannitol and betaine also remediated the effects of salinity on F_V/F_M to lesser extents. All the tested polyamines and compatible solutes caused an initial increase in the rETR of isolated chloroplasts treated with salinity, but caused significant negative effects after 100 min of treatment. The antioxidant thiourea was able to remediate the effects of salinity on the F_V/F_M of isolated chloroplasts, while DMSO had no effect. In conclusion, to chemically confer salinity tolerance, a very tight control over the accumulation of both compatible solutes and polyamines in the chloroplasts of stressed plants is required.

Key words: polyamines, compatible solutes, chlorophyll fluorescence, PAM, reactive oxygen species, antioxidant

To be submitted to: *Environmental and Experimental Botany*

Introduction

Salinity costs agriculture in excess of $12 billion p.a. in lost production globaly. Remediation of agricultural land through management practices based on land engineering and removal of salts from the soil is expensive and not economically viable, leaving only two practical ways to overcome detrimental effects of salinity on crop production. The first option is breeding/engineering plants for salinity tolerance; the second alternative is a chemical amelioration of toxicity effects. Both processes require a comprehensive understanding of how salinity stress affects plant metabolic and physiological processes (Ashraf and Foolad 2007; Shabala et al. 2012).

There has been growing interest in engineering plants to increase production of compatible solutes (low molecular weight organic molecules that can accumulate in the cytoplasm with out affecting metabolic processes in the cell), including polyols and amino acids (Ashraf and Foolad 2007), antioxidative enzymes (Pilon et al. 2011) and other metabolites including polyamines (Hussain et al. 2011) that may have beneficial effects under salinity stress. Each of these attempts has achieved only a limited success. A range of genes have been manipulated with the aim of improving salinity tolerance in a wide range of plant species. Genes including *codA* (Hayashi et al. 1997; Huang et al. 2000), *cox*, and *betA* have all been studied in relation to salinity stress in a diverse group of plants (Chen and Murata 2002). Transformations increased the concentration of glycine betaine, in some cases leading to increased salinity tolerance (Hayashi et al. 1997; Huang et al. 2000). However, not all transgenic betaine producers show increased salinity tolerance (Huang et al. 2000) and one side effect to the increased production of betaine is reduced yield in non-salinity treated plants (Wang et al. 2013; Huang et al. 2000). Last but not least, a

generally negative public perception of GMO hampers the prospects of using transgenic crops for food production in marginal and saline lands.

A viable alternative to creating transgenic crops with improved salinity tolerance is ameliorating salinity stress symptoms by chemical means. There are a variety of different classes of chemicals used for amelioration; these can be separated into four broad categories (Shabala and Munns 2012): (i) mineral nutrients, (ii) compatible solutes, (iii) polyamines and (iv) hormones.

Mineral nutrients can be used for ameliorative purposes in two ways: applied directly to the soil before sowing, or added later to the foliage. Ca^{2+} and other divalent cations are generally applied to the soil improving soil structure (Sumner 1993) and beneficially effecting plant growth. However, beneficial effects of supplemental Ca^{2+} were also observed in hydroponic systems (Shabala et al. 2003), suggesting that alleviation of salt stress symptoms is not limited to improved soil structure but is causally related to altered plant physiology. Ca^{2+} was shown to reduce Na^+ uptake in Arabidopsis roots (Reid and Smith 2000) by inhibiting uptake through NSCC (Demidchik and Tester 2002). Ca^{2+} also restricts Na^+-induced K^+ efflux in roots and leaves (Shabala et al. 2006). It is also possible that increased soil Ca^{2+} leads to higher cytosolic concentrations of Ca^{2+}, resulting in increased SOS1 activity (White and Broardley 2003). Elevated cytosolic Ca^{2+} may also enhance Na^+ sequestration in vacuole through greater NHX activity (Zhu 2003).

Foliar application of nutrients can be used to overcome salinity-induced nutrient imbalances (Yildirim et al. 2009). The foliar application of potassium resulted in increased growth in tomato under saline conditions, which was accompanied by increased chlorophyll content (Kaya et al. 2001). Growth increases were also observed in salt-grown strawberry with the foliar application of KNO_3 and $Ca(NO_3)_2$ (Yildirim et al. 2009). Other foliar nutrient applications including $Ca(NO_3)_2$, $MgSO_4$ and K_2HPO_4 have increased salinity tolerance in rice with the observation of enhancing plant photosynthetic parameters (Sultana et al. 2001). While these applications of nutrients improved plant growth under saline conditions, the practicality of this approach is still questionable, as applications should be given every few days. A better understanding of timing for the foliar applications of

fertilisers to minimise the number of applications would be highly beneficial. Better yet would be to apply other substances that can help the plants to remediate nutrient imbalances through changing nutrient uptake.

The foliar application and genetic manipulation of polyamine metabolism can improve plant salt tolerance under certain conditions. Polyamines have many different effects on plants such as; controlling plant senescence (Pandey et al. 2000), regulation of ion transport (Shabala et al. 2007), direct structural protection of the photosystems (Demetriou et al. 2007) and ROS scavenging (Ha et al. 1998). When applied as foliar sprays, putrescine, spermine and spermidine improve ion homeostasis of saline-grown plants (Krishnamurthy 1991; Chattopadhayay et al. 2002; Lakra et al. 2006; Wang et al. 2007). They also may prevent the degradation of chlorophyll and alleviate some other metabolic effects of salt stress (Krishnamurthy 1991; Chattopadhayay et al. 2002). However, an equally large number of reports on exogenously applied polyamines showed no beneficial (or even negative) effects on plant performance under salinity stress (Suleiman et al. 2002). With most reports being merely phenomenological and conducted at the whole-plant level, little is known about the effects of polyamines on photosynthesis under salinity stress at a subcellular level. This understanding is fundamental to develop better protocols for polyamine application.

When exposed to salinity, many plants increase accumulation of compatible solutes (Serraj and Sinclair 2002), and higher accumulation has been traditionally associated with enhanced salinity tolerance (Shen et al. 1997b; Chen and Murata 2002; Chen et al. 2007). Numerous attempts were also undertaken to exogenously apply compatible solutes to different plant species to ameliorate stress symptoms (Ashraf and Foolad 2007; Athar et al. 2009; Shabala and Munns 2012). The increases in salinity tolerance have been attributed to antioxidative effects (Smirnoff and Cumbes 1989; Keunen et al. 2013), enzyme stabilisation (Mutrata et al. 2007) and the ability of the solutes to act as molecular chaperones (Hare et al. 1998; Chen and Murata 2002). However, the direct effects of compatible solutes on photosynthesis in salinity stressed chloroplasts remain largely unexplored.

Plant hormones have been demonstrated to increase salinity tolerance in a variety of plants through both foliar sprays (Gadallah 1999a) and seed priming (Gao et al. 1999; Iqbal and Ashraf 2007). There is little evidence as to the actual mechanisms of this induced tolerance, however it has been suggested hormone application improves ion homeostasis reducing Na^+ and Cl^- uptake while maintaining adequate K^+ nutrition (Gadallah 1999a; Iqbal and Ashraf 2005). Increased tolerance has also been attributed changes in antioxidant activity (Zhang et al. 2007; Eisvand et al. 2010).

The exogenous application of antioxidants such as ascorbic acid (Athar et al. 2008) or thiourea (Khan and Ungar 2001; Kaya et al. 2013; Anjum et al. 2008) may provide a practical way of dealing with salinity stress. Antioxidants were found to increase photosynthesis (Anjum et al. 2008; Athar et al. 2008), improve K^+ nutrition (Kaya et al. 2013; Athar et al. 2008), enhance seed germination (Khan and Ungar 2001) and increase yield (Anjum et al. 2008). The effects of different antioxidants seem to be species- and cultivar- specific. In wheat, one salt-treated cultivar was observed to have increased yield with exogenously ascorbic acid while the other cultivar's yield was unaffected despite improved photosynthetic rate (Athar et al. 2008). The exact mechanisms by which antioxidants increase the efficiency photosynthesis remains unknown, nor are the differences between cultivars understood. Thus, further investigation into the direct effects on photosynthesis is needed before the application of antioxidants can be considered as a viable agronomic option.

Most of the work investigating the addition of exogenous ameliorative substances has been observational, with little evidence of the precise mechanisms through which stress tolerance is conferred (Ashraf and Foolad 2007; Shabala et al. 2012). Before these substances can be used extensively in the field, more fundamental knowledge is required about the direct effects of these chemicals on plants, dosage required, and the timing and mode of application (Ashraf and Foolad 2007; Shabala and Munns 2012).

Regardless of the nature of the chemical agent used, amelioration of salinity stress is ultimately linked to improved ionic relations at a whole plant level (Krishnamurthy 1991; Chattopadhayay et al. 2002; Garg et al. 2002; Athar et al. 2008). Salinity stress causes perturbations to ion homeostasis, with increased Na^+ and Cl^- being accompanied by reductions in plant potassium content. An inverse relationship exists

between salinity induced potassium efflux from plant roots and tolerance in many species including wheat (Cuin et al. 2008), barley (Chen et al. 2005), lucerne (Smethurst et al. 2008) and poplar (Sun et al. 2009). Changes in ionic relations through genetic manipulation have resulted in improved tolerance (Zhang and Blumwald 2001). Increased tolerance was achieved by the over-expression of a Na^+/H^+ antiporter NHX on the vacuolar membrane resulting in increased Na^+ sequestration in the vacuole (Zhang and Blumwald 2001). Plants that can maintain higher potassium concentrations in the cytosol are more salinity tolerant than their counterparts (Flowers and Hajibagheri 2001; James et al. 2006).

As commented above, most studies of ameliorative substances to improve plant performance under saline conditions have been conducted at the whole plant level. Studies at the cellular levels are less frequent and are mostly limited to control of Na^+ and K^+ transport across the plasma membrane. It was shown that compatible solutes (Cuin and Shabala 2005), antioxidants (Chapter 2) and polyamines (Shabala et al. 2007) can all reduce Na^+ induced K^+ efflux in plant tissues. While beneficial effects of exogenously applied antioxidants has been attributed to their ability to prevent activation of the plasma membrane non-selective cation channels (NSCC) by reactive oxygen species (Demidchik et al. 2010; Chapter 2), polyamines directly interact with ion channels preventing K^+ efflux and possibly restrict Na^+ influx (Shabala et al. 2007). Polyamines may also be involved in the activation of ATPases (Reggiani et al. 1992) responsible for Ca^{2+} signalling and the maintenance of membrane potential which are both important under salinity stress.

The knowledge of mechanisms underlying the ameliorative effects of the above substances at subcellular level remains rather rudimentary, due to both the applied nature of most research and the lack of appropriate techniques to study these processes. Meanwhile, such knowledge may be absolutely essential for developing more resistant plants. One such organelle is the chloroplast. The chloroplast is the "work horse" of the plant. Without its proper function, the whole plant will not grow to its full potential. The exact functions that ameliorative substances have on chloroplasts under salt stress are still not well understood. Polyamines were shown to have a range of effects on plants from altering ionic homeostasis to binding to the photosystems and acting as molecular chaperones (Lakra et al. 2006; Roychoudhury

et al. 2011; Hamdani et al. 2011), but to what extent these mechanisms help chloroplasts under salinity stress remains to be answered. It is also known that compatible solutes have the ability to change the oxidative balance in the chloroplast (Hare and Cress 1997; Gadallah 1999a; Seckin et al. 2008) and some of these solutes have been shown to stabilise certain enzymes under salt stress (Papageorgiou and Murata 1995). However, to the best of our knowledge, direct effects of these solutes on photochemical characteristics of chloroplasts under salt stress conditions have not been investigated.

The aim of this study was to gain a better understanding of whether, and how, the aforementioned chemical compounds ameliorate detrimental effects of salinity on leaf photochemistry and primary photosynthetic processes in chloroplasts, using broad beans as a model species. We have used chlorophyll fluorescence, a very powerful tool for assessing photochemical "health", to fill the existing gaps in our knowledge and to answer some of the questions outlined above. These measurements showed why a "spray and pray" approach is not sufficient and what considerations need to be made prior to the chemical application or genetic engineering for the production of ameliorative substances. The majority of ameliorative substances tested remediate the effects of salinity stress on F_V/F_M to varying extents, demonstrating their ability to protect PSII in both whole cells and isolated chloroplasts. Proline was the most effective ameliorative agent for F_V/F_M causing a 34% increase. All compatible solutes increased F_V/F_M of salinity treated chloroplasts. The effects of ameliorative substances on rETR were more complex; all polyamines caused an initial increase in rETR this increase was most pronounced in the first 5 min however after 100 min all polyamines had a negative impact on F_V/F_M.

Materials and methods

Plant material

Broad beans (*Vicia faba* L cv Oswald; Hollander Imports, Hobart, Australia) were grown in 0.5 L pots of potting mix (70% composted pine bark, 20% course sand, 10% sphagnum peat (pH 6.0) which was fertilised with 1.8 kg m^{-3} Limil, 1.8 kg m^{-3}

dolomite, 6.0 kg m^{-3} osmocote plus and 0.5 kg m^{-3} ferrous sulphate) from seed. The glasshouse conditions were 19 ^{o}C to 26 ^{o}C with a day length of between 12-14 h and an average humidity of ~65%. Only the youngest, fully expanded leaves were used for measurements.

Chlorophyll fluorescence measurements from leaf mesophyll

Mesophyll tissue experiments were undertaken essentially as described in Chapter 2. The newest fully expanded leaves were removed from plants and taken to the laboratory. The abaxial epidermis was removed using fine forceps; the pealed leaves were then cut into 5x7 mm segments. The segments were left floating pealed sided down on basic salt media (1 mM NaCl, 0.5 mM KCl, 1 mM $CaCl_2$, pH 5.7 non-buffered) in 35 mm Petri dishes. Samples were left in the dark for 12 h to recover from pealing before treatment. For treatment, leaf segments were transferred onto treatment media in a fresh Petri dish. For measurements, samples were placed on a microscope slide, with pealed side up, and suspended in their treatment solutions. A cover slip was placed over the specimen. The sample was then focused under a dim green light in the Microscopy-PAM (Walz, Effeltrich, Germany). The microscope light was turned off immediately after focusing, and a black curtain was pulled around the microscope to allow 5 min of dark adaption before fluorescence measurements were taken.

Chloroplast isolation

Chloroplasts were isolated as described previously in Chapter 3. In brief, three broad bean leaves were put into 60ml of isolation media (described below). The leaf material was blended in a kitchen blender (Breville Power Max 550 w model BBL200) for 30 sec then strained through four layers of kitchen cloth (CHUX® Superwipes® original). The solution was then diluted 1 mL to 9 mL of isolation media to give required concentration for water PAM measurements. Chloroplasts were isolated in treatment media for all PAM measurements except for transient fluorescence measurements where they were isolated in control media (150 mM KCl, 1mM ATP and made up to 570 mmol.kg^{-1} with sucrose, and adjusted to pH 7.1 using KOH). All isolation media was isotonic and pH adjusted to 7.1.

Chloroplast PAM fluorescence measurements

All samples were dark adapted for 30 min prior to measurement with Water-PAM (Walz, Effeltrich, Germany). The maximum photochemical efficiency of photosystem II (F_V/F_M), relative electron transport rate (rETR) and transient fluorescence responses were measured using blue light essentially as described in Chapter 3.

Chloroplast treatment with ameliorating agents

Polyamines, compatible solutes, and antioxidants were each added to the isolation media prior to pH and osmotic adjustment. The measurements of F_V/F_M and rETR were undertaken in the concentrations listed in Table 1 (see above for details of measurements). For measurement of florescence transients, ameliorating agents were added at the time of salt treatment in pH and osmotically adjusted solutions.

Table 1. Ameliorating agents and their concentrations used.

Type of solute	Ameliorative agent	Concentration
Polyamine	Putrescine	1 mM
	Spermine	100 μM
	Spermidine	100 μM
Compatible solute	Trehalose	50 mM
	Betaine	50 mM
	Proline	50 mM
	Mannitol	50 mM
Antioxidants	Thiourea	10 mM
	DMSO	0.3 % w/v

Results

Polyamines

Putrescine and spermine both increased the Fv/Fm of salinity treated chloroplasts (P=0.04 & 0.02 respectively), while spermidine had no significant effect on Fv/Fm at 100 min (Fig. 1). Putrescine also has a positive effect on the Fv/Fm of non-salinity treated chloroplasts (P=0.01).

Effect of polyamines on the rETR showed a clear time-dependence (Fig. 2). For the first 25 min all three polyamines had a positive effect, increasing the electron transport rate of salinity-treated chloroplasts (Fig. 2). Under control conditions, spermidine increased rETR for the first 25 min (P<0.05). After 25 min, spermine and spermidine had no significant effect on rETR under control conditions. All three polyamines caused a significant decline in the electron transport rate after 100 min of treatment in both salinity-treated and non-saline chloroplasts (P<0.05)(Fig. 2).

Compatible solutes

The four compatible solutes tested all increased the F_V/F_M of isolated salinity-treated chloroplasts 100 min after treatment (P<0.05)(Fig. 3). Proline had the greatest effect followed by trehalose > mannitol > betaine. Proline and mannitol both had some positive effects on non-salinity treated chloroplasts. At 25 min after treatment trehalose had a significant (P<0.05) negative effect on both control and salinity-treated chloroplasts. The other compatible solutes had no significant effect on F_V/F_M at this time. By 100 min after treatment trehalose had no significant effect on the F_V/F_M of control chloroplasts.

The effect of compatible solutes on the rETR of isolated chloroplasts was solute- and time-specific (Fig. 4). All four compatible solutes reduced rETR of isolated non-salinity treated chloroplasts at 5 min (P<0.05). At 25 min, both betaine and trehalose significantly (P<0.05) increased rETR, while mannitol and proline had negative effects (P<0.05). After 100 min of treatment there was no longer a significant (P<0.05) positive effect of any compatible solutes, and both proline and trehalose had negative effects at this time (P<0.05). For salt treated chloroplasts mannitol increased

the rETR 5 min after treatment, while the other three solutes had no significant effect ($P < 0.05$). At 25 min all compatible solutes except trehalose had significant positive effects on rETR. At 100 min all the compatible solutes caused significant reductions in rETR, with the strongest effect being observed for trehalose ($P<0.05$) (Fig. 4).

Antioxidants

Thiourea caused an increase in the F_V/F_M of salt treated chloroplasts ($P <0.05$) while dimethyl sulfoxide (DMSO) had no significant effect on F_V/F_M. DMSO had a significant negative effect on non-salt treated isolated chloroplasts by reducing F_V/F_M. Both thiourea and DMSO increased the transient fluorescence of salt treated chloroplasts. DMSO also caused an increase in the transient fluorescence of non-salinity treated chloroplasts (Fig. 5).

Ameliorative effects at the tissue level

While all the above measurements have been taken from isolated chloroplasts, ameliorative effects of these agents may also exist at the whole-cell level. Additional experiments involved measuring chlorophyll fluorescent characteristics from leaf mesophyll.

Salinity treatment resulted in reduced F_V/F_M and rETR of mesophyll tissue ($P<0.05$) (Fig. 6). DMSO and betaine both increased the F_V/F_M and rETR of salinity treated mesophyll tissue ($P<0.05$) (Fig. 6)

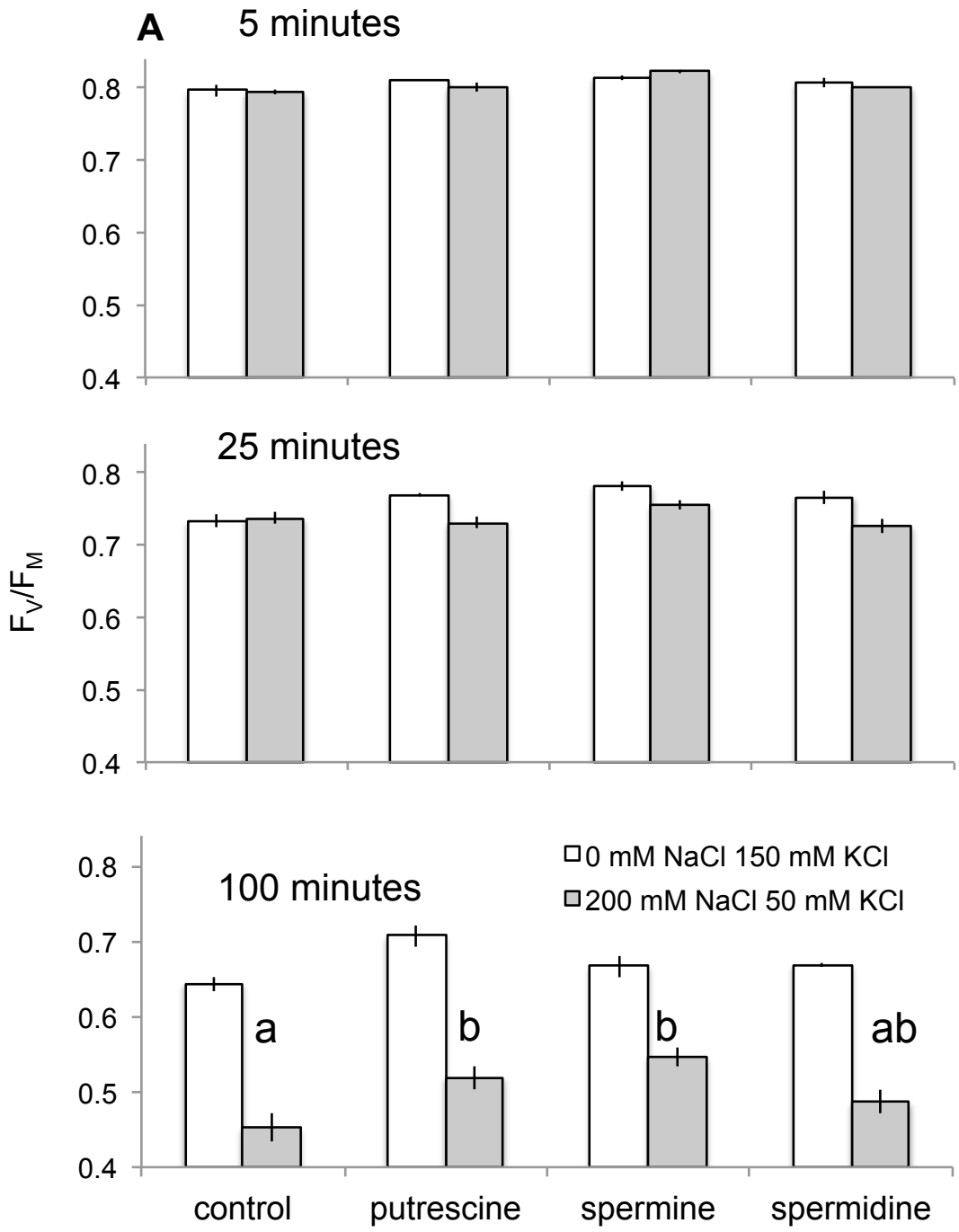

Fig. 1 Effect of the interaction between polyamines and salinity stress on chloroplast F_V/F_M over a 100 min time frame. Salinity treatment contained 200 mM NaCl, 50 mM KCl, 1 mM H_2KPO_4, 1 mM ATP; control 150 mM KCl 1 mM H_2KPO_4, 1 mM ATP; both solutions were adjusted to pH 7.10 using KOH and an osmolality of 570mmol.kg-1 using sucrose. Error bars ± SE (n=3)

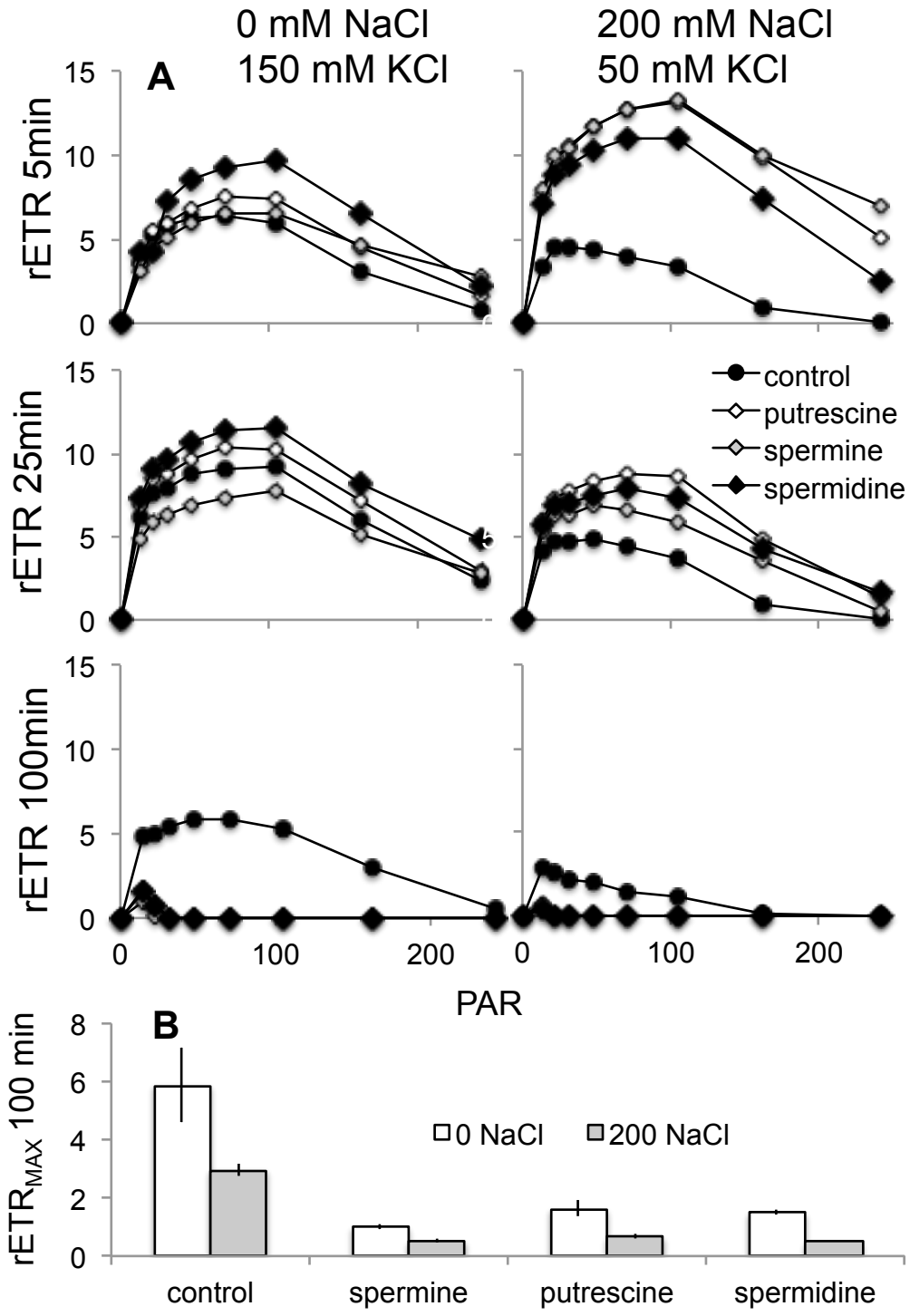

Fig. 2 Effect of the interaction between polyamines and salinity stress on chloroplast electron transport chain over a 100 minute time frame(**A**; light curves rETR, **B**; $rETR_{MAX}$). Salinity treatment contained 200mM NaCl, 50 mM KCl, 1 mM H_2KPO_4, 1 mM ATP; control 150 mM KCl 1 mM H_2KPO_4, 1 mM ATP; both solutions were adjusted to pH 7.10 using KOH and an osmolality of 570mmol.kg-1 using sucrose. Error bars ± SE (n=3)

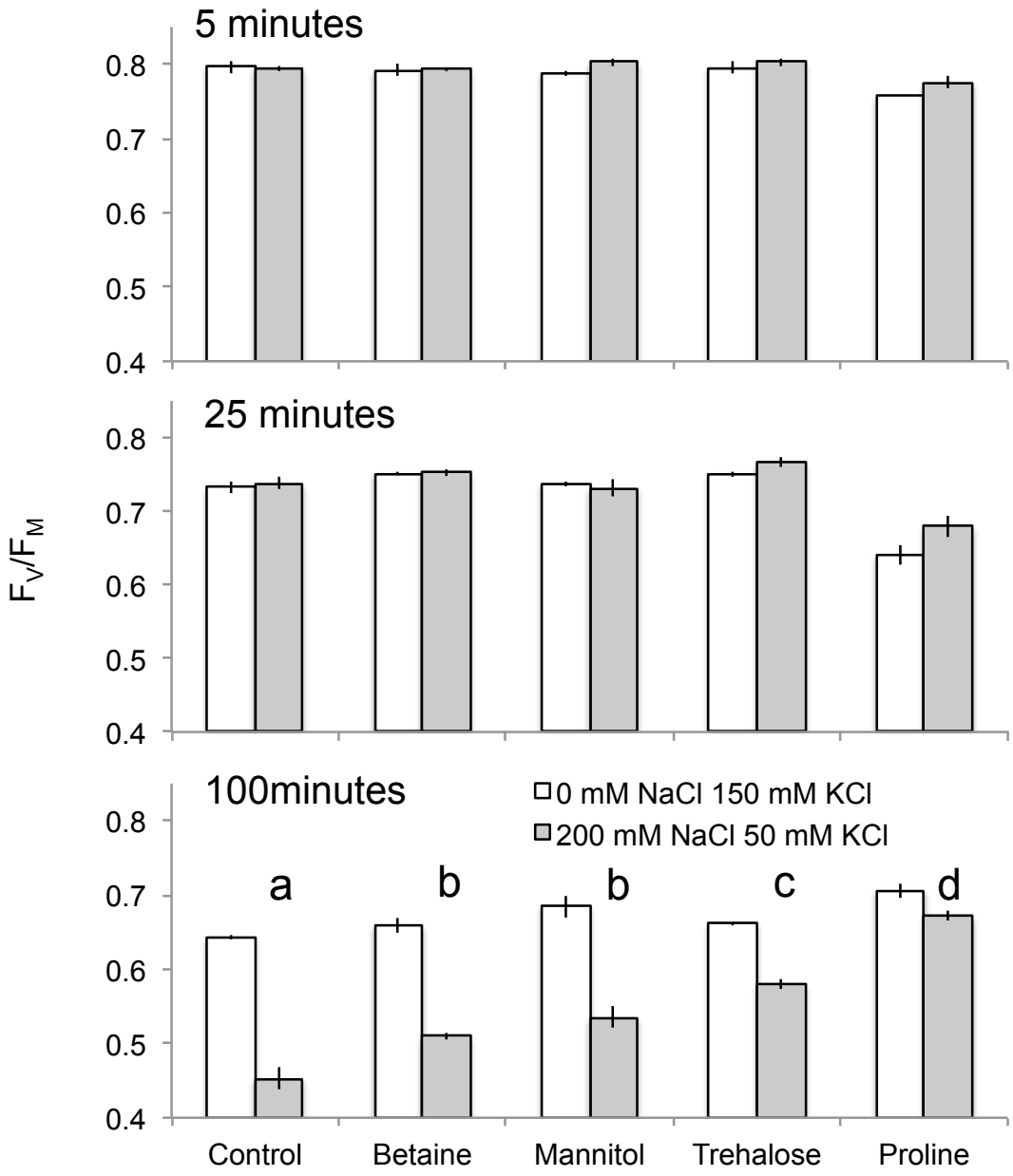

Fig. 3 Effect of the interaction between compatible solutes and salinity stress on chloroplast F_V/F_M over a 100 min time frame. Salinity treatment contained 200 mM NaCl, 50 mM KCl, 1 mM H_2KPO_4, 1 mM ATP; control 150 mM KCl 1 mM H_2KPO_4, 1 mM ATP; both solutions were adjusted to pH 7.10 using KOH and an osmolality of 570 mmol.kg-1 using sucrose. Error bars ± SE (n=3)

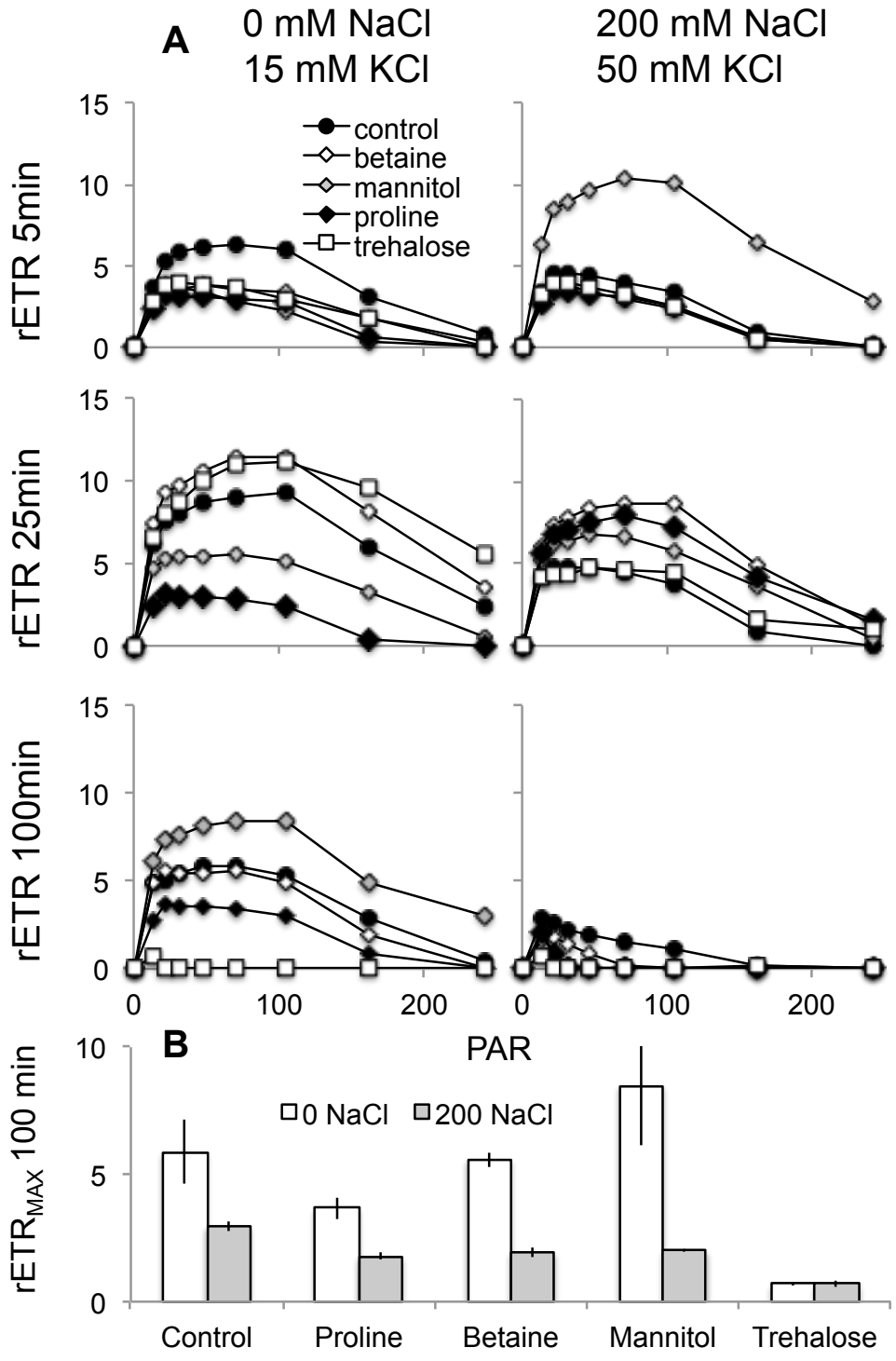

Fig. 4 Effect of the interaction between compatible solutes and salinity stress on chloroplast electron transport chain over a 100 minute time frame(**A**; light curves rETR, **B**; $rETR_{MAX}$). Salinity treatment contained 200mM NaCl, 50mM KCl, 1mM H_2KPO_4, 1mM ATP; control 150mM KCl 1mM H_2KPO_4, 1mM ATP; both solutions were adjusted to pH 7.10 using KOH and an osmolality of 570mmol.kg-1 using sucrose. Error bars ± SE (n=3)

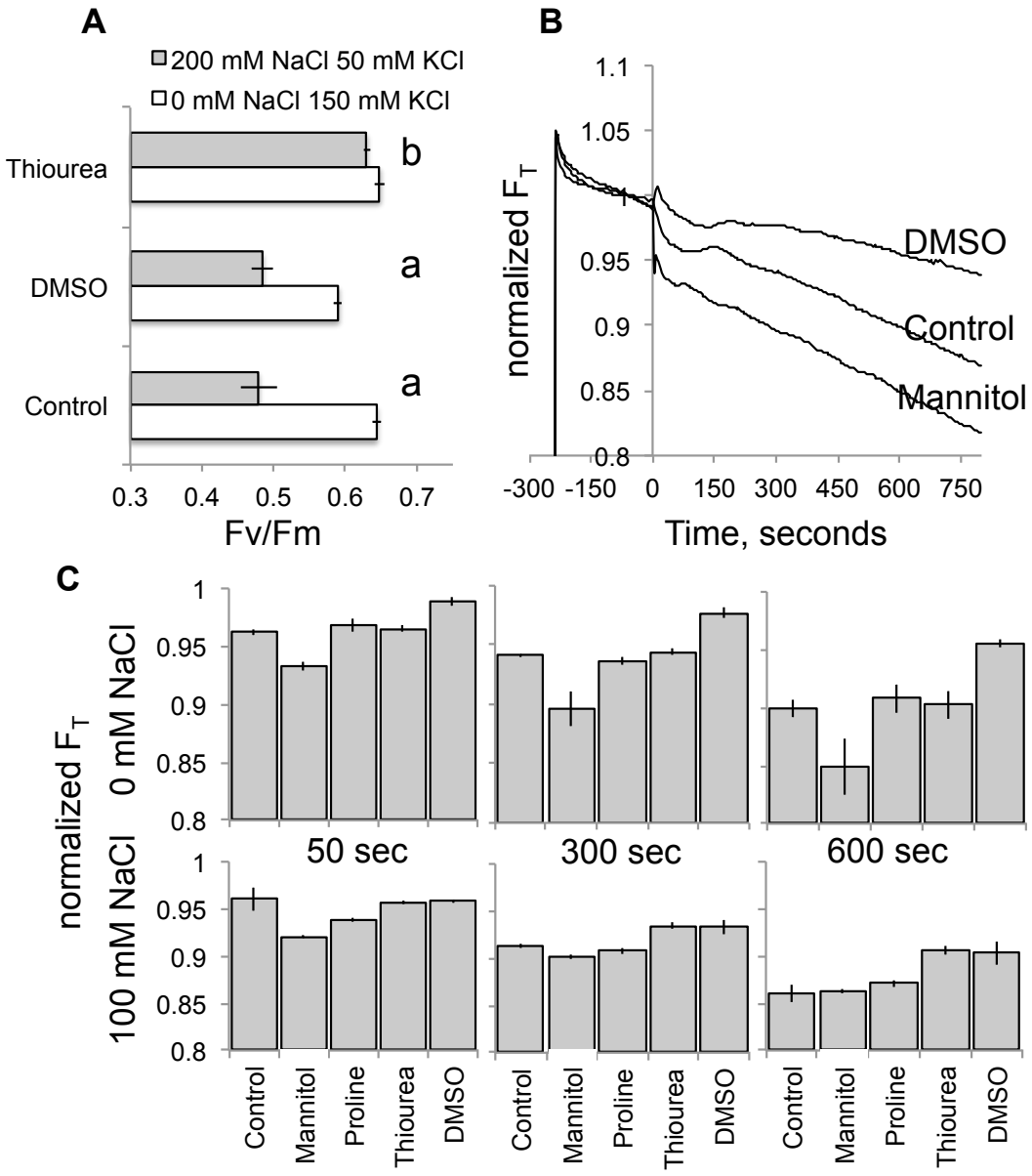

Fig. 5 A; effect of 2 antioxidants the on Fv/Fm of isolated chloroplasts after 100 minutes B; Effect of compatible solutes on normalised transient florescence (F_T) of non-salt treated chloroplast suspensions. **C**; specific time points of demonstrating the effect of compatible solutes ± salt stress on normalised transient florescence of chloroplast suspensions. Actinic light was turned on at 60sec. 200µL of either 4molL^{-1} NaCl or isolation media was added at 300 seconds. This data was first normalised for concentration using a formula similar to F_V/F_M. A 5 second average of F_t from 50 to 55 seconds was taken as F_0 and Ft was used as FM. The data was further normalised to account for different leaf health. The data was divided by the average Ft between 200 and 205 seconds. 3 biological reps were averaged.

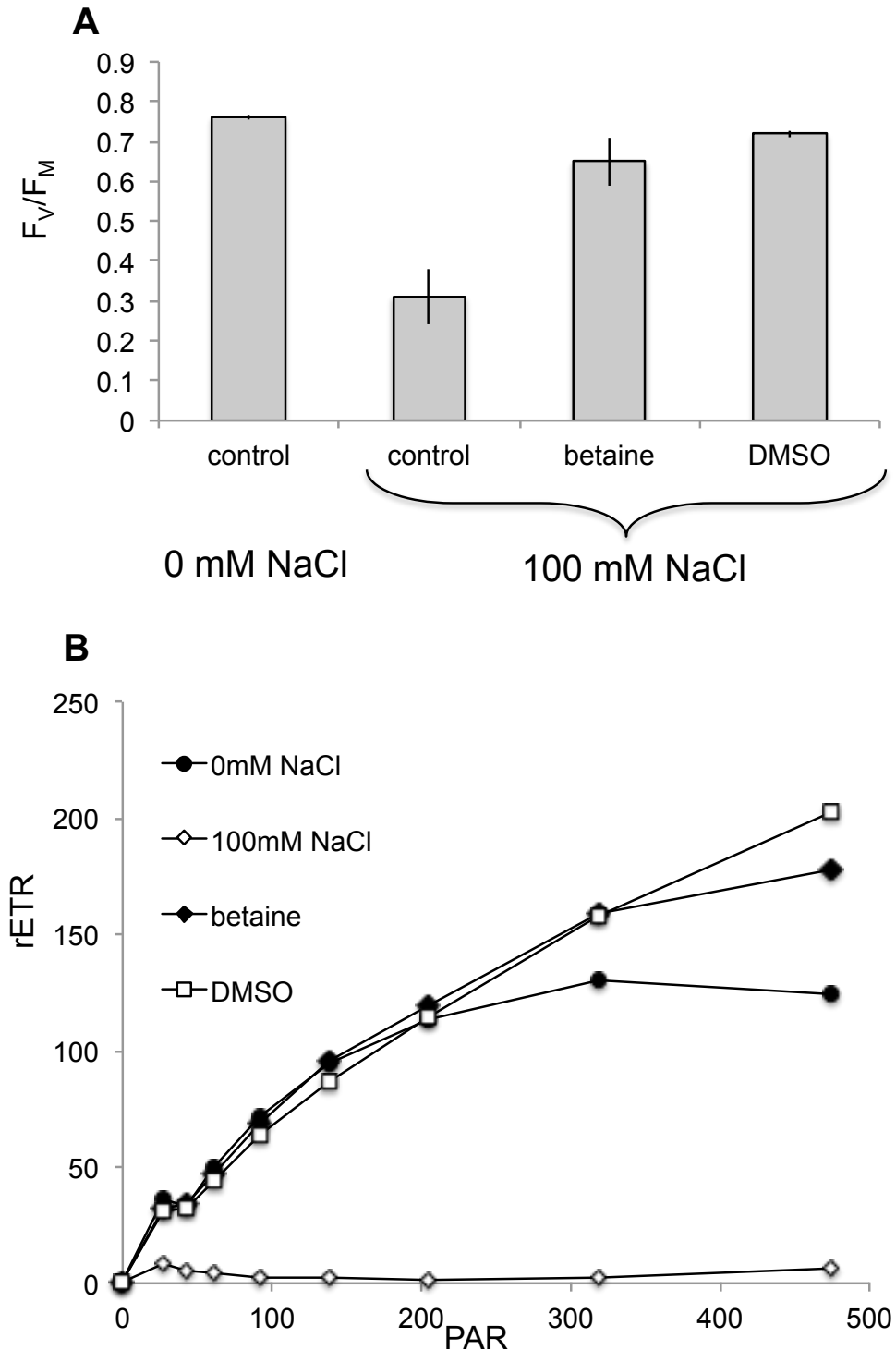

Fig. 7

A; F_V/F_M and **B**; rETR of mesophyll tissue treated with 1 compatible solute betaine and one antioxidant DMSO. These measurements were taken 72hr after treatment with pharmacological agents ± salinity stress. Salinity stress 100mM NaCl. Error bars ± SE (n=3)

Discussion

Polyamines

The exogenous application of polyamines (PA) either as foliar sprays, or by means of seed priming can increase salinity tolerance (Chattopadhayay et al. 2002; Iqbal and Ashraf 2005; Lakra et al. 2006; Wang et al. 2007; Roychoudhury et al. 2011). Plants treated with PA maintain ion homeostasis to a greater extent than their untreated counterparts, excluding Na^+ and retaining K^+ (Lakra et al. 2006; Roychoudhury et al. 2011) and Ca^{2+} (Wang et al. 2007). The mechanisms by which polyamines protect plants from salinity stress are numerous and include protein stabilisation, ion channel modulation (Lopatin et al. 1994; Liu et al. 2000), ATPase activation (Reggiani et al. 1992) and ROS scavenging (Ha et al. 1998). Until now, it remained to be answered which of these mechanisms operate in chloroplasts.

Putrescine and spermine caused an increased F_V/F_M in salinity treated chloroplasts, while spermidine had no significant effect (Fig. 1). This increase suggests that PA binding to various components of PSII may mitigate salinity damage to photosynthetic apparatus. The specificity of PA effects may be explained by the preferential binding of different PA to different parts of the photosynthetic apparatus (Hamdani et al. 2011). Spermine has been shown to be more effective than putrescine and spermidine at preventing damage to chlorophyll and electron transport between PSII and PSI under stress conditions (Subhan and Murthy 2001; Ioannidis and Kotzabasis 2007). At the same time, putrescine preferentially binds to the light harvesting antenna complexs of PSII and stabilises them (Ioannidis and Kotzabasis 2007).

The effects of PA on rETR are more complex than the effects on F_V/F_M. After 100 min of treatment PA have a detrimental effect on both salt treated and non-salt treated chloroplasts while in the first 25 min of treatment the PA have a distinct beneficial effect (Fig. 2). High concentrations of PA have been shown to cause reductions in PSII activity and overall oxygen yield (Hamdani et al. 2011). This time-dependence may be explained based on an assumption that PA excerpt their function from the

luminal side of chloroplasts, and that PA transport across chloroplast membranes requires some time. At low concentrations, PA could play the role of molecular chaperone and thus protect key chloroplast structures. When accumulated at higher quantities within the lumen (with time), detrimental effects start to dominate. PA in the lumen at high enough concentrations cause inhibitory effects disrupting the quinone acceptors of PSII (Beauchemin et al. 2007).

In the light of above, it is highly likely the positive effects seen for the first 25 min are before the PA cross the thylakoid membrane into the lumen. Before this point all three PA had positive effects increasing the $rETR_{MAX}$ and increasing the amount of light required to saturate the electron transport chain. These positive effects are likely caused by stabilisation of the electron transport chain. It is less likely as a result of the stabilisation of the light-harvesting complex or the stabilisation of PSII as the detrimental effect of Na^+ on rETR occurs before F_V/F_M (Figs. 1 & 2). Over the first 25 min there was no Na^+ induced reduction in F_V/F_M, however there was a 54% reduction in $rETR_{MAX}$. After 25 min the PAs are most likely accumulated to high concentrations within the chloroplasts entering the lumen and causing damage. At high PA concentrations, PA protection of the photosystem is reversed by the modification of protein secondary structure. This structural modification is due to the strong interaction of PAs with polypeptide C=O, C-N and N-H groups leading to the inhibition of photosynthetic processes (Hamdani et al. 2011). All three PA have a significant negative effect on both salinity and non-salinity treated chloroplasts in long-term exposures (Fig. 2).

Additional to the direct effects of PA on the photosynthetic apparatus, it is likely that PAs also affect the cation channels of the chloroplast. PAs have been shown to block non selective cation channels in the plasma membrane (Shabala et al. 2007) and fast-activating and slow-activating cation channels in vasculor membranes (Brüggemann et al. 1998; Dobrovinskaya et al. 1999). As PAs are potent blockers of cation channels it is likely that they would also block channels in the thylakoid. The thylakoid membrane contains a cation channel with little selectivity between different cations (Pottosin and Schönknecht 1996) and a K^+ selective channel (TPK3) (Checchetto et al. 2012). These channels are essential for charge balancing during photosynthesis, without which photosynthesis is inhibited due to a build-up of a charge across the

thylakoid membrane inhibiting the CF_0F_1 ATP synthase (Checchetto et al. 2013; Carraretto et al. 2013). The blockage of ion channels with PAs increases with time (Shabala et al. 2007) and this blockage of ion channels is one possible explanation for the negative effect of PAs on rETR after 100 min (Fig. 2).

PAs decrease both superoxide radical and H_2O_2 concentrations in salt stressed plant leaves (Verma and Mishra 2005). This decrease may be attributed to several possible mechanisms including direct scavenging of ROS (Ha et al. 1998), increased effectiveness of antioxidant enzymes including peroxidase and catalase in PA-treated tissues (Verma and Mishra 2005), and limiting production of ROS through the above mentioned stabilisation of the photosystems and improved ionic relations preventing unwanted oxidation of non-target species (Gill and Tuteja 2010a). All these antioxidative mechanisms may potentially be responsible for amelioration of detrimental salinity effects on F_V/F_M and rETR.

Compatible solutes

Betaine

Betaine accumulation has been associated with increased salinity tolerance, further to this, the exogenous application of betaine (Gadallah 1999b; Mäkelä et al. 1999; Hoque et al. 2007; Athar et al. 2009; Chen et al. 2009; Hossain and Fujita 2010; Hu et al. 2012) and increased accumulation through genetic manipulation (Sakamoto et al. 1998; Holmström et al. 2000; Fan et al. 2012; Wang et al. 2013) has been demonstrated to improve salinity tolerance in plants and suspension cell cultures. Some transgenes have also caused reduced growth associated with the over production of betaine (Wang et al. 2013); this reduced growth is normally associated with the increased metabolic cost of betaine production. Betaine has been observed to reduce salt-induced membrane injury, improve potassium nutrition, increase photosynthetic rate, increase the activity of a range of antioxidative enzymes (including SOD, CAT, peroxidase and APX) and reduce photorespiration (Gadallah 1999b; Mäkelä et al. 1999; Hoque et al. 2007; Hu et al. 2012). Betaine however cannot directly scavenge ROS (Smirnoff and Cumbes 1989; Hoque et al. 2007) despite reducing ROS concentrations and lipid peroxidation within cells (Demiral and Türkan 2004).

Betaine caused a significant increase in both F_V/F_M and rETR in salinity treated mesophyll tissue after 3 days of treatment with 100 mM NaCl (Fig. 6). It has been demonstrated leaf segments have the ability to take up and accumulate betaine within cells (Gibon et al. 1997). These increased photosynthetic parameters were also observed in isolated chloroplasts, however, betaine had a detrimental effect on rETR after 100 min (Fig. 3 & 4). In chloroplasts, it is evident that betaine protects PSII more than it does the rest of the photosystem. Betaine stabilises the PSII oxygen evolving complex (Papageorgiou and Murata 1995; Murata et al. 2007) in particular the Cyt*b599* , D1 and D2 protein complexes (Allakhverdiev et al. 2003). The oxygen evolving complex is one of the major sites of ROS production (Pospíšil 2009). This stabilisation of PSII is one of the likely causes of the increased F_V/F_M of isolated chloroplasts (Fig. 3). Unlike PSII, which is not affected by osmotic stress (Chapter 3), PSI is easily perturbed by osmotic disturbances (Wignarajah and Baker 1981). Betaine increased the rETR in salinity chloroplasts, an increase that could be due to either osmotic compensation by betaine, improved antioxidative enzyme function (Gadallah 1999b; Mäkelä et al. 1999; Hoque et al. 2007; Hu et al. 2012) or reduced ROS production due to the stabilisation of PSII.

It is known that very high concentrations of betaine are inhibitory to numerous processes including protein synthesis in leaf discs (Gibon et al. 1997; Sulpice et al. 1998). At high concentrations under stress conditions, betaine can destabilise membranes (Hincha 2006), therefore, the observed reduction in rETR after 100 min of treatment (Fig. 4) could be caused by destabilisation of the thylakoid membrane. Another reason for the negative effect of betaine on the electron transport chain is its effect on polyamine metabolism (Sulpice et al. 1998; Larher et al. 2003). When 5 mM betaine was supplied to whole cells there were no negative effects on rETR (Fig. 6) suggesting that betaine does not reach concentrations high enough to cause membrane destabilisation under this treatment.

Mannitol

Mannitol is accumulated in some plants under stress conditions (reviewed in Stoop et al. 1996), and its exogenous application has been shown to increase growth under salinity stress (Kaya et al. 2001; Seckin et al. 2008). When plants are genetically

engineered to overproduce mannitol they exhibited higher salinity tolerance (Karakas et al. 1997; Shen et al. 1997a; Zhifang and Loescher 2003; Abebe et al. 2003; Tang et al. 2005; Hu et al. 2005; Pujni et al. 2007).

When the transgenic accumulation of mannitol is targeted in the chloroplast the plants exhibit greater salinity tolerance than when it is produced in the cytosol (Shen et al. 1997a). The increased tolerance to plants accumulating mannitol or exogenously supplied with mannitol is likely associated with its effects on chloroplasts. Mannitol significantly increased the F_V/F_M of isolated chloroplasts under salinity treatment (Fig. 3), and increased rETR for the first 25 min (Fig. 4). In most circumstances, the ROS scavenging properties of mannitol (Smirnoff and Cumbes 1989) are the likely cause of its protective function, as mannitol does not normally reach concentrations where it would significantly contribute to the osmotic potential of the cell (Abebe et al. 2003). The increases in rETR (Fig. 4) are likely associated with this ROS scavenging function of mannitol which has been shown to protect ferredoxin and other thio-regulated enzymes which are integral parts of the electron transport chain from oxidation by OH* radicals (Keunen et al. 2013). The effects of mannitol on the electron transport chain are complex with no significant effect on unstressed chloroplasts at 100 min, and a significant negative effect in salt-treated chloroplasts (Fig. 4).

Proline

Proline significantly increased the F_V/F_M and rETR of isolated chloroplasts ameliorating the negative effects of salinity treatment (Figs. 3 & 4). Changes in proline's metabolism have been often associated with the salinity stress responses in plants. Under non-stressed conditions proline is normally synthesised in the cytosol and stored in the vacuole (Gagneul et al. 2007). With the onset of salinity stress proline is relocated from the vacuole to the cytosol (Gagneul et al. 2007). Proline synthesis also occurs in the chloroplast (Rayapati et al. 1989; Székely et al. 2008). Exogenous application of proline (Gadallah 1999b; Okuma et al. 2000; Ehsanpour and Fatahian 2003; Ali et al. 2007; Hoque et al. 2007) and the over expression of genes for the production of proline (Nanjo et al. 1999; Hong et al. 2000) both increase salinity tolerance. Proline protects plant cells in a few ways, acting as an antioxidant

itself (Smirnoff and Cumbes 1989; Alia et al. 1997; Matysik et al. 2002) and also increasing the activity of antioxidant enzymes including methylglyoxal, peroxidase, glutathione-S-transferase, superoxide dismutase and catalase (Hoque et al. 2008; Islam et al. 2009; Szabados and Savouré 2010). Other protective properties of proline include; stabilizing proteins (Rajendrakumar et al. 1994), buffering cellular redox potential (Hare and Cress 1997) and osmotic adjustment (Handa et al. 1986). Under high light stress proline has also been shown to reduce the production of $^1O_2$ and to detoxify ROS species leading to reduced thylakoid oxidation (Alia et al. 1997).

Oversupply of exogenous proline has been demonstrated to reduce the growth of plants (Nanjo et al. 1999; Yamada et al. 2005). Proline toxicity is caused by feedback mechanisms altering the $NADP^+$/NADPH ratio (Szabados and Savouré 2010) which results in damage to chlorophyll. It may therefore be suggested that transport of proline into the lumen may be a reason for the observed reduced rETR after 100 min of proline treatment (Fig. 4).

Trehalose

Trehalose is naturally accumulated to high concentrations in a small subset of plants, mainly resurrection plants (Paul et al. 2008; Fernandez et al. 2010) where it functions as a compatible solute having the ability to stabilise membranes through hydrogen bonding with proteins and lipids (Crowe et al. 1998; Brumfiel 2004). Many other plants also have the genes for trehalose production, (Paul et al. 2008; Fernandez et al. 2010) where trehalose is thought to play a regulatory role rather than a role as a compatible solute (Leyman et al. 2001; Fernandez et al. 2010). The exogenous application of trehalose has been demonstrated to increase the salt tolerance of treated plants when applied at the right concentration, but over supply resulted in reduced growth and toxicity (Garcia et al. 1997; Chang et al. 2014). The engineering of increased trehalose accumulation in a range of plants has been demonstrated to increase salinity tolerance with various effects on overall plant growth (Yeo et al. 2000; Garg et al. 2002; Jang et al. 2003).

Trehalose had a significant positive effect on the F_V/F_M (Fig. 3) of salinity treated chloroplasts and a negative effect on rETR (Fig. 4). The positive effect on F_V/F_M could be due to the antioxidative (da Costa Morato Nery et al. 2008), and membrane

stabilising (Crowe et al. 1998; Brumfiel 2004) properties of trehalose. Another positive effect that has been reported with the exogenous application of trehalose at the whole plant level is changed ionic homeostasis (reduced Na^+ uptake along with reduced K^+ loss) and it is possible this effect could also be contributing to increased F_V/F_M. The negative effects of trehalose on rETR (Fig. 4) are possibly one of the reasons for reduced growth with over supply of exogenous trehalose (Garcia et al. 1997; Chang et al. 2014) and changes in plant morphology, chlorosis and reduced growth in some transgenic plants (Yeo et al. 2000). The molecular mechanisms of trehalose toxicity could be related to the prevention of chaperone-assisted protein folding (Leyman et al. 2001). Another possible mechanism for reduced growth and changes in morphology could be related to the increased production of other compatible solutes when exogenous trehalose is supplied (Chang et al. 2014). The effects on isolated chloroplast electron transport are pronounced in both non-salt treated and salt treated chloroplasts suggesting direct interference with the electron transport chain (Fig. 4).

Antioxidants

The use of exogenous antioxidants has been shown to reduce the effects of salinity stress (Anjum et al. 2008; Athar et al. 2008; Srivastava et al. 2010; Anjum et al. 2011; Kaya et al. 2013). Exogenously applied antioxidants have been demonstrated to increase photosynthetic rates (Anjum et al. 2008) and improve ionic homeostasis (Athar et al. 2008; Kaya et al. 2013). The direct effects of thiourea and DMSO on photosynthesis chloroplasts have not been studied, despite chloroplasts being the primary sight of ROS production in plants.

Oxidative damage to PSII is evident in salinity treated chloroplasts as thiourea increases Fv/Fm (Fig. 5). It is also evident oxidation and reduction reactions are essential for normal plant function as DMSO, a known ROS scavenger (Sanmartín-Suárez et al. 2011), reduced the F_V/F_M of non-saline chloroplasts without affecting the F_V/F_M of salt-treated chloroplasts (Fig. 5). The likely reason for the difference in response between the two antioxidants is that DMSO is highly soluble in both lipid and aqueous environments while thiourea is not. DMSO may have entered the lipid systems and disrupted electron transport through PSII or other parts of the

photosystems. As thiourea is not soluble in lipids this disruption could not happen. Instead resulting in scavenging in the compartments within the chloroplast.

There are a few different sources of ROS in the chloroplast. PSI can donate electrons to O_2 to form $O_2^{\cdot-}$ (superoxide) via the Mehler reaction when the $NADP^+$ pool is depleted (Gill and Tuteja 2010a). Chlorophyll molecules in the light harvesting complex II (LHCII) can react directly with O_2 to generate very reactive singlet oxygen (Gill and Tuteja 2010a). Both lipids (Moran et al. 1994) and proteins (Tambussi et al. 2000) are the targets of oxidative damage. The D_1 proteins of PSII are also subject to photo-oxidative damage (Zhang et al. 2011). It is possible that thiourea is protecting the D_1 proteins and enhancing Fv/Fm (Fig. 5). LHCII is the most abundant protein in the chloroplast and it is not easily oxidised although it is the sight of singlet oxygen production. This is probably due to LHCII proteins being encased in lipids and chlorophyll molecules (Siefermann-Harms and Angerhofer 1998).

Both DMSO and thiourea significantly increase the F_T of salinity treated chloroplasts (Fig. 5), demonstrating lights involvement in oxidative damage under salinity stress, as DMSO did not increase F_V/F_M where there is minimal light exposure.

Conclusion

Salinity treatment results in reduced F_V/F_M and rETR in mesophyll tissue over a period of 72 hr. It is possible to remediate these effects using antioxidants and compatible solutes. The effects on isolated chloroplasts are more complex than on mesophyll tissue and likely to explain the difference between different treatments on different species reported in the literature. All compatible solutes increased the F_V/F_M of salinity treated chloroplasts and the rETR in the short term, however the over-accumulation of these solutes in the chloroplasts with time resulted in reduced rETR. This shows a need to tightly control the concentration of these ameliorative substances in the chloroplast and their transport within the chloroplast.

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Chapter 5: Linking growth with mesophyll ion transport under saline conditions in three species contrasting salinity tolerance

William J. Percey[1], Lana Shabala[1], Michael C. Breadmore[2], Rosanne M. Guijt[3], Jayakumar Bose[1], Sergey Shabala[1]

[1]School of Land and Food, University of Tasmania, Hobart, Tasmania 7001, [2]Australian Centre for Research on Separation Science (ACROSS), School of Chemistry, University of Tasmania 7001 and [3]School of Pharmacy, University of Tasmania, Hobart, Tasmania 7001

Abstract

The relationship between ion transport in mesophyll and salinity tolerance was investigated by assessing growth, photosynthetic parameters and ion fluxes (H^+ and K^+) in response to salinity treatment in three species with contrasting salinity tolerance were measured one halophyte (quinoa), one moderately salt tolerant species (sugar beet) and a salt sensitive species (broad bean). A 300 mM NaCl stress for 20 days resulted in all three species having reduced growth with broad bean being most affected. Broad bean leaves of all ages had significantly reduced maximum photochemical efficiency of photosystem II (F_V/F_M) and reduced chlorophyll content, while the F_V/F_M was unaffected in the two more tolerant species; also, chlorophyll content increased following salinity application. These changes in photosynthetic ability are linked to the ability of mesophyll cells to retain K^+ under salinity treatment. Over the first 24 h of salinity treatment, bean mesophyll loses 2.6 fold more K^+ compared with quinoa and beet mesophyll. This decline is likely linked to the reduced maximum photochemical efficiency of photosystem II, chlorophyll content and growth observed in beans. In addition to reduced K^+ efflux, the more tolerant species also exhibited reduced H^+ efflux, which is interpreted as an energy-saving strategy allowing more resources to be redirected towards plant growth. Differences were also

observed in the ability of the different species to modulate their H^+ and K^+ fluxes in response to light/dark fluctuations. These changes are most likely linked to membrane depolarisation and the amount of available energy due to differences in H^+-ATPase activation under salinity stress. It is concluded that the ability of mesophyll to retain K^+ in mesophyll without a need to activate plasma membrane H^+-ATPase is an essential component of salinity tolerance in halophytes and halophytic crop plants.

Submitted to: *Plant and Soil*

Introduction

Globally, over 800 million hectares of agricultural land is negatively affected by salinity (Rengasamy 2010). The exclusion of Na^+ from the cell cytosol has been the major focus of research over the last decades (Zhu 2001; Apse and Blumwald 2007). Despite significant breakthroughs in our understanding mechanisms of Na^+ transport and signalling (Apse and Blumwald 2007; Kronzucker and Britto 2010; Ji et al. 2013; Kronzucker et al. 2013; Benito et al. 2014), there have however, been no major improvements in the salinity tolerance of commercial crops. It has become more and more evident that manipulating the expression level/function of merely one gene has little chance to make a significant change in the overall salinity stress tolerance, and a "pyramiding approach" was advocated (Flowers and Yeo 1995; Yamaguchi and Blumwald 2005; Tester and Langridge 2010; Shabala 2013). The question remains as to which traits should be combined, especially because some of the favourable traits are mutually exclusive.

Possible clues may come from studying natural variation in salinity tolerance among plant species and, specifically, from exploiting mechanisms of salinity tolerance in halophytes (Flowers et al. 2010; Shabala and Mackay 2011). Once these mechanisms are revealed, plant breeders may exploit them to produce tolerant crops. Importantly, the differences in tolerance between naturally salt tolerant plants (halophytes) and salt sensitive pants (glycophytes) comes from physiological differences rather than from unique anatomical features (Shabala and Mackay 2011). Traits such as better control

over cytosolic ion homeostasis (Shabala and Mackay 2011), efficient osmotic adjustment (Flowers and Colmer 2008; Hariadi et al. 2011) and better ROS scavenging (Bose et al. 2013) are important for both halophytes and glycophytes; the difference is in the extent and efficiency of these processes.

There is one trait, however, that is considered a hallmark of all halophyte species and is observed only in a very limited number of crops. This trait is related to the means by which plants achieve their osmotic adjustment under saline conditions. Most glycophytes exclude Na^+ from uptake and synthesise compatible solutes (low molecular weight organic compounds such as polyols and amino acids that do not interfere with metabolic processes in the cell) to maintain cell turgor under hyperosomotic saline conditions (Munns and Tester 2008). This comes with substantial yield penalties, because for the production of one mol of compatible solute, plants spend between 50 and 70 mols of ATP (Raven 1985), at the expense of other metabolic and growth-related processes. On the contrary, all halophytes are Na^+ includers; they generally have higher Na^+ contents than other plants in non-saline environments, and in saline environments they further increase their Na^+ concentration to maintain shoot turgor using Na^+ as a cheap osmoticum (Santa-Cruz et al. 1999; Flowers and Colmer 2008; Shabala and Mackay 2011). For example, in a halophyte pseudo-cereal quinoa up to 95% of osmotic adjustment in saline environments is achieved by the accumulation of inorganic ions (Hariadi et al. 2011). This mechanism of osmotic adjustment, however, comes with strict control over cytosolic Na^+ and K^+ homeostasis in a trait termed as “tissue tolerance”.

The “classical” component of tissue tolerance is vacuolar Na^+ sequestration mediated by tonoplast Na^+/H^+ NHX exchangers (Apse and Blumwald 2007; Munns and Tester 2008). However, higher NHX expression levels by themselves do not always result in increased salinity tolerance (Getnet Adem and Stuart Roy, personal communication). At the very least, these NHX exchangers should be energized by the tonoplast H^+-pumps (Shabala 2013). Two major types of such pumps are known: tonoplast H^+-ATPases that use ATP (Schumacher and Krebs 2010) and H^+-PPases that use inorganic pyrophosphate (Gaxiola et al. 2007) to acidify the vacuole to provide the driving electrochemical force for Na^+ transport across the tonoplast membrane against concentration and electrochemical gradients. Given reduced ATP availability under

saline conditions (Shabala 2013), it is reasonable to suggest H^+-PPase should play a major role in fuelling tonoplast Na^+/H^+ exchangers. Indeed, under conditions of salt stress the expression and activity of H^+-PPase increase (Colombo and Cerana 1993; Zingarelli et al. 1994; Fukuda 2004). Increased H^+-PPase and NHX expression on their own induce salinity tolerance. However, when these genes are co-expressed greater salinity tolerance is observed (Bhaskaran and Savithramma 2011; Bao et al. 2014; Shen et al. 2014). H^+-ATPase expression has also been shown in increase under salt stress (Popova and Golldack 2007). This upregulation H^+-ATPase is linked to the SOS pathway (Batelli et al. 2007) and is likely a key determinated of salinity tolerance.

Type 1 H^+-PPase activity is strongly dependent on K^+ activity in the cytosol (Belogurov and Lahti 2002; Gaxiola et al. 2007), implying the importance of cytosolic K^+ retention as a component of the tissue tolerance mechanism. Salt-tolerant barley varieties have a greater ability to retain K^+ in mesophyll with increased apoplastic salinity (Wu et al. 2013). Increased apoplastic salinity causes a rapid and sustained K^+ efflux from bean mesophyll resulting in a dramatic decline in cytosolic K^+ concentrations from an estimated 150 mM to 59 mM (Chapter 2). This decline in cytosolic K^+ concentration results in reduced photosynthetic performance in chloroplasts (Chapter 3). Thus, the differences between halophytes and glycophytes abilities to handle high NaCl concentrations could be ultimately related to their ability to retain K^+ in the cytosols of mesophyll cells. This hypothesis, however, is yet to be tested in direct experiments.

The majority of Na^+ induced K^+ efflux from bean mesophyll occurs through outward rectifying depolarization-activating KOR channels (chapter 2). KOR channels are voltage gated (Hedrich 2012) and as such are initially activated by plasma membrane depolarisation under salinity stress due to the rapid entry of Na^+ into the cytosol (Shabala and Cuin 2008). Another pathway for K^+ leak is via K^+-permeable non-selective cation channels (NSCC) activated by reactive oxygen species (ROS) produced in plants under saline conditions (Demidchik et al. 2002; Demidchik et al. 2010; Chapter 2). It remains to be answered, however, if the same scenario is applicable to halophytes, and what is the relative contribution of KOR and NSCC in salt-induced K^+ leak from mesophyll in this species?

Fluctuations in light intensity modulate ion transport across a variety of cellular membranes in leaves including the plasma membrane of epidermal (Elzenga et al. 1995; Shabala and Newman 1999), mesophyll (Elzenga et al. 1995; Shabala and Newman 1999) and guard cells (Schroeder 1988; Kinoshita and Shimazaki 1999), as well as across the chloroplast envelope (Kreimer et al. 1985) and thylakoid membranes (Spetea and Schoefs 2010). These changes were shown to be causally related to both photosynthetic (Checchetto et al. 2013; Carraretto et al. 2013) and developmental (Dodd et al. 2010) processes, as well as to leaf expansion growth (Blum et al. 1992; Staal et al. 1994; Živanović et al. 2005). In bean, the ability to respond to light with ion fluxes was diminished by salinity stress (Chapter 2) and, thus, could be used as an indicator of adverse salinity effects on cell metabolism.

In this work, the non-invasive MIFE technique was used to fill some of the above gaps in our knowledge by linking growth and photosynthetic parameters with ion transport in mesophyll. Specifically, two hypotheses were tested: that salt tolerant plants (i) have a better ability to retain potassium in mesophyll and (ii) their ability to respond to light is less affected by salinity treatment.

Materials and methods

Growth experiment plant material

Broad beans (*Vicia faba* L cv Oswald), sugar beet (*Beta vulgaris*; Hollander Imports, Hobart, Australia) and quinoa (*Chenopodium quinoa* cv 19) were grown from seed under controlled greenhouse conditions (temperature between 19 ^{o}C and 26 ^{o}C; day length 12-14 h; average humidity ~65%) at the University of Tasmania in 0.5 L plastic pots (3 plants per pot). The potting mixture was composed of 70% composted pine bark, 20% course sand, 10% sphagnum peat (pH 6.0) which was fertilised with 1.8 kg m^{-3} Limil, 1.8 kg m^{-3} dolomite, 6.0 kg m^{-3} osmocote plus™ and 0.5 kg m^{-3} ferrous sulphate. Plants were watered once daily with tap water to maintain full field capacity for 18 days. For the next 20 days plants were watered daily with treatment

solutions (tap water, 100 mM NaCl and 300 mM NaCl), 3 pots per treatment per species.

Photochemical and growth measurements

Maximum photochemical efficiency (F_V/F_M) and relative chlorophyll content (SPAD) were measured on leaves of three different ages: old (lowest leaf on plant), intermediate and young (not yet fully expanded leaf) in the case of quinoa and bean and two different leaf ages in the case of beet (old and young). F_V/F_M measurements were taken after 30 min dark adaption using a chlorophyll fluorometer (Chlorophyll fluorometer OS-30p, Opti Sciences, USA) which were followed by SPAD measurements that were taken with a Minolta Chlorophyll Meter SPAD-502 (Minolta, http://www.konicaminolta.com/).

At the end of the experiment, shoot fresh (FW) and dry weights (DW) were recorded Relative water content was calculated for each individual plant.

Plant material for MIFE measurements

Broad beans, sugar beet and quinoa were grown from seed under controlled greenhouse conditions as described above. Plants were watered daily with tap water and the newest fully expanded leaves were harvested from plants between four to six weeks old.

Ion flux measurements

Net fluxes of H^+ and K^+ were measured non-invasively using vibrating ion-selective microelectrodes (the MIFE technique; University of Tasmania, Hobart, Australia), as described previously in Chapter2.

Ion flux measuring protocols

The protocols for measuring ion fluxes from mesophyll are identical to those outlined in Chapter 2. In brief, the first full-expanded leaf was removed from the plant and the abaxial surface was peeled off using very fine forceps. Peeled leaves were cut into 5x7 mm segments. These segments were then placed on a shallow layer of basic salt media (BSM, 1 mM NaCl; 0.5 mM KCl; 0.1 mM CaCl pH 5.7 unbuffered) in 35 mm

petri dishes overnight prior to measurement to allow recovery from confounding wounding responses (see Shabala 2003; Živanović et al. 2005 for justification). The segments were mounted in Perspex sample holders 1 h before measurement and placed in a 6 ml sample chamber containing 4 ml BSM. Two different types of measurement were taken from samples prepared in this way. The first were transient fluxes in response to the sudden application of 100 mM NaCl. For these measurements 2 ml of BSM containing the required concentration of NaCl was added to the 4 mL of BSM in the chamber and mixed briefly. Other measurements were performed on samples that had been exposed to various concentrations of salinity for up to 24 h. In these experiments, samples were exposed to 5 min/5 min rhythmical light-dark (white light from an optic fibre light source 100 Wm^{-2}) fluctuations for 90 min until a stable change between light and dark fluxes was achieved.

Results

Salinity treatment (300 mM NaCl applied to glasshouse-grown plants for 20 days) significantly reduced plant growth (Fig 1) and affected whole plant photosynthetic characteristics (Fig 2). Quinoa was the most tolerant of the three species tested, showing 39% and 40% reductions in shoot fresh and dry weight, respectively (Fig. 1). Beet (53 and 45% reductions) was the next most tolerant species followed by bean (83 and 52% reductions), (Fig. 1A, B, C and D). Bean was the only species where relative water content (RWC) was reduced significantly (Fig. 1 E and F). In addition to the reductions in RWC observed in beans there was a significant decline in F_V/F_M and chlorophyll content in leaves of all ages (Fig. 2A and B). Both quinoa and beet had significantly ($P<0.05$) increased chlorophyll content in old leaves under 300 mM NaCl treatment. Quinoa also had increased chlorophyll content in leaves of an

intermediate age.

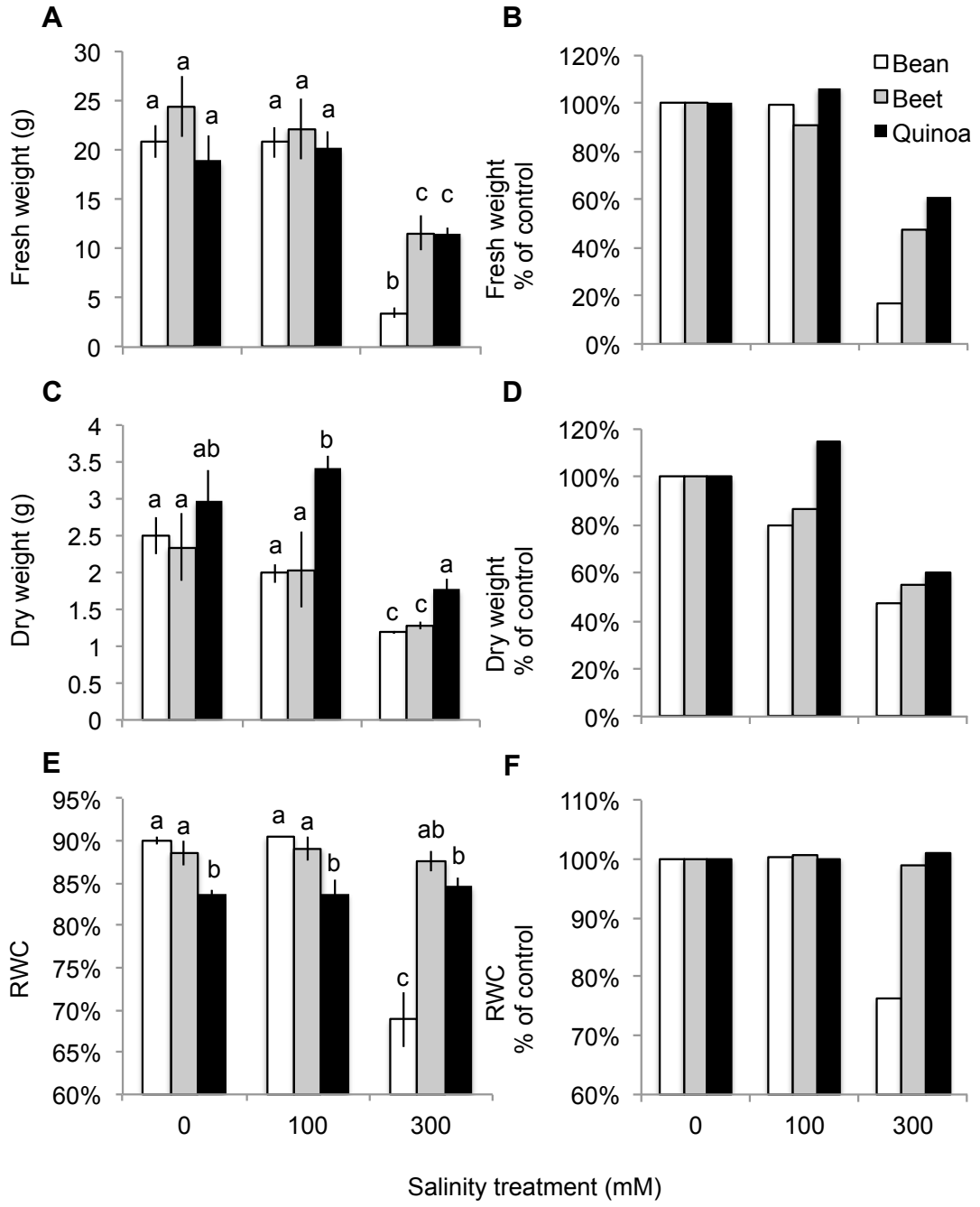

Fig. 1 Salinity stress effects on plant weight and RWC in three species with contrasting salinity tolerance. Fresh weight expressed grams (A), and as 5 of control (B). Dry weight expressed in grams (C), and as % of control (D). RWC (E), expressed as percentage of control (F). Results are given for 38-day-old plants grown under three levels of salt stress (0, 100 and 300 mM) for 20 days. Letters denote significants at $P<0.05$ (average ± SE, $n = 4$-6)

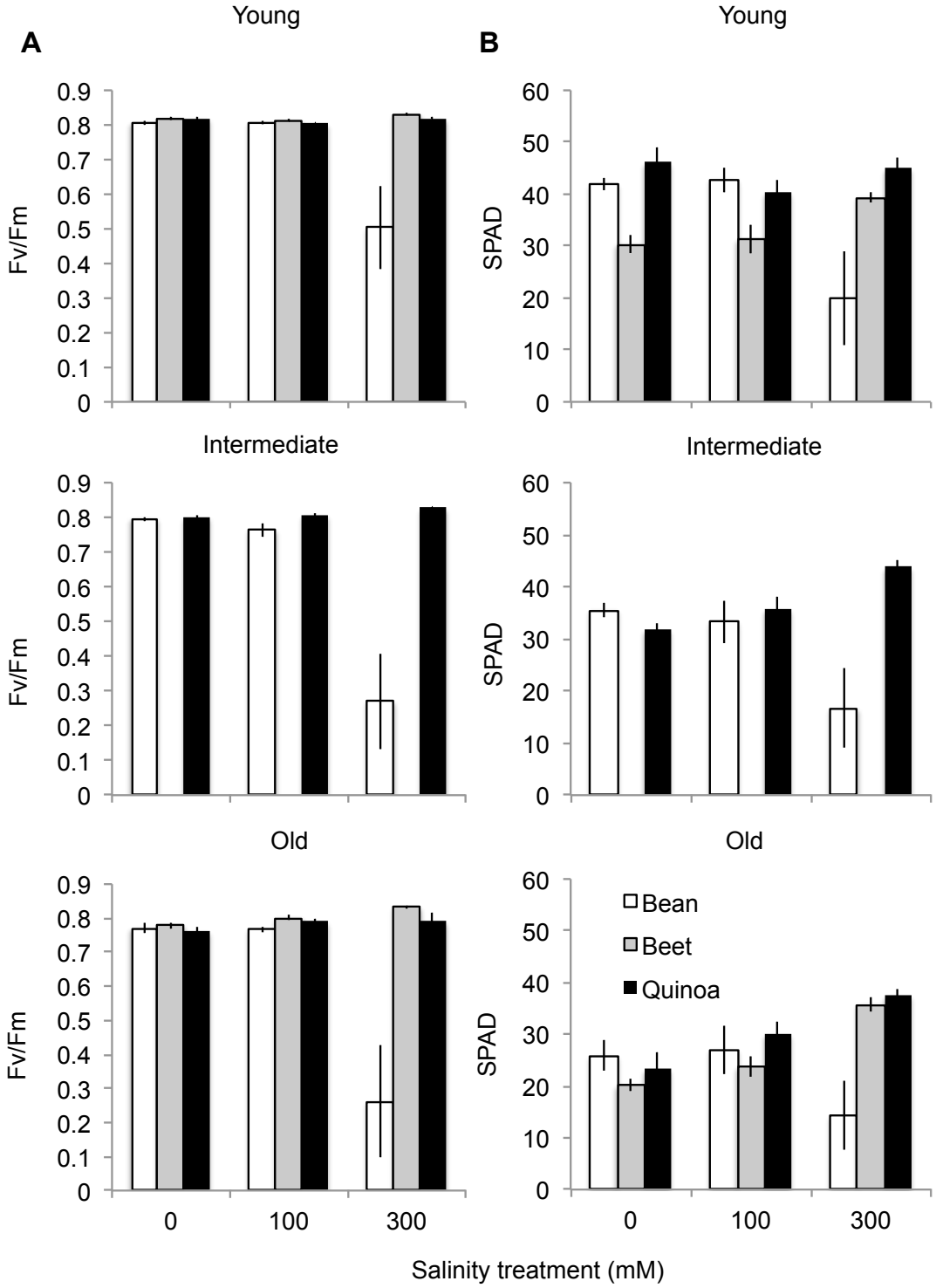

Fig. 2 Effects of salinity stress on maximum photochemical efficiency (F_V/F_M) (A) and relative chlorophyll content (SPAD) (B) in three species with contrasting salinity tolerance. Results are given for three different leaf ages from 38-day-old plants grown under three levels of salt stress (0, 100 and 300 mM) for 20 days. Letters denote significants at $P<0.05$ (average ± SE, n = 4-6)

Depending on external NaCl concentration and duration of exposure, leaf sap NaCl concentrations can reach over 100 mM, both in halophytes (Shabala et al. 2013) and glycophytes (Bose et al. 2014). Accordingly, in this work we have tested effects of 100 mM NaCl on ion transport in leaf mesophyll. Acute 100 mM NaCl treatment caused a large K^+ efflux from all three species tested (Fig. 3A). The largest efflux was recoded from the mesophyll tissue of bean (glycophyte). In this species, both total efflux and peak flux were significantly higher ($P<0.05$) than in either quinoa (halophyte) or beet (halophylic) species (Table. 1). This trend of bean having the greatest K^+ efflux continued for at least 24 h of 100 mM NaCl treatment (Fig. 3A), and the overall K^+ loss from its mesophyll over the first 24 h was 2.6 fold higher compared with quinoa and beet.

Table. 1 Summary of K^+ flux statistics from Fig. 3A showing the total amount of K^+ efflux and peak efflux of mesophyll tissue in the first 40 min after 100 mM NaCl treatment from three different species contrasting in their salinity tolerance (letters denote significants at p = 0.05, *n=3-5*).

Species	Total efflux (mmol)	SE	Peak flux (nmol m^{-2} s^{-1})	SE
Bean	-136 [a]	23	-89 [a]	6
Beet	-94 [b]	7	-52 [b]	4
Quinoa	-91 [b]	14	-51 [b]	6

Treatment with 100 mM NaCl resulted in increased net H^+ efflux from leaf mesophyll in all species tested (Fig. 4). The increase in net H^+ efflux from bean was much greater compared with two more tolerant species, both in terms of the peak flux and a greater total H^+ efflux and in the first 40 min after treatment (Table. 2). After 2 h of 100 mM NaCl treatment there is no significant difference between the H^+ efflux from bean and quinoa (Fig. 4B), while the H^+ efflux from beet was significantly less than the efflux from the other two species. By 24 h after salinity treatment quinoa has the smallest H^+ efflux followed by beet then bean (Fig. 4B).

Table. 2 Summary of H^+ flux statistics from Fig. 4A showing the total amount of H^+ efflux and the peak efflux from the mesophyll tissue in the first 40 min after 100 mM NaCl treatment of three species contrasting in salinity tolerance (letters denote significants at p = 0.05, *n=3-5*).

Species	Total efflux (mmol)	SE	Peak flux (nmol m^{-2} s^{-1})	SE
Bean	-395 [a]	80	-195 [a]	36
Beet	-153 [b]	3	-79 [b]	4
Quinoa	-87 [c]	15	-53 [c]	5

Light/dark transitions resulted in a strong modulation of kinetics of net K^+ and H^+ fluxes across the mesophyll cell plasma membrane (Fig. 5). These patterns were significantly altered by the application of salinity stress (Fig. 6). The effect of salinity on the light-induced K^+ flux response from mesophyll was not significant in either quinoa or beet (Fig. 6A). In beans the ability of mesophyll to respond to light by modulating K^+ flux was reduced already after 2 h salinity treatment (Fig. 6A). On the contrary, the magnitude of light/dark modulation of net H^+ flux increased dramatically in beans but much less in other (halophyte) species (Fig. 6B).

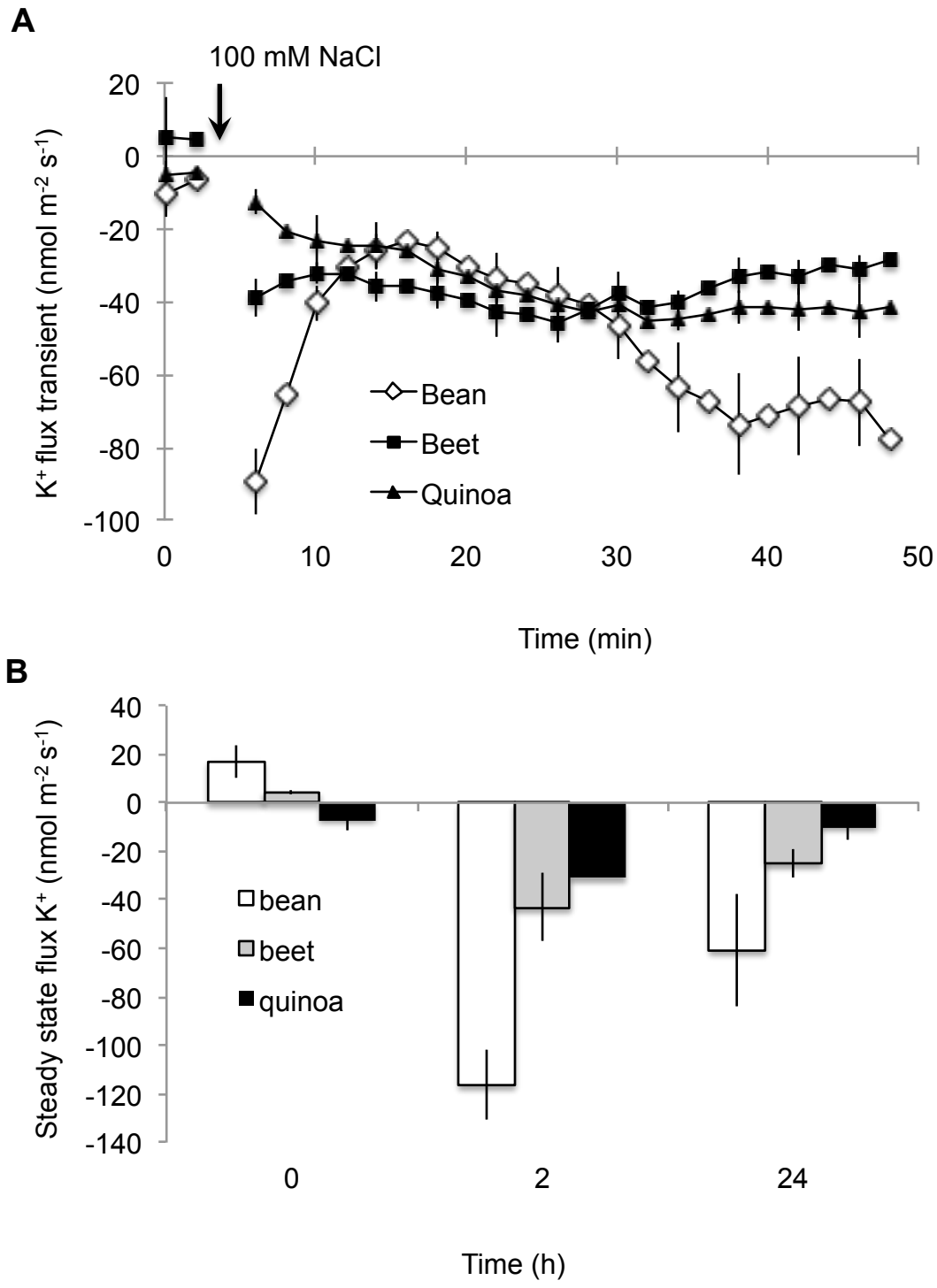

Fig. 3 Transient K^+ flux (A) and steady state flux (B) from mesophyll tissue from three species with contrasting salinity tolerance in response to treatment with 100 mM NaCl. (average ± *n*= 3-5)

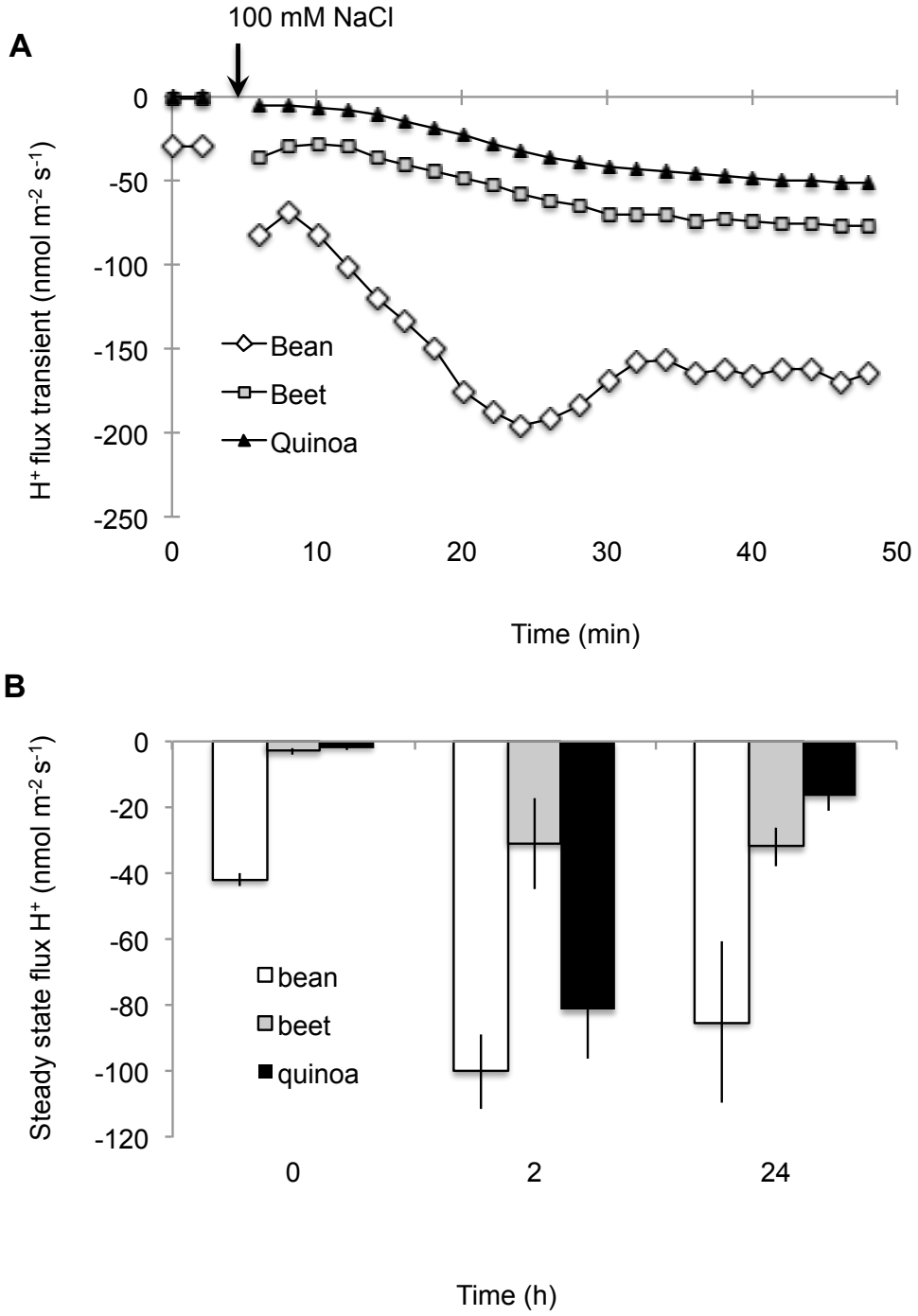

Fig. 4 Transient H^+ flux from mesophyll tissue from three species with contrasting salinity tolerance in response to treatment with 100 mM (A) NaCl. (average ± SE, *n* = 3-5)

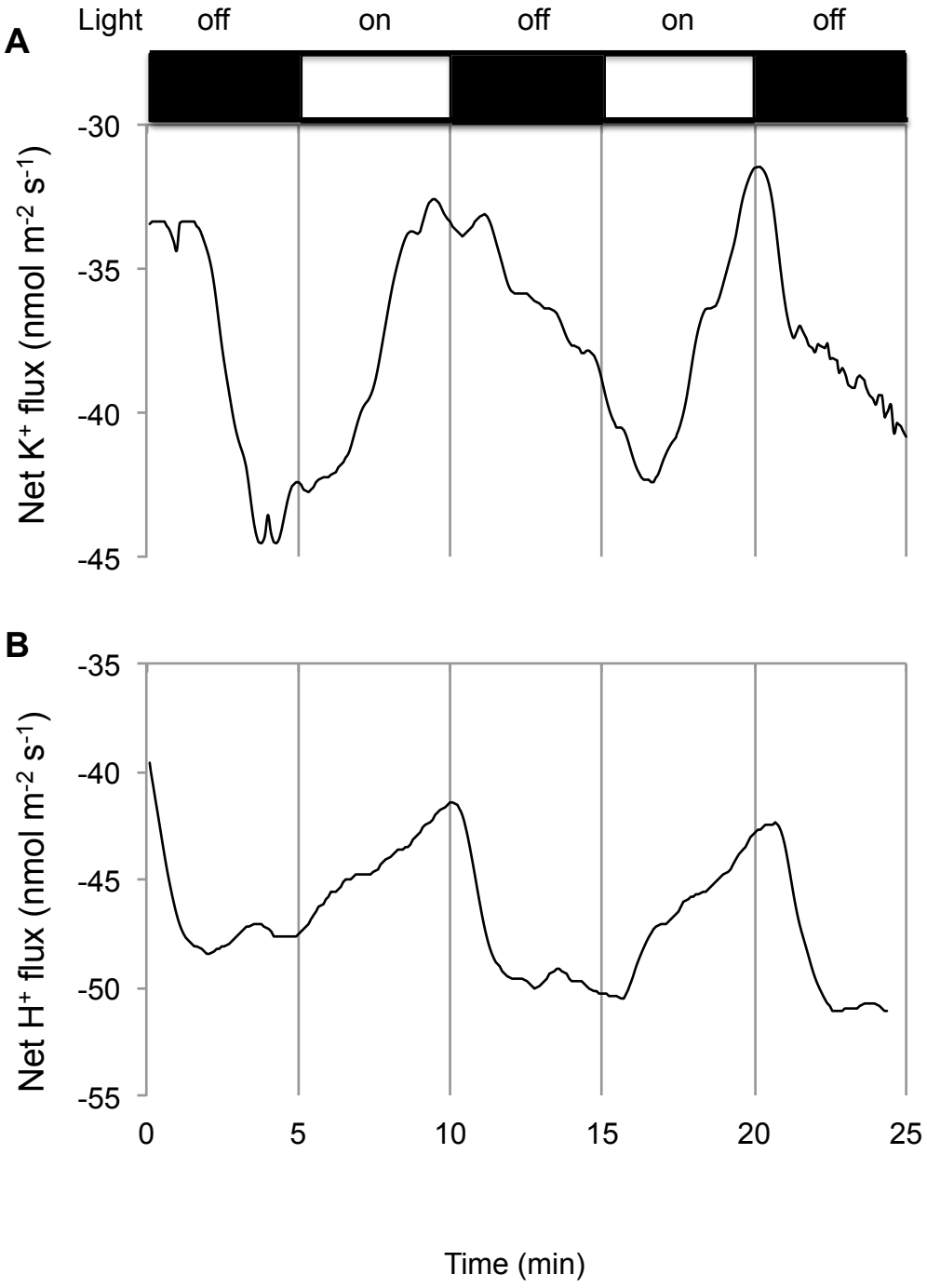

Fig. 5 example fluxes showing the response of K^+ (A) and H^+ (B) fluxes from beet mesophyll treated with 100 mM NaCl in response to light.

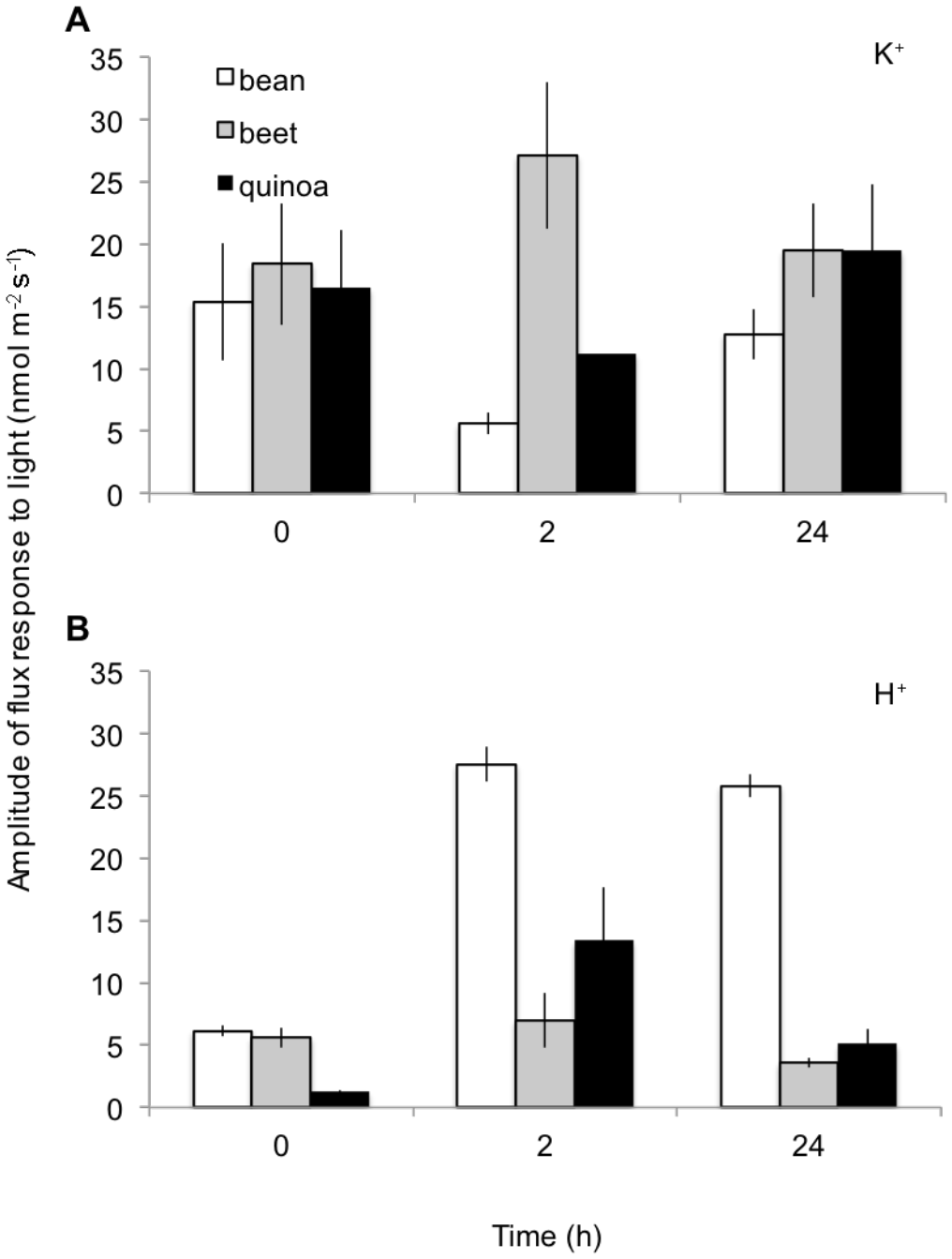

Fig. 6 Amplitude of K^+ (A) and H^+ responses to light in mesophyll segments from three species with contrasting salinity tolerance exposed to 100 mM NaCl between 0 and 24 h (average ± SE, *n* = 3-5)

Discussion

The three species selected for this study were quinoa (a true halophyte whose optimal growth is observed at about 150 mM NaCl; Hariadi et al 2011), sugar beet (optimal growth at about 50 mM NaCl; Greenway and Munns 1980) and salt-sensitive glycophyte broad bean. Accordingly, whole-plant physiological and agronomical performance also clearly followed quinoa>beet>bean pattern (Figs. 1 and 2). Salinity stress had major impact on beans reducing fresh and dry weight, relative water content (RWC), F_V/F_M and chlorophyll content. While 300 mM NaCl exceeded the optimal levels and caused reduced growth in beet and quinoa, there were no detrimental effects on RWC, F_V/F_M or chlorophyll content (Figs. 1 and 2). Moreover, in these two later species the chlorophyll content even increased significantly ($P< 0.05$; Fig. 2) in plants grown at high NaCl. The overall differences in salinity tolerance/damage between the species are attributed to the differential ability of mesophyll cells to control cytosolic K^+ homeostasis under salinity stress conditions.

In Chapter 3 it was shown that cytosolic K^+ was absolutely essential to maintain photosynthetic activity in isolated chloroplasts. An increase in Na^+ content in the incubation media (mimicking stress-induced elevation in cytosolic Na^+) did not affect photosynthetic capacity in isolated chloroplasts. At the same time, reduced K^+ content from 50 to 10 mM resulted in a 20% decline in F_V/F_M. At the tissue level, the efflux of K^+ from bean mesophyll was large enough to result in declines in F_V/F_M (Chapter 2). These declines in photosynthetic performance can be seen here in bean plants at the whole plant level (Fig. 2), with declines in both F_V/F_M and chlorophyll content. Bean has the largest Na^+ induced efflux of K^+ from mesophyll tissue of the three species measured (Fig. 3 and Table 1). The ability of the two more salt tolerant species to retain K^+ in mesophyll cells results in neither quinoa nor beet plants showing declines in photosynthetic parameters under salinity stress (Fig. 2).

Over the last decade, cytosolic K^+ retention has emerged as one of the main determinants of salinity tolerance in a range of glycophyte species (Chen et al. 2005; Cuin et al. 2008; Wu et al. 2013). Here we measured a larger increased K^+ efflux

under salinity stress in salt sensitive (glycophyte) species in comparison with salt tolerant (halophyte) species (Fig 3A, Table 1).

In the first 45 min of salinity treatment beet and quinoa loose respectively 30 and 33% less K^+ from their mesophyll tissue than bean (Table 1). The efflux of K^+ from bean over this time has been shown to be predominately through KOR channels (Chapter 2). KOR channels are Shaker-type channels that are activated by membrane depolarisation (Shabala and Pottosin 2014). Na^+ induced depolarisation of the plasma membrane of bean results in the initiation of a vanadate sensitive H^+ efflux (Chapter 2). Thus, it may be suggested that an increased rate of H^+-ATPase pumping is required as a negative feedback mechanism aimed at preventing K^+ efflux through the depolarisation activated KOR channels. As both quinoa and beet show much less K^+ efflux, they also require much less activation of H^+-ATPase (Figs. 3A and 4A and Tables 1 and 2). This comes with substantial energy cost-benefits.

Treatment with 50 mM NaCl results in long lasting membrane depolarisation in bean mesophyll (Chapter 2), accompanied by increased vanadate-sensitive H^+ efflux. Generating this H^+ flux (by H^+-ATPase) is energy demanding and may result in growth penalties (Bose et al. 2014). This appears to be the case for beans (Fig 1) but not for quinoa and beet. Both quinoa and beet are able to retain K^+ within mesophyll tissue (Fig. 3) without sustained large increases in H^+ efflux (Fig. 4).

For every mol of H^+ transported 1 mol of ATP is consumed (Michelet and Boutry 1995). Thus, direct yield penalty due to increased H^+-ATPase activity can be calculated on an assumption that 9 mol of ATP is required to produce 1 mol of glyceraldehyde-3-phosphate (Raven et al. 2005).

For any specific H^+ flux the amount of ATP (in mol) consumed by the H^+-ATPase in one day to drive H^+ transport across membrane surface of 1 m^2 can be calculated as

$$n(ATP)/day = n(H^+)/day = n(H^+)\ nmol^{-2}\ s^{-1} * 86400\ s * 10^{-9}$$

This will be equivalent to the amount (in mol) of glyceraldehyde-3-phosphate ($C_3H_6O_3$) that could have been produced from the ATP consumed

$$n(C_3H_6O_3)/day = n(ATP)/day/9$$

or if expressed in grams

$$m(C_3H_6O_3)/\ day = n(C_3H_6O_3)/day \times 90.08\ g\ mol^{-1}$$

Assuming the H^+ efflux after 24 h of salt treatment is has reached a steady state and putting the numbers from Fig 4 into above equations, bean plants will incur a penalty of 0.073 g of (otherwise fixed) carbon per day per square meter of leaf area, while beet and quinoa would lose only 0.027 g and 0.014 g respectively. Although these numbers would not account for the vast majority of the yield penalty observed in beans under salinity stress they are nonetheless significant and would make up a proportion of lost productivity.

Increased H^+-ATPase activity provides a driving force for the exclusion of Na^+ from the cytosol back into the apoplast (Ayala et al. 1996). The SOS1 Na^+/H^+ exchanger in mesophyll tissues is responsible for the removal of Na^+ from the cytosol back into the apoplast; however, this mechanism is effective only when there are high activities of the H^+-ATPase (Shabala 2013). Despite this the up regulation of the plasma membrane H^+-ATPases is not an essential feature for conferring salt tolerance in halophytes (Shabala and Mackay 2011).

The more tolerant plants do not induce such a large H^+ efflux across the plasma membrane (Fig. 4), and it is plausible to suggest that they instead use the available ATP pool to energize the activity of the vacuolar H^+-ATPase to sequester Na^+ into the vacuole. The activity of vacuolar H^+-ATPases is increase under salinity stress in

halophytes while in glycophytes the activity stays the same or declines (Wang et al. 2001).

Sequestration of Na^+ in the vacuole has many advantages over removal of Na^+ into the apoplast, including increased turgor pressure and allowing cells to relocate K^+ from the vacuole into the cytosol. It is also highly beneficial from a cost-benefit point of view, as plants do not have to invest heavily into production of compatible solutes, at the expense of other energy-demanding processes. At the same time, vacuolar Na^+ sequestration still achieves the goal of maintaining membrane potential (by removing positive charge from the cytosol), thus enabling K^+ retention. Both the up regulation of NHX (Apse et al. 1999) and vacuolar H^+-PPase (Gaxiola et al. 2001) increase salinity tolerance in *Arabidopsis*, demonstrating the importance of vacuolar Na^+ sequestration.

After 2 h of salinity treatment the H^+ efflux in quinoa increases to the point that it is not significantly different to that of bean (Fig. 4B). This could be due to an initial saturation of the ability of quinoa to sequester Na^+ in the vacuoles of mesophyll cells. For vacuolar sequestration to be effective under salinity treatment cells must be able to maintain a 4- to 5-fold Na^+ concentration gradient across the vaculor membrane which is not thermodynamically favourable (Shabala and Mackay 2011). To achieve this Na^+ must be actively transported across the vacuolar membrane with minimal re-entry of Na^+ from the vacuole back into the cytosol. Quinoa has the ability to change the transport properties of the vacuolar membrane under salt stress to limit the release of Na^+ from the vacuole into the cytosol allowing the build-up of higher concentrations of Na^+ in the vacuole (Bonales-Alatorre et al. 2013). The increased H^+ efflux is likely to help compensate for a short-term increase in cytosolic Na^+ concentration that would otherwise cause membrane depolarisation and the efflux of potassium. After 24 h it would seem that depolarisation in quinoa mesophyll cells is no longer a problem as H^+ efflux is reduced significantly from the levels 2 h after treatment (Fig. 4B).

The Na^+ induced K^+ leak in bean mesophyll exposed to 100 mM NaCl is halved 50 min after treatment with the addition of DMSO (Chapter 2). This suggests that, in the longer-term, control of the ROS-induced K^+ efflux becomes essential for maintaining

cytosolic potassium homeostasis. ROS are known to cause the opening of both NSCC (Demidchik 2003) and KOR (Demidchik et al. 2010) channels. A comparative study between the relatively salt tolerant barley and salt sensitive peas showed that lower ROS sensitivity of NSCC in the root apex has been essential to confer differential salt sensitivity between these species (Bose et al. 2014). NSCC not only partially mediate the efflux of K^+ from mesophyll cells (Chapter 2) but are also responsible for the accumulation of Na^+ within the cell (Demidchik and Tester 2002; Demidchik and Maathuis 2007) leading to further membrane depolarisation. There are four ways that quinoa and beet may be preventing the efflux of K^+ though ROS activated NSCC. These include: (i) halophytes having better ROS detoxification mechanisms (Bose et al. 2013) thereby reducing the open probability of NSCC; (ii) halophytes may have a better ability to maintain normal photosynthesis under altered ion homeostasis (Chapter 3) resulting in less ROS production (Hafsi et al. 2010); (iii) halophytes may possess a different population of NSCC (e. g. a smaller fraction of those activated by ROS); or (iv) halophytes may have reduced ROS production resulting from maintenance of higher levels of K^+ in cytosol (Fig. 3A and Table 1; Kim et al. 2010; Hafsi et al. 2010) due to prevention of membrane depolarisation. All four of these mechanisms are likely to play important roles in mediating K^+ loss through NSCC.

Differences in the expression of KOR channels could also be responsible, at least partially, for differences in the Na^+ induced K^+ efflux observed between species. *Aribidopsis gork* mutants had a much smaller Na^+ induced K^+ efflux from their roots than there wild type counter parts (Shabala and Cuin 2008). It is not likely that the majority of the difference in K^+ efflux comes from this mechanism as KOR play essential roles in plant growth. Further to this argument no relationship was found between *gork* expression in barley and salinity tolerance (Shabala 2003). It is therefore likely the control over ion channels, mediating the K^+ efflux, rather than the number of channels, is essential for conferring salinity tolerance in leaf mesophyll.

Light/dark transitions strongly modulate the kinetics of net K^+ and H^+ fluxes across the mesophyll cell plasma membrane (Fig. 5). These patterns were significantly altered by the application of salinity stress (Fig. 6). The effect of salinity on the light-induced K^+ flux response from mesophyll was not significant in either quinoa or beet (Fig. 6A) but in beans the ability of mesophyll to respond to light by modulating K^+

flux was reduced after 2 h salinity treatment (Fig. 6A). On the contrary, the magnitude of light/dark modulation of net H^+ flux increased dramatically in beans and much less so in other more tolerant species (Fig. 6B).

Mesophyll tissue responds to light with changes in both K^+ and H^+ fluxes (Fig. 5; Elzenga et al. 1995; Shabala and Newman 1999). Salinity stress diminishes the flux response to light in bean mesophyll (Chapter 2) and could be expected to have less effect on the more tolerant species that have better control over their ion transport under saline conditions. In the case of K^+ flux responses this is true 2 h after 100 mM treatment, where the K^+ flux response of beet and quinoa is not significantly altered while the response of bean is diminished. Light induced changes in ion fluxes are modulated by changes in membrane potential brought about by Ca^{2+} influx into the cell upon illumination with light (Shabala and Newman 1999). Salinity stress causes membrane depolarisation resulting in the opening of KOR channels; it is likely that both salinity and light have the same mechanism of inducing K^+ efflux. Salinity may be nearly saturating this mechanism in bean resulting in a reduced response, while beet and bean do not experience the same extent of salinity induced membrane depolarisation over this time period. The light induced Ca^{2+} influx in bean may also not be as strong under saline conditions as there would be less ATP available for Ca^{2+}-ATPase activity due to the increased H^+-ATPase activity under saline conditions (Fig. 4). Both quinoa and beet would most likely have more ATP available for Ca^{2+}-ATPase activity due to lower H^+-ATPase activity over the preceding 2 h. In addition to higher energy consumption by H^+-ATPase bean has a reduced ability to photosynthesis and produce energy under saline conditions (Chapters 2 and 3), while more tolerant species are not as effected by altered ionic homeostasis (Chapter 3).

The H^+ flux response of bean is most affected by salinity at both 2 h and 24 h after salinity treatment. The observed changes in H^+ flux from mesophyll tissue under light in the short term (first 3 to 5 min) are generated due to changes in pH because of CO_2 uptake for photosynthesis (Shabala and Newman 1999). The light induced changes in H^+ fluxes under control conditions in all three species could be attributed to this effect. The increased H^+ flux response in bean under salinity treatment could be attributed to the diversion of energy away from the H^+-ATPase that is activated under salinity stress to cope with membrane depolarisation (Chapter 2) for other light induced

processes. H^+-ATPase activity is energy intensive (Briskin and Hanson 1992; Gaxiola et al. 2007), under light other energy intensive process including the above mentioned Ca^{2+}-ATPase activity are required. So the enhanced decrease in H^+ efflux in salinity treated bean (Gig. 6 B) is likely due to energy constraints that are not seen in the more salinity tolerant species that have lower H^+ efflux under saline conditions (Fig. 4). Again the changes in flux could be related to a reduced ability to produce energy from photosynthesis under conditions of altered ionic homeostasis (Chapter 3).

Conclusion

The ability of quinoa and beet to retain potassium in their mesophyll tissue when exposed to increased apoplastic salinity is an overriding mechanism which results in greater salt tolerance, both in terms of growth and photosynthetic parameters. The reduced photosynthetic parameters in bean may be at least partially related to poor mesophyll K^+ retention ability under salinity stress. It is likely that the higher level of K^+ retention in quinoa and beet is related to a better ability to sequester Na^+ in vacuoles therefore avoiding plasma membrane depolarisation and the resultant K^+ leak. In its turn, better K^+ retention is essential to minimise ROS production (Kim et al. 2010; Hafsi et al. 2010) and thus may prevent a further increase in K^+ leak mediated by ROS-activated NACC channels, operating in a positive-feedback manner, and thus exacerbating detrimental effects of salinity on cell metabolism. K^+ retention, without vast increase in H^+ fluxes, is an important trait of salinity tolerance, most likely due to energy conservation. Differences in light responses between species affected by salinity are also likely to be related to the amount of energy available in salt-stricken plants.

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Chapter 6: General discussion

Salinity causes vast reductions in plant productivity and, as such, results in massive penalties to agriculture worldwide. There has been a large focus on producing salinity tolerant crops over the past few decades in an attempt to mitigate these losses. This focus has been met with only limited progress due to salinity tolerance being a complex multigenic trait.

Most of the research effort to date has been focused on roots. This is because roots are the organs of the plant that first come into contact with salinity. Na^+ exclusion from roots has long been considered as an essential component of salinity tolerance in glycophytes and has received much attention (Greenway and Munns 1980; Munns and Tester 2008), including the over-expression of SOS1 Na^+/H^+ antiporter responsible for the extrusion of Na^+ from the cytosol. Over expression of SOS1 has been found to reduce Na^+ accumulation and improve salinity tolerance in transgenic *Arabidopsis* (Shi et al. 2003). No reports are available, however, suggesting that higher SOS1 activity may confer superior salinity tolerance in crop species, despite the fact that the presence of SOS1-homologues have been shown at both molecular (Mullan et al. 2007) and functional (Cuin et al. 2011) levels. This is most likely due to this strategy not taking into account the osmotic effects of salinity.

To overcome the osmotic effects of salinity in the most energetically favourable way it is necessary to have Na^+ uptake into the shoot (Shabala and Shabala 2011). This implies a need for an efficient tissue tolerance mechanism in the leaf mesophyll. The majority of the efforts to increase tissue tolerance have focused on Na^+ sequestration in the vacuoles of cells away from the metabolically active cytosol (Munns and Tester 2008). These efforts have produced plants that are more tolerant to salinity in laboratory/ glasshouse conditions (Apse et al. 1999; Zhang and Blumwald 2001). However, to the best of my knowledge, these pivotal studies still have not resulted in salt tolerant cultivars in farmers' fields. Thus, it appears that there is more to tissue tolerance than merely vacuolar sequestration.

Insights into salt tolerance mechanisms can be gained from halophytes. One of the hallmark traits exhibited in halophytes is much higher Na^+ concentrations in their shoots (Flowers and Yeo 1995; Yamaguchi and Blumwald 2005; Shabala 2013) compared with less tolerant plants. This provides the required osmotic adjustment for plant growth (Shabala and Mackay 2011; Hariadi et al. 2011). Elevated Na^+ concentrations in the apoplast of leaves, however, alter ion transport in mesophyll tissue (Chapter 2 and 5), more so in non-salinity tolerant species (Chapter 5). The maintenance of cytosolic K^+ homeostasis in mesophyll cells is an essential component of salinity tolerance (Chapters 2, 3 and 5). It also appears that maintenance of high cytosolic K^+ (~100-150 mM) concentrations are more important than reducing Na^+ entry into the cytosol for the maintenance of photosynthesis (Chapter 3). This can be explained by the fact that K^+ is essential for the activation of over 50 enzymes (Marschner 1995) including enzymes involved in carbon fixation (Jin et al. 2011), protein synthesis (Weber et al. 1977) and H^+-PPases (Belogurov and Lahti 2002; Gaxiola et al. 2007).

Salinity induces a K^+ efflux from a range of plant tissues in many species (Chen et al. 2005; Smethurst et al. 2008; Cuin et al. 2008; Wu et al. 2013; Chapter 5). The extent of this efflux is Na^+ concentration dependant with higher Na^+ concentrations causing greater K^+ efflux (Chapter 2). Na^+ induced K^+ efflux has been negatively correlated with salinity tolerance in the mesophyll of wheat and barley (Wu et al. 2013) and, indeed, this relationship was also found to be true across species contrasting in salinity tolerance (Chapter 5).

Salinity damage to the photosynthetic machinery of bean mesophyll tissue takes longer before it is noticeable than salinity damage to bean chloroplasts (Chapters 2 and 3). This is because mesophyll cells have the ability to delay the reduction in cytosolic K^+ concentration by redrawing available K^+ from the vacuolar pool. Under normal non-saline conditions the majority of K^+ within cells is stored in the vacuole (Greenway and Munns 1980; Cuin et al. 2003; Munns and Tester 2008). Salinity stress induces K^+ efflux across the plasma membrane resulting in reduced cellular K^+ concentrations. The vaculor store of K^+ is used to replenish any loss of K^+ from the cytosol (Cuin et al. 2003; Shi et al. 2003). Over time the amount of K^+ in the vacuole is diminished to a point where it can no longer replenish the K^+ lost from the cytosol.

Such a mechanism is not available in chloroplasts. Na^+ has the ability replace K^+ functionally in the vacuole maintaining cell turgor however does not have the ability to replace the functionality of K^+ in the cytosol for enzyme activation (Mullan et al. 2007; Wakeel et al. 2011).

K^+ to Na^+ ratios in the cytosol have long been considered the key to salinity tissue tolerance (Maathuis and Amtmann 1999; Cuin et al. 2011). However there have been few direct measurements of cytosolic K^+ concentrations and even less measurements of cytosolic Na^+ concentrations and even less measurements of K^+:Na^+ ratios (Shabala and Shabala 2011; Kronzucker et al. 2013). There is no direct evidence supporting the cytosolic ratio between K^+ and Na^+ being the key determinate of tissue tolerance in the literature. Recently it has been suggested that K^+ retention is the key to tissue tolerance and Na^+ concentrations are secondary and don't have a big effect on metabolism (Munns and Tester 2008; Kronzucker et al. 2013). Some salinity tolerant plants have been found to have high concentration of Na^+ in the cytosol (Weber et al. 1977; Apse et al. 1999; Zhang and Blumwald 2001; Belogurov and Lahti 2002; Gaxiola et al. 2007; Flowers and Colmer 2008; Jin et al. 2011; Kronzucker and Britto 2011). Backing up this are the findings in chapter 3 that K^+ retention in the cytosol is much more important for the maintenance of photosynthesis than the exclusion of Na^+. A drop from 50 mM K^+ to 10 mM K^+ simulating a drop in cytosolic concentration resulted in a 21% decline in F_V/F_M in isolated chloroplasts. Increasing Na^+ concentrations even up to 100 mM increased F_V/F_M especially when there were low concentrations of K^+ (Chapter 3, Fig.5).

Upon the induction of salinity stress Na^+ rapidly enters the cytosol through non-selective cation channels (NSCC) causing membrane depolarisation. The vast majority of the Na^+ induced K^+ efflux from bean occurs due to this depolarisation of the plasma membrane. Depolarisation results in the opening of K^+ outward rectifying (KOR) channels (Flowers and Yeo 1995; Yamaguchi and Blumwald 2005; Hedrich 2012; Shabala 2013) which are responsible for 84% of the K^+ efflux from bean mesophyll (Chapter 2) and ~70% of the efflux from barley mesophyll (Shabala and Mackay 2011; Hariadi et al. 2011; Wu et al. 2013). The remainder of the observed K^+ efflux occurs through NSCC (Marschner 1995; Chen et al. 2005; Smethurst et al. 2008; Cuin et al. 2008; Wu et al. 2013).

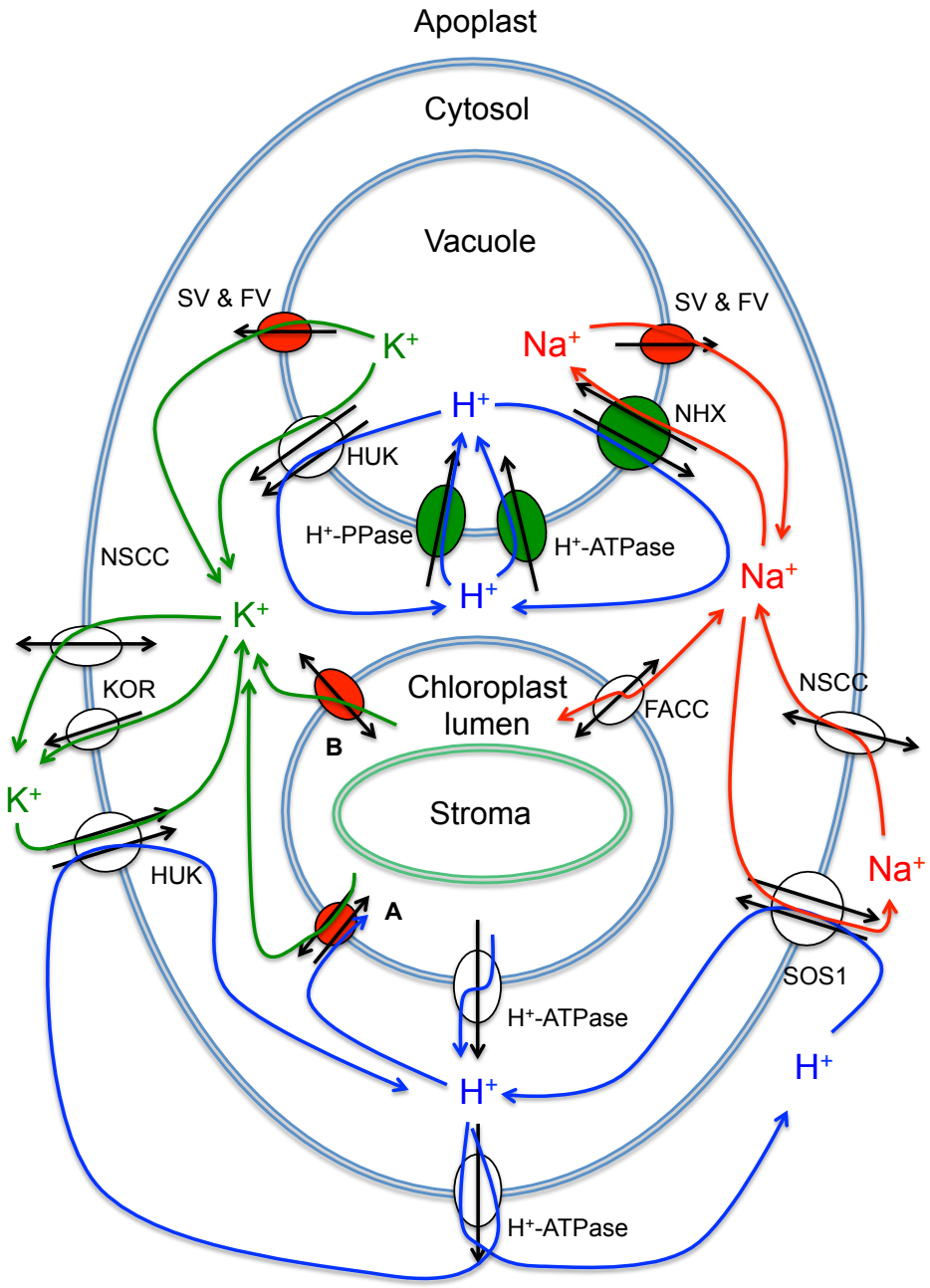

Fig. 1 Summary of ion transport in mesophyll cells under salinity stress. Green lines denote K^+, red lines Na^+, and blue lines H^+. Transporters marked in red may be down regulated to confer salinity tolerance while those marked in green may be up regulated for increased salinity tolerance. The molecular identity of transporters **A** and B is yet not known. The possible candidates may be either CHX23 or KEA1 for transporter A (monovalent cation H^+ antiporters), and some voltage gated potassium channel for **B.**

Many transporters mediate Na^+ and K^+ transport through the plasma and tonoplast membrane (Jin et al. 2011; Hedrich 2012); the most critical ones are summarised in Figure 1. Given that TEA reduced K^+ efflux from bean mesophyll (Chapter 2) and increased photosynthetic parameters (Fig. 2) it might be suggested that the down regulation of KOR channels would be beneficial for salinity tolerance. Indeed the down regulation of GORK (a major type of KOR found in *Arabidopsis*) resulted in reduced K^+ efflux and reduced symptoms of salt stress including programmed cell death and ROS generation in *Arabidopsis* roots (Demidchik et al. 2010).

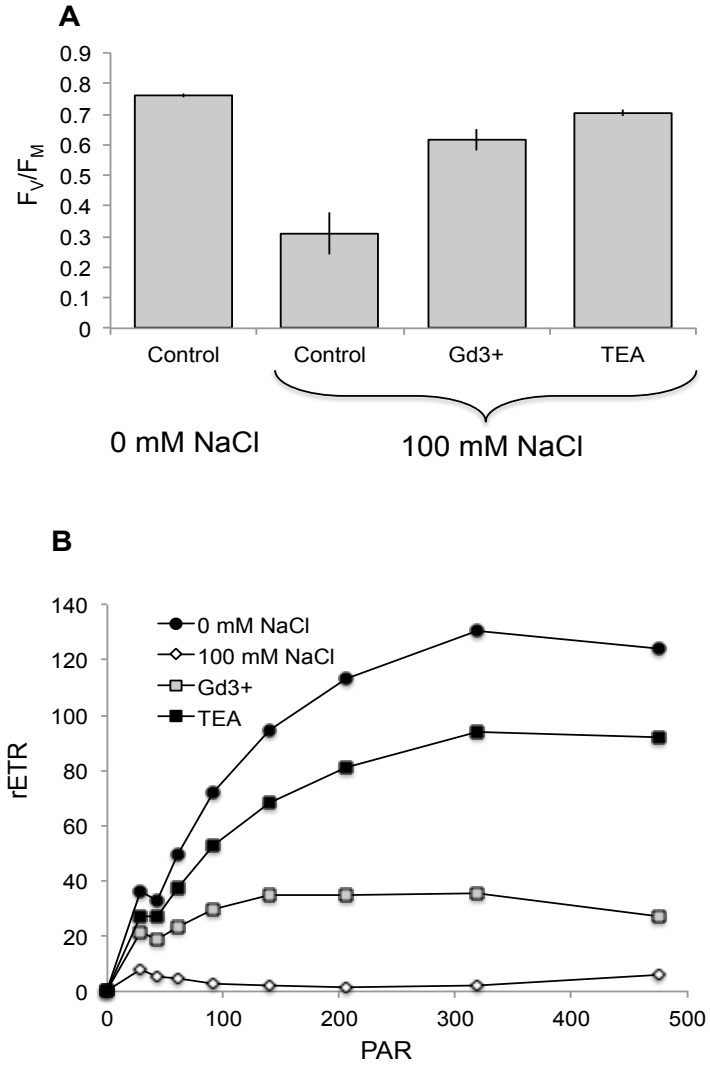

Fig. 2 A; F_V/F_M and **B**; rETR of mesophyll tissue treated with the channel modulators Gd^{3+} and TEA. These measurements were taken 72hr after treatment with pharmacological agents ± salinity stress. Salinity stress 100mM NaCl. Error bars ± SE (n=3)

The next logical step is to look at the NSCC which are responsible for the remainder of K^+ leak (Weber et al. 1977; Cuin et al. 2003; Wu et al. 2013) and the entry of Na^+ into the cell (Demidchik and Tester 2002; Belogurov and Lahti 2002; Cuin et al. 2003; Gaxiola et al. 2007; Demidchik and Maathuis 2007). Again, the blockage of these channels with Gd^{3+} results in reduced K^+ efflux (Chapter 2) and reduced damage to photosynthetic parameters (Fig. 2). NSCC are a group of cation channels of which their molecular identity is not clear and there are no known pharmacological agents that can differentiate them (Chen et al. 2005; Smethurst et al. 2008 Chapter 5; Cuin et al. 2008; Wakeel et al. 2011; Wu et al. 2013; Pottosin and Dobrovinskaya 2014). All this makes NSCCs a very difficult target to manipulate in breeding programs. An approach of reduced NSCC conductivity to increase salinity tolerance, however, would likely be ineffective as Na^+ exclusion from plants, and cells within plants, causes problems with cell turgor. NSCC also are responsible for a variety of essential physiological functions within the plant (Maathuis and Amtmann 1999; Wu et al. 2013; Pottosin and Dobrovinskaya 2014). With further investigation it may be possible to improve the regulation or expression of some NSCCs to improve salinity tolerance, through reducing K^+ leak.

Salinity induces a vanadate sensitive increase in H^+ efflux, suggesting that this efflux is mediated by the plasma membrane H^+-ATPases (Chapter 2). The increased activity of the H^+-ATPase at the plasma membrane of salinity treated mesophyll tissue plays a role in repolarising the plasma membrane. This strategy to restore membrane polarity helps reduce K^+ efflux from mesophyll. Inhibition of the H^+-ATPases with vanadate increased K^+ efflux in both bean (Chapter 2) and barley mesophyll (Wu et al. 2013). Increased plasma membrane H^+-ATPase expression has been shown to increase salinity tolerance, however, the increased expression resulted in abnormal growth and development (Gevaudant et al. 2007). The activity of H^+-ATPases is energy intensive and comes with yield penalties. Thus, it was suggested that species possessing an ability of maintaining cytosolic K^+ homeostasis with reduced reliance on H^+-ATPase activity might have an adaptive advantage (Bose et al. 2014). This notion is supported by the findings in chapter 5 that more tolerant species have less H^+ efflux from mesophyll tissue under salinity treatment than sensitive species.

The classical view of salt tolerance has been one of Na^+ exclusion from the photosynthetic areas (Munns and Tester 2008). The SOS1 plasma membrane Na^+/H^+ antiporter has received much attention in this respect. The SOS1 antiporter is energised by a H^+ gradient to move Na^+ against the electrochemical gradient out of the cytosol into the apoplast (Blumwald et al. 2000). Increased plasma membrane Na^+/ H^+ antiporter expression has been reported to increase the salt tolerance of *Arabidopsis* (Gao et al. 2003; Shi et al. 2003; Hedrich 2012; Feki et al. 2014) and rice (Wu et al. 2005). This mechanism for Na^+ removal relies on H^+ pumping which, as previously mentioned, results in ATP depletion. It would also lead to the futile cycling of Na^+ out of the cytoplasm into the apoplast from which Na^+ entered in the first place (Fig. 1). The approach of SOS1 exclusion of Na^+ from the cytosols of mesophyll cells would be energy intensive and ineffective as it would start a Na^+ relocation cycle and not resolve the problem of turgor maintenance.

At the plasma membrane of mesophyll cells there are not many viable options for conferring high levels of salt tolerance. As such the tonoplast membrane may hold mechanisms that can confer salinity tolerance. The vacuole makes up over 90% of the cell volume and, as such, can be used for the storage of ions including K^+ and Na^+. Plants have the ability to relocate K^+ from the vacuole into the cytosol to maintain cytosolic K^+ levels near optimum, for as long as possible, under stress conditions (Cuin et al. 2003; Britto and Kronzucker 2008). At the same time the membrane depolarising effects of accumulated Na^+ can be reduced through Na^+ sequestration in the vacuole thereby limiting K^+ efflux through KOR channels.

The sequestration of Na^+ into the vacuole is not energetically favourable (Shabala and Mackay 2011) and is mediated by a Na^+/H^+ antiporter (NHX). Increased NHX expression in a range of plants has been shown to increase salinity tolerance (Apse et al. 1999; Zhang and Blumwald 2001). The increased NHX expression results in increased plant growth under saline conditions and increased Na^+ concentrations within tissues including leaves (Zhang and Blumwald 2001). However increasing NHX expression is only part of the equation. These antiporters rely on H^+ gradients across the tonoplast membrane. These H^+ gradients are generated by two active transporters, V-H^+-ATPase and the H^+-PPase. Increased expression of either of these active transporters results in increased salinity tolerance (Bhaskaran and Savithramma

2011; Bao et al. 2014; Shen et al. 2014) and higher Na^+ concentrations in leaves (Bhaskaran and Savithramma 2011; Bao et al. 2014). The overexpression of these active transporters in conjunction with NHX overexpression leads to an even greater level of salinity tolerance (Bhaskaran and Savithramma 2011; Bao et al. 2014; Shen et al. 2014).

In addition to getting Na^+ into the vacuole, retaining Na^+ is also an important aspect of salinity tolerance, as Na^+ can leave the vacuole and re-enter the cytosol through two types of channels: fast activating (FV) and slow activating (SV) vacuolar channels (Bonales-Alatorre et al. 2013a Fig. 1). These are non-selective cation channels showing little if any preference between the transport of K^+ and Na^+. It was shown that the halophyte quinoa species reduce the conductance of SV and FV channels 5 to 7 fold (depending on cultivar and leaf age) when grown under saline conditions (Bonales-Alatorre et al. 2013b) . The reduction in leak of Na^+ from the vacuole into the cytosol reduces the need for Na^+ translocation due to reduced futile cycling and therefore energy intensive H^+ pumping (Fig. 1).

Halophytes not only have the ability to retain more K^+ but they also maintain their ability to respond normally to light with both K^+ and H^+ fluxes across the plasma membrane (Chapter 5). These two attributes are inextricably linked for many reasons. The first and most likely predominate reason in the case of light induced fluxes of K^+ is that they are caused by membrane depolarisation due to an increase in cytosolic Ca^{2+} concentrations (Shabala and Newman 1999), while salinity induced K^+ efflux is also caused by membrane depolarisation (Shabala et al. 2000 Chapter 2). This overlap means that plants with a greater salinity induced membrane depolarisation have a diminished K^+ light response (Chapter 5). More salinity sensitive species also exhibit an increased light induced H^+ flux response under salinity treatment (Chapter 5). Under normal non-saline conditions the initial H^+ flux response to light is dominated by CO_2 uptake causing changes in H^+ concentrations (Shabala and Newman 1999). CO_2 uptake is measured as a H^+ influx and seen as a reduction in H^+ efflux. It is unlikely that the increased reduction in H^+ flux observed in the salinity treated bean mesophyll (Chapters 2 and 5) is due to increased photosynthesis, as under salinity stress bean has reduced photosynthetic parameters (Chapters 2, 3 and 5). It is more

likely that other energy dependant processes initiated by light are diverting ATP away from the H^+-ATPase.

In addition to more salinity tolerant plants having a greater ability to maintain ionic homeostasis in saline conditions (Chapter 5) they also have a greater ability to photosynthesize with altered cytosolic ionic homeostasis (Chapter 3, Figs 6 & 7). It is unlikely that the enzymes of halophytes are more tolerant to changed ionic conditions as the sensitivity of enzymes to Na^+ and K^+ concentrations does not vary between species (Greenway and Osmond 1972; Flowers and Colmer 2008). There are a few possible reasons for the difference between species. More tolerant species might be better able to control transport across the inner envelope of the chloroplast, therefore maintaining ionic homeostasis within the chloroplast. They may have better antioxidative defences and/or high concentrations of compatible solutes and polyamines to protect the photosynthetic structures within the chloroplast.

The maintenance of ion homeostasis in chloroplasts is very important. There are many different processes that are activated by potassium (e.g. RuBisCo activity or the synthesis of the co-enzyme NADPH; Jin et al. 2011). K^+ is also important for the production of ATP via ATP-synthase as cytochrome b_6f generates a H^+ gradient across the thylakoid membrane, which requires charge balancing. Charge balancing is not an ion specific task and Na^+ and K^+ would most likely be interchangeable (Hind et al. 1974; Chow et al. 1976; Krause 1977). This interchange ability is most likely the cause of increased F_V/F_M of chloroplasts in low K^+ supplied with Na^+ (Chapter 3, Fig. 5).

Modifying the ion transport properties of the chloroplast with different pharmacological agents can increase the F_V/F_M of chloroplasts under salinity stress (Chapter 3, Fig. 8), opening prospects for amelioration of salinity stress in field-grown plants by foliar sprays. Vanadate, unsurprisingly, reduces the F_V/F_M of isolated chloroplasts regardless of salinity treatment. Vanadate is a P-type ATPase inhibitor and inhibits the Ca^{2+}-ATPase and H^+-ATPase of the inner envelope as well as ATPsynthase. This would result in a reduced ability to maintain membrane potential across the inner envelope as well as a reduced ability for calcium signalling. It would

also reduce photochemical efficiency due to the build up of H^+ in the lumen thus reducing the efficiency of cytochrome b_6f (Rott et al. 2011)

The blockage of the non-selective cation channels in the chloroplast with Gd^{3+} did not increase F_V/F_M. There are two known non-selective cation channels in the chloroplast. One in the inner envelope and one in the thylakoid membrane (Pottosin and Schönknecht 1996; Pottosin et al. 2005). The non-selective cation channel of the inner envelope (Fast-Activated Cation Channel, FACC) is the most likely candidate for Na^+ entry into the chloroplast. This demonstrates that Na^+ entry into the chloroplast is not likely a cause of reduced photochemical efficiency. The voltage gated cation channel of the thylakoid is likely used in charge balancing (Pottosin and Schönknecht 1996). The blockage of this channel with Gd^{3+} is unlikely to affect F_V/F_M as F_V/F_M is only measured using a very short pulse of light that would not generate a significant build-up of charge across the thylakoid membrane.

The positive effects of TEA on the F_V/F_M of salinity treated chloroplasts (Chapter 3) are likely linked to K^+ retention within the lumen. TEA might block the voltage independent K^+ channel in the chloroplast inner envelope due to the sequence homology of the channel with other K^+ channels (Mi et al. 1994). This channel may potentially mediate a similar K^+ leak from chloroplasts (Fig. 1) to that observed from mesophyll tissue upon exposure to salinity treatment.

Amiloride, a known inhibitor of cation/H^+ antiporters (Blumwald and Poole 1985; Cuin et al. 2011), also increased the F_V/F_M of salinity treated chloroplasts (Chapter 3). Chloroplasts have multiple types of $K^+(Na^+)/H^+$ antiporters in the inner envelope membrane (Song et al. 2004; Evans et al. 2012; Pfeil et al. 2014) as well as antiporters in the thylakoid membrane (Ettinger et al. 1999). The above positive effect could be attributed to the effects at the inner envelope membrane; the antiporters of the inner envelope are responsible for K^+ and Na^+ extrusion from the stroma into the cytoplasmic space. Therefore blocking these channels would minimise K^+ leak from the stroma maintaining K^+ homeostasis and benefiting photosynthesis.

These pharmacological experiments demonstrate that differences in chloroplast membrane transport can result in differences between the ability of chloroplasts to photosynthesis in altered ionic environments similar to that of salinity stress.

There has been a large focus on the involvement of compatible solutes in salinity tolerance, with reports suggesting that increased compatible solute production in the chloroplast can lead to increased salt tolerance (Chen and Murata 2002; Zhifang and Loescher 2003; Chen et al. 2007; Chang et al. 2014). Compatible solutes have also been used exogenously to increase the salinity tolerance and yield of different crop plants (Ali et al. 2007; Ashraf and Foolad 2007). The application of exogenous betaine has been shown to be an economically viable option to improve the tolerance of cotton to drought stress (Naidu et al. 1998). Despite the economic benefits shown for cotton under drought stress, there have been mixed reports as to the beneficial effects of the application of both betaine and proline to a variety of species. With many reports of increased growth under salinity stress there are also a few reports of no change in growth or even detrimental effects (Ashraf and Foolad 2007). These differences could be due to differences between species or due to differences in treatment (Ashraf and Foolad 2007). It is known that the localisation of compatible solutes in the chloroplast can help increase salinity tolerance (Sakamoto and Murata 2000). What is less clear is how these compatible solutes increase salinity tolerance. The findings in chapter 4 have assisted to fill some gaps in this knowledge. The compatible solute betaine increases both the rETR and F_V/F_M of salinity treated mesophyll tissue (Chapter 4, Fig. 6). Compatible solutes also have the ability to increase the F_V/F_M of isolated chloroplasts with proline being the most effective followed, in order, by trehalose, mannitol and betaine (Chapter 4, Fig. 3). After 25 min salinity treatment proline, mannitol and betaine all had significant positive effects on $rETR_{MAX}$, while trehalose had no effect. After 100 min treatment all the compatible solutes had a negative effect on $rETR_{MAX}$ (Chapter 4, Fig. 4). While compatible solutes generally increase salinity tolerance when applied exogenously, or when overexpressed, the control of over expression and localisation within the cytosol and possibly within the chloroplast, is very important. Overexpressing mutants often display reduced growth. Much of this has previously been associated with the energy cost of compatible solute production and associated yield penalties. However, there is evidence that compatible solutes, when over accumulated, have adverse effects on plants (Garcia et al. 1997; Gibon et al. 1997; Nanjo et al. 1999; Sulpice et al. 2003; Yamada et al. 2005; Szabados and Savouré 2010; Chang et al. 2014), including interfering with photosynthesis (Chapter 4). This demonstrates that compatible solutes

need to be used with caution as they may cause reduced plant growth due to interference with the electron transport change in chloroplasts.

Polyamines have been considered as attractive targets for conferring salinity tolerance both through genetic engineering and exogenous application (Groppa and Benavides 2008; Gill and Tuteja 2010; Hamdani et al. 2011). Polyamines have numerous roles within plants; being potent channel modulators (Lopatin et al. 1994; Dobrovinskaya et al. 1999), antioxidants (Ha et al. 1998; Hussain et al. 2011), and having capacity to stabilise proteins (Kotzabasis et al. 1993; Hamdani et al. 2011). Polyamines have been shown to decrease Na^+ induced K^+ efflux from mesophyll tissues (Shabala et al. 2007). If the same is true for chloroplasts then polyamine-reduced K^+ may be the reason for some of the positive effects of putrescine and spermine on F_V/F_M and rETR. Another probable explanation is that polyamines are known to bind with different proteins of the photosystems and electron transport chain (Kotzabasis et al. 1993; Hamdani et al. 2011). Polyamines have multiple positive charges that interact with the negative charges of proteins providing support and stability. The same positive charges that provide protection can also cause damage to proteins (Hamdani et al. 2011). This is the likely explanation for all three polyamines causing declines in the rETR of isolated chloroplasts after 100 min treatment. Polyamines are known to do damage to the quinone receptors of photosystem II within the lumen of chloroplasts (Beauchemin et al. 2007). When polyamines have been applied exogenously to plants in attempts to increase salinity tolerance there have been mixed reports of success including adverse effects (Subhan and Murthy 2001; Wang et al. 2006; Ndayiragije and Lutts 2006; Roychoudhury et al. 2011). These mixed reports are likely due to differences in concentrations and timings of treatments leading to either the positive benefits seen on rETR in the first 25 min or the detrimental effects seen after 100 min of treatment (Chapter 4 Fig. 2). If polyamines are to be used as ameliorative chemicals it is evident that much more needs to be known about their effects on the photosystems. Although the binding of polyamines to photosysytems is well understood, their effects on photosynthesis under stress conditions is less well investigated. It is clear that they can have both positive and negative effects on both photosystem II and the electron transport chain (Chapter 4).

Salinity stress induces ROS production and toxicity. Halophytes have better antioxidative mechanisms than glycophytes to deal with these increases in ROS (Bose et al. 2013; Baxter et al. 2014), preventing damage to key structures within the cell. In photosynthesising plant tissues the majority of ROS are produced in chloroplasts (Asada 2006; Pospíšil 2009; Kozuleva et al. 2011; Bose et al. 2013). The damage to photosystems observed under salinity stress increases ROS production in chloroplasts, due to the oxidation of non-target species (Pospíšil 2009; Kozuleva et al. 2011). This is a self-propagating problem without effective antioxidative strategies as the photosynthetic machinery and membranes sustain oxidative damage. The application of the antioxidant DMSO to bean mesophyll tissue results in reduced damage to both F_V/F_M and rETR (Chapter 4; Fig. 6). DMSO and thiourea have different effects on chloroplasts. Both are beneficial when chloroplasts are exposed to salinity and light simultaneously; while DMSO is not beneficial to dark-adapted chloroplasts exposed to salinity (Chapter 4, Fig. 5). This shows that different antioxidant pools in different species can interact in their own way in chloroplasts to provide distinct benefits. When using antioxidants to ameliorate the effects of salinity it is important to consider which antioxidant to use.

The positive effects of DMSO and betaine on whole bean mesophyll tissue (Chapter 4, Fig. 6) may be related to K^+ nutrition. DMSO increased K^+ retention after the first 50 min of salinity treatment, presumably by reducing the oxidative load in the cytosol and therefore reducing the conductance of ROS-activated NSCC (Chapter 2). Although not an antioxidant in its own right, betaine is also known to reduce the amount of ROS in cells. Possible mechanisms may include both promoting ROS detoxification and reducing ROS generation. Compatible solutes have been demonstrated to reduce Na^+ induced K^+ efflux from roots (Cuin and Shabala 2005), and it appears that this would also be the case for mesophyll cells.

Salinity induced increases in ROS production in chloroplasts results from inhibition of photosynthesis. Photosynthesis under salinity stress is inhibited by reductions in cytosolic K^+ concentrations and reductions in K^+ concentrations within chloroplasts (Chapter 3). This method of ROS production explains the increased ROS production observed under salinity stress and plants suffering from K^+ deficiency (Kim et al. 2010; Hafsi et al. 2010). The ability of chloroplasts from species with increased

salinity tolerance to be able to photosynthesize better under saline conditions would presumably reduce ROS production. This reduced ROS production should result in less K^+ loss from the cytosol as both NSCC and KOR are activated by ROS (Demidchik and Tester 2002; Demidchik et al. 2010). ROS production in chloroplasts due to K^+ loss would result in positive feedback amplification, as more ROS is produced more K^+ is lost. Further to this point, plants with better compatible solute, polyamine and/or antioxidant properties in the chloroplast that stimulate more efficient photosynthesis would be expected to retain more K^+. Reducing ROS levels within mesophyll reduces Na^+ induced K^+ efflux (Chapter 2)

Conclusions and Prospects

It appears form the work in chapter 3 that K^+ retention in the cytosol is more important for photosynthesis than Na^+ exclusion from this compartment. Following this, halophytes have better control over K^+ homeostasis in mesophyll tissue under saline conditions than glycophytes. In addition to having better K^+ retention under salinity stress, halophytes are also able to maintain photosynthetic "health" under altered ionic homeostasis, similar to that which occurs under salinity stress. Halophytes are able to maintain K^+ concentrations in the cytosol under salinity stress without vast increases in energy expensive H^+-ATPase activity leaving more energy for other energy demanding metabolic and growth processes.

There are many transporters involved in maintaining K^+ and Na^+ homeostasis within cells (summarised in Fig. 1). Changing the transport properties of the plasma membrane is fraught with danger. Halophytes tend to change the properties of tonoplast transport in response to salinity stress by up-regulating vacuolar H^+-PPase and H^+-ATPase activity to energise the sequestration of Na^+ into the vacuole, via the NHX Na^+/H^+ antiporter, which is also up-regulated. In addition to the increased ability to transport Na^+ into the vacuole they also reduce the activity of slow (SV)- and fast (FV)-activating tonoplast channels, thus preventing back-leak of Na^+ from the vacuole into the cytosol. The changes in vaculor ion transport are summarised in Fig. 1, with beneficially up-regulated channels and pumps being shown in green while beneficially down-regulated channels shown in red. These changes in vacuolar ion transport result in changes in the plasma membrane transport. As Na^+ is moved from

the cytosol into the vacuole plasma membrane depolarisation is avoided, resulting in less K^+ loss through KOR. The reduced loss of K^+ results in less ROS production and, therefore, less ROS-induced NSCC and KOR activity. This further reduces K^+ leak and Na^+ uptake through NSCC.

Not only is K^+ retention in the cytosol an essential part of salinity tolerance but K^+ retention in chloroplasts, under altered ionic conditions, seems also to play an important role in salinity tolerance. It is also evident that better control over compatible solute and polyamine accumulation in chloroplasts can play vital roles in conferring salt tolerance and maintaining optimal photosynthesis under salinity stress. Having the right antioxidant pools available is also essential to confer salinity tolerance.

Future work to improve salinity tolerance in crops should be focused on mechanisms that allow increases in shoot Na^+ concentrations (for energy-efficient osmotic adjustment) without detrimental effects on metabolic activity in leaf mesophyll. Although Na^+ itself does not seem to inhibit photosynthesis, cytosolic Na^+ causes membrane depolarisation and K^+ loss. This means that future work should focus on ways to sequester Na^+ efficiently into vacuoles to prevent Na^+ induced plasma membrane depolarisation. Additionally, K^+ retention within chloroplasts may provide another way to decrease the negative impacts of salinity stress. Further investigation of the inner envelope transport properties under salinity stress, may result in other strategies for producing tolerant plants.

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